New concepts for risk and safety assessment of botanicals and botanical preparations including plant food supplements (PFS)

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Suzanne Johanna Petronella Lena van den Berg

Thesis

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Chapter 1

General introduction

Short description and aim of the present thesis

Botanicals and botanical preparations such as plant food supplements (PFS) are widely marketed within the European Union (EU). However, procedures for safety assessments of PFS have not been legally harmonized at the EU level and are therefore governed by national legislation and manufacturer's procedures (Silano *et al.*, 2011). To adequately support the safe use of PFS, the validity of existing procedures for risk and safety assessment need to be demonstrated and procedures need to be harmonized at the European level. While many consumers equal 'natural' with 'safe', PFS may contain compounds that are of concern for human health. One of the major bottlenecks in the safety assessment of botanicals and botanical ingredients, and thus also for PFS, is the fact that some botanicals may contain compounds that are both genotoxic and carcinogenic. The risk assessment of such compounds is complicated and an international scientific agreement on the best practices for risk assessment of these type of compounds is currently lacking (EFSA, 2005). Therefore, the present thesis aimed at testing new concepts that could be of use for the risk and safety assessment of PFS focusing on finding adequate ways to judge the risk or safety of PFS that may contain compounds that are both genotoxic and carcinogenic. The concepts tested include the Margin of Exposure (MOE) approach, a mode of action based approach and a matrix effect approach. These concepts, described below, address the most important issues in risk and safety assessment of PFS according to dedicated bodies such as the European Food Safety Authority (EFSA). Considering that risk management actions can only be based on adequate safety evaluations, this work should ultimately give a better idea on when risk management actions would be needed, but also for which PFS there is no reason for concern even though they do contain limited amounts of compounds that may be genotoxic and carcinogenic.

Plant Food Supplements (PFS)

Definition of PFS

According to the EU Directive on Food Supplements (2002/46/EC), the term "food supplement" covers a wide range of nutrients and other ingredients such as vitamins and minerals, amino acids, essential fatty acids, fiber and

various plants and herbal extracts. Food supplements can be defined as "… foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities" (Directive 2002/46/EC). Food supplements prepared from botanicals are generally referred to as PFS (acronym of plant food supplements).

Market structure and consumer behavior towards PFS

To date, the market volume for botanicals and botanical preparations including PFS is rapidly expanding. Reportedly, in 1999 the global market for herbal supplements was worth $15 billion (Glaser, 1999) and the predicted sales of herbal supplements and remedies will reach $93 billion by 2015 (Vargas-Murga *et al.*, 2011). This robust growth has been linked to the fact that PFS are within easy reach of consumers as they are sold over the counter in supermarkets, drugstores, health-food shops, pharmacies and via Internet (EFSA, 2004). In fact, the number of web shops marketing PFS online is still increasing and the sale of PFS via Internet is expected to be of growing importance (Vargas-Murga *et al.*, 2011). Resulting from the increasing market volume of PFS, including the sale of PFS from countries were PFS are not regulated, the need for stricter control and safety testing of such products becomes more important in order to protect consumers against adverse effects.

Additional causes of the increasing market volume are the ageing population, a lack of confidence in conventional medical practice, increasing awareness of consumers to take charge of their health and well-being by self-medication and the fact that botanicals and products made thereof generally have a high consumer acceptance (Egan *et al.*, 2011; Vargas-Murga *et al.*, 2011). Although available consumer studies focus predominantly on the use of dietary supplements in general, and as a result data on consumer attitudes and beliefs about PFS are limited, the available studies suggest that the people consuming PSF, for example products consisting of echinacea, ginkgo biloba, ginseng, green tea extract, St. John's Wort and valerian amongst others, are generally female, have a higher education and relatively high income and are middle-aged (Egan *et al.*, 2011 and references therein).

Concerns related to the quality and safety of PFS

Although botanicals and products made thereof including PFS are widely marketed and generally have a high consumer acceptance, some botanicals and/or botanical preparations may contain toxic compounds that are of concern for human health. Previously, EFSA expressed some general concerns related to the quality and safety of botanicals and botanical preparations such as PFS (EFSA, 2004). It was stated, amongst others, that chemical and microbial contamination of botanicals and botanical preparations, for example those originating from Asia, can be a problem. In fact, a variety of selected traditional Ayurverdic herbal preparations on the Dutch market sampled between December 2004 and June 2007 were found to be contaminated with mercury, lead and arsenic resulting in daily intakes at levels that would exceed the established safety limits following use at dose levels recommended by the respective manufacturer (Martena *et al.*, 2010). In addition, Martena *et al.* (2007) reported that despite the ban on aristolochic acids in herbal preparations worldwide, a number of Chinese traditional herbal preparations on the Dutch market were found to be contaminated with aristolochic acids. In fact, relatively high levels up to 1676 mg aristolochic acid/kg herbal preparation were found (Martena *et al.*, 2007).

The claimed quantity and stability of the active ingredients in botanicals and products made thereof is another possible concern (EFSA, 2004). EFSA indicated that the occurrence of any efficacy following the use of botanical products can often be ascribed to the presence of one or more secondary plant metabolites. However, the patterns and concentrations of such secondary metabolites are highly dependent on biotic and abiotic factors causing stress (EFSA, 2004). As a result, differences can occur between botanicals that belong to the same species and variety, especially for botanicals grown in the wild. Such differences make it difficult to guarantee consistency of the composition of the botanical or the product made thereof (EFSA, 2004).

Another concern expressed by EFSA is the presence of naturally occurring toxic compounds in botanicals (EFSA, 2004). Especially the use of highly concentrated plant material is of concern. Additionally, some botanicals or their ingredients are of possible concern because of their interactions with prescription drugs. Such contra-indications are often not mentioned on the label of botanical products including PFS (EFSA, 2004). Another issue of concern is the intentional addition of synthetic drugs to botanical preparations. An example is the intentional addition of sildenafil, the active ingredient of Viagra®,

to dietary supplements used for erectile dysfunction (FDA, 2009). However, the presence of Viagra® or its active ingredient might be of potential concern for human health especially for men taking prescription drugs that contain nitrates. Nitrate-containing drugs are often prescribed to persons suffering from diabetes, high blood pressure, high cholesterol, or heart diseases which are common conditions in persons with erectile dysfunction (FDA, 2009). The concomitant use of prescription drugs rich in nitrates and sildenafil might result in a marked lowering of blood pressure and should therefore be avoided (FDA, 2009). These existing concerns related to the quality and safety of PFS, underline the need for uniform and well accepted procedures for risk and safety assessment of PFS as well as for quality control.

Concepts tested in the present thesis

MOE approach

Several approaches exist for the safety evaluation of compounds that are both genotoxic and carcinogenic, which may be applied in the risk and safety assessment of botanicals and/or botanical products containing these type of compounds. However, a general framework for the risk and safety assessment of PFS is not in place and rules for safety testing of PFS are subjected to national law. An overview of different approaches available for the risk and safety assessment of PFS is presented in **Chapter 2** of the present thesis and includes the history of safe use, the tiered approach proposed by EFSA, the Threshold of Toxicological Concern (TTC) and the MOE concept.

Regulatory and advisory bodies might use qualitative or quantitative approaches in the risk assessment of genotoxic carcinogens (Barlow and Schlatter, 2010). In the quantitative risk assessment of carcinogenic food-borne chemicals, animal data are often used to predict the risk for human health as data from human epidemiological studies are often not available or are difficult to obtain. Thus, the assessment of human cancer risk includes extrapolation from data obtained in animal studies and this may introduce uncertainty in the interpretation of the human cancer risk.

One of the approaches to quantitatively assess the potential risk for human health, is linear extrapolation of the animal carcinogenicity data to obtain a virtual safe dose (VSD) at which the additional lifetime cancer risk would

be one in a million. When extrapolating animal data to a VSD that gives a one in a million extra risk for developing cancer upon lifetime exposure, a variety of models can be used including models that are based on simple extrapolation and models that are mathematically more complicated (EFSA, 2005). However, low-dose linear cancer risk extrapolation has been widely used and has been reported to be a worst case extrapolation method resulting in the lowest possible VSD but is, in spite of this, currently not generally accepted (EFSA, 2005). Moreover, it is often unknown if the selected model adequately describes the underlying biological process associated with tumor development and risk estimates of a particular compound may vary orders of magnitude depending on the model applied (EFSA, 2005; O'Brien *et al.*, 2006). Moreover, the effect of species differences in metabolism and the effects of genetic polymorphisms and lifestyle factors are not taken into account since rodent tumor data are converted to a VSD without incorporating uncertainty factors for interspecies or interindividual variation (Rietjens *et al.*, 2011). Thus, better approaches are required for cancer risk assessment of compounds that are both genotoxic and carcinogenic.

One of such approaches is based on the so-called Margin of Exposure (MOE). EFSA previously prepared an opinion on the MOE as a harmonized approach in the risk assessment of compounds that are both genotoxic and carcinogenic (EFSA, 2005). The MOE is a dimensionless ratio between (1) a reference point obtained from epidemiologic or experimental data on tumor incidence and (2) the estimated daily intake in humans (Barlow *et al.*, 2006; EFSA, 2005). The BenchMark Dose (BMD) approach is the preferred method to derive the reference point. The BMD can be obtained by fitting mathematical models to the observed data without extrapolation (Barlow *et al.*, 2006). The BMDL, that is the one-sided 95% lower confidence limit of the BMD, is considered to be the preferred reference point, because it takes statistical uncertainties in the value of the BMD into consideration (Barlow *et al.*, 2006). Using the BMD approach, reference points can be obtained for treatment-related tumor types occurring at a specific site or for the total number of tumors (Barlow *et al.*, 2006). BMD and BMDL values can be calculated for a pre-defined increased tumor incidence compared to the background level, that is, the BenchMark Response (BMR), including a 1%, 5% or 10% incidence of tumors (EPA, 1995). Generally, the use of a $BMDL_{10}$ (*i.e.* a 10% increase in tumor incidence) is preferred since the use of lower incidences will increase uncertainty as 1% and 5% may be outside

the experimentally observed incidences (Barlow *et al.*, 2006). Figure 1 illustrates how the BMD and BMDL values are derived when a BMR of 10% is selected. To allow calculation of a BMDL value, studies are required that are performed using a control group and at least two exposure levels for which a treatment-related dose-response relationship is observed. Whenever carcinogenicity data are insufficient to derive a BMDL, also other relative potency estimates can be used as an alternative to derive a reference point allowing the calculation of an MOE (EFSA, 2005). An example of such an alternative is the T25, that is, a 25% tumor incidence in tested animals after lifetime exposure (Dybing *et al.*, 1997).

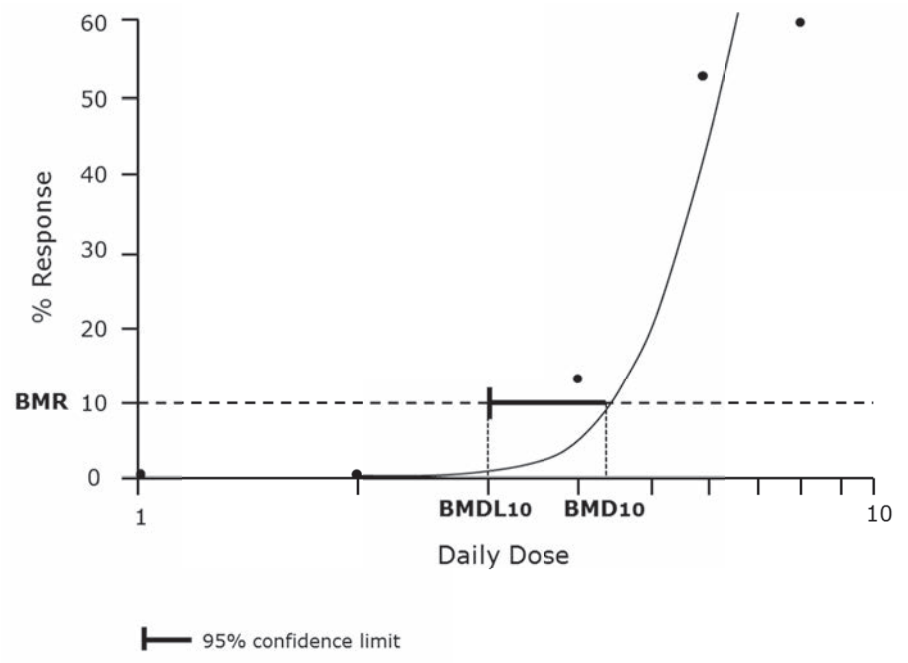

Figure 1. Dose-response curve illustrating how the BenchMark Dose (BMD) and BMDL value (the one-sided 95% lower confidence limit of the BMD) are obtained for a BenchMark Response (BMR) of 10%.

Previously, several regulatory and advisory bodies including EFSA, the World Health Organization (WHO) and the International Life Sciences Institute, European Branch (ILSI Europe), concluded that the MOE concept is the preferred approach in risk assessment of compounds that are both genotoxic and carcinogenic (Barlow *et al.*, 2006; EFSA, 2005; JECFA, 2005; O'Brien *et al.*, 2006).

In fact, during an international conference organized by EFSA, the WHO and ILSI, it was concluded that "… the MOE approach was a useful and pragmatic option for risk assessment of substances that are both genotoxic and carcinogenic. It has the potential to improve the advice provided to risk managers, since it allows comparison between compounds and prioritization of risk management actions, especially if the MOE is accompanied by an appropriate narrative explaining inherent uncertainties" (Barlow *et al.*, 2006).

EFSA has suggested that an MOE lower than 10,000 is considered as a priority for risk management actions and would be of concern for human health provided that the MOE is based on a $BMDL_{10}$ from animal data (EFSA, 2005). The value of 10,000 takes into account uncertainties in the MOE including (1) a factor 100 for species differences and human variability in kinetics and dynamics, (2) a factor 10 for inter-individual human variability in cell cycle control and DNA repair, and (3) a factor 10 for the reason that the $BMDL_{10}$ when used as a reference point is not identical to a no observed adverse effect level (NOAEL) (EFSA, 2005). When using a reference point other than a $BMDL_{10}$ value, the MOE should be interpreted differently. For example, the T25 value represents an increased tumor incidence of 25% compared to the background incidence while the $BMDL_{10}$ value is related to a 10% increased cancer incidence. In other words, the T25 value indicates a risk that is at least 2.5 times higher than the risk represented by the $BMDL_{10}$ (Dybing *et al.*, 2008). Therefore, an MOE value of 25,000 is proposed to be used to denote the level of concern when comparing a T25 value with estimated daily intake levels instead of an MOE of 10,000 (Dybing *et al.*, 2008). It is important to note that EFSA previously stated that risk managers, and not risk assessors, are responsible to judge what MOE value would be considered acceptable (EFSA, 2005). In addition, whenever MOE values are determined to be 10,000 or higher, this should not preclude the application of risk management actions to reduce human exposure (EFSA, 2005).

In addition to the carcinogenicity data that are required for calculating $BMDL_{10}$ values, adequate human exposure data are required as well to calculate the MOE. Moreover, MOE values can be calculated for different exposure scenarios. For example, estimated daily intakes, and thus the MOE, can be calculated for the whole population, the mean and median intakes, or individuals that are highly exposed (EFSA, 2005). The MOE approach can thus be applied to compare the potential risk for human health resulting from different exposure

scenarios *e.g.* exposure to a botanical compound from the use of herbs and spices, compared to the use of PFS.

Mode of action based approaches

A major limitation of the MOE approach is the fact that this approach requires carcinogenicity data to define the $BMDL_{10}$, whereas for many botanical ingredients such carcinogenicity data are not available. Therefore, mode of action based approaches may be used to perform a read across from one compound to another and still perform a risk assessment for compounds for which tumor data are absent. The US Environmental Protection Agency (EPA) previously defined the term mode of action as "…a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation. A "key event" is an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element." (EPA, 2005). It is important to note that genotoxic compounds can exert different modes of action. For example, such compounds can bind directly to the molecular target or it might require bioactivation to a reactive metabolite that can interact with the molecular target (Paini, 2012). To describe key events leading to the development of tumors, one should take into account physical, chemical, and biological information (EPA, 2005) including kinetics (absorption, distribution, metabolism and excretion) and dynamics (events leading to tumor formation).

To derive a mode of action based approach, physiologically based kinetic (PBK) modeling may be used. PBK models allow to mathematically describe the absorption, distribution, metabolism and excretion (ADME) of chemicals. These mathematical equations are principally based on physiological parameters (*e.g.* tissue blood flow rates, tissue volumes and cardiac output), physico-chemical parameters (*e.g.* blood-tissue coefficients) and metabolic parameters (*e.g.* kinetic constants for metabolic conversions) (Chiu *et al.*, 2007; Clewell and Clewell, 2008; Krewski *et al.*, 1994; Rietjens *et al.*, 2011). The outcomes of these equations can give insight in possible dose-dependent changes in metabolism with increasing levels of exposure to a chemical (Rietjens *et al.*, 2011). Moreover, blood and tissue concentrations of a particular chemical or its active metabolite(s) can be monitored over time (Rietjens *et al.*, 2011). The parameters used in PBK modeling can be substituted for parameters

for a species of interest allowing interspecies extrapolation (Andersen, 1994). Additionally, PBK modeling can give an insight in interindividual differences by including kinetic constants for metabolic conversion by human individuals and for specific isoenzymes (Bogaards *et al.*, 2000; Punt *et al.*, 2010; Rietjens *et al.*, 2011; Rostami-Hodjegan and Tucker, 2007). The development of PBK models consists of six different steps *i.e.* (1) definition of the conceptual model, (2) translation into a mathematical model, (3) defining parameter values, (4) solving the equations, (5) evaluation of model performance and (6) making predictions, which are extensively described by Rietjens *et al.* (2011). In short, in the first step several compartments should be defined representing organs and tissues that are relevant for the ADME characteristics of the compound of interest. The remaining organs and tissues can be pooled in compartments representing slowly (*e.g.* bone, muscle and skin) or richly (*e.g.* adrenals, brain and heart) perfused tissues. Moreover, an additional compartment for fat can be added if the compound of interest is relatively lipophilic. All compartments will be mutually connected through the systemic circulation. Figure 2 provides a schematic overview of a basic PBK model consisting of several compartments. In the next step, mathematical equations need to be defined describing the kinetic processes, including ADME characteristics, of the compound of interest. For each compartment, a set of mathematic equations needs to be defined. In addition to the definition of mathematical equations for each compartment, also parameter values need to be defined. Such parameters include (1) physiological and anatomical parameters, (2) physico-chemical parameters and (3) kinetic parameters as described above. After the mathematical model has been defined, the equations need to be solved to obtain concentrations of the compound of interest and its metabolite(s) in the relevant compartment or the body. This can be done using a variety of software packages such as Berkeley Madonna (Macey and Oster, UC Berkeley, CA, USA), acslXtreme (AEgis Technologies Group), and MATLAB (The MathWorks). Subsequently, an evaluation of model performance has to be made. This can be done by comparing the model outcomes to available in vivo data supporting the validity of the model. Moreover, a sensitivity analysis can be performed to provide a quantitative evaluation of how the input parameters of the model influence model output. Based on such an analysis it can be concluded whether the model output is directly or inversely related to a specific parameter. When the PBK model adequately predicts in vivo observed data, the model can be used to make predictions for different exposure scenarios.

For example, the concentration of an unstable reactive intermediate can be predicted which generally remains undetected in conventional toxicity studies. To describe the dynamic processes resulting in cancer formation, PBK models can even be extended to physiologically based dynamic (PBD) models. Some examples of dynamic characteristics that can be included in the PBD model to describe the mode of action of the compound of interest are DNA-adduct formation and DNA repair and ultimately tumor formation (Paini, 2012).

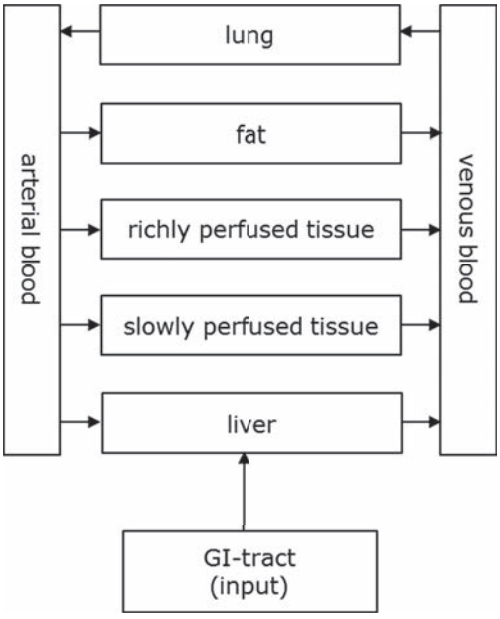

Figure 2. Schematic overview of a PBK model.

Matrix effect approach

Generally, risk assessment of botanicals and botanical preparations is performed using data obtained from rodent studies which use high dose levels of the botanical compound administered by gavage in the absence of a natural botanical matrix (Rietjens *et al.*, 2008). However, the presence of a botanical matrix might alter the toxicity of the botanical compound(s) of interest (Rietjens *et al.*, 2008). In fact, the bioavailability of the compound of interest may be reduced when consumed in the presence of a matrix resulting from a slow or

incomplete release of the active principle from the matrix (Schilter *et al.*, 2003). Another example is that specific intestinal carriers involved in active uptake of an ingredient might be inhibited by the matrix (Rietjens *et al.*, 2008). These examples indicate that the ADME characteristics of a botanical compound of interest might be modulated by additional compounds present in the botanical or the product made thereof. As a result, the use of data from rodent studies conducted using the pure compound may not represent an adequate basis for risk assessment (Rietjens *et al.*, 2008). This suggests that (experimental) data are essential to demonstrate the importance of matrix-derived combination effects for risk assessment of individual compounds. However, marked differences in composition can occur between botanicals that belong to the same species and variety, especially for botanicals grown in the wild (EFSA, 2004). Such differences make it difficult to guarantee consistency of the composition of the botanical or the product made thereof (EFSA, 2004). In addition, a wide variety of manufacturing methods for preparing PFS are available resulting in compositional differences of the final product. Clearly, this complicates the performance of risk and safety assessments of such botanical products in a generic manner. Therefore, a full toxicological assessment should preferably be performed for each individual botanical or botanical product. However, it would be impossible to perform toxicity studies for all specific botanicals and botanical preparations available. It is therefore of importance to develop and validate new science-based approach(es) to take matrix-derived combination effects into account without the need of animal testing.

Previously, Rietjens *et al.* (2008) proposed to study the matrix-derived combination effects on a case-by-case basis based on analytical characterization of the major compound(s) responsible for this effect. Recently, this proposed approach was applied by Alhusainy and colleagues who identified the major compounds responsible for the matrix modulation of the bioactivation of the alkenylbenzene estragole based on in vitro data (Alhusainy *et al.*, 2012). In addition, the possible in vivo effects were predicted by means of PBK modeling taking into account the relative ratio between the active principle (*i.e.* estragole) and the major compound responsible for this effect (*i.e.* nevadensin). Moreover, the importance of the matrix-derived combination effect in vivo was validated and confirmed in an in vivo rat study (Alhusainy *et al.*, 2013).

Alkenylbenzenes, the model compounds tested in the present thesis

Background

To test the use of the MOE concept, the mode of action concept and the matrix concept for the risk and safety assessment of PFS, compounds from the group of alkenylbenzenes were selected as model compounds. **Chapter 3** of the present thesis describes the process underlying this selection of the alkenylbenzenes, as an important group of compounds that may be present in PFS and that exert genotoxic and/or carcinogenic properties. In short, this selection was based on (1) information on genotoxic and carcinogenic botanical ingredients as listed in the EFSA compendium (EFSA, 2012), (2) data from studies performed by the National Toxicology Program (NTP) providing evidence of genotoxicity and/or carcinogenicity of botanical compounds, and (3) the expert judgment from partners collaborating under the PlantLIBRA project (acronym PLANT food supplements: Levels of Intake, Benefit and Risk Assessment), a European collaborative project under the Seventh Framework Programme. Important compounds that were judged to be present in PFS, and are genotoxic and carcinogenic, were found to belong to the group of alkenylbenzenes (*e.g.* estragole, methyleugenol, safrole, β-asarone and elemicin) and the group of unsaturated pyrrolizidine alkaloids (*e.g.* riddelliine and monocrotaline). To date, compounds belonging to the group of unsaturated pyrrolizidine alkaloids are regulated and not allowed in PFS. For this reason, compounds belonging to the group of alkenylbenzenes were selected as the model compounds tested in the present thesis.

Alkenylbenzenes including estragole, methyleugenol, safrole and β-asarone but also elemicin, apiol, myristicin, trans-anethole, eugenol and isosafrole (Figure 3) are naturally occurring compounds that contribute to the characteristic taste and fragrance of herbs and spices. Several herbs and spices such as anise, nutmeg, fennel, mace and basil contain combinations of alkenylbenzenes (SCF, 2001a, 2001b, 2002). However, within the same botanical, differences may occur in the levels of the different alkenylbenzenes present depending on geographical origin, the plant maturity at harvest and techniques used for harvesting, storage conditions, processing methods such as drying, and the distribution of alkenylbenzenes within the plant (Smith *et al.*, 2002). Although humans are widely exposed to alkenylbenzenes through

the consumption of traditional foods, not much is known concerning the possible adverse impact on human health. A number of rodent carcinogenicity assays reveal the hepatocarcinogenic effects of the alkenylbenzenes estragole, methyleugenol and safrole (SCF, 2001a, 2001b, 2002). Moreover, a genotoxic mode of action has been suggested for these compounds (SCF, 2001a, 2001b, 2002). These adverse effects have been linked to the conversion of the alkenylbenzenes to their carcinogenic metabolites. Figure 4 presents an overview of the metabolic pathways of estragole which are also relevant for the other alkenylbenzenes. The observed genotoxic and carcinogenic effects of alkenylbenzenes have been linked to their bioactivation by cytochrome P450 enzymes and sulfotransferase (SULT)-enzymes, resulting in the formation of 1'-sulfoxymetabolites and corresponding carbocations formed upon degradation of the 1'-sulfoxymetabolites (Anthony, 1987; Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Sangster *et al.*, 1987; Solheim and Scheline, 1973; Wiseman *et al.*, 1987).

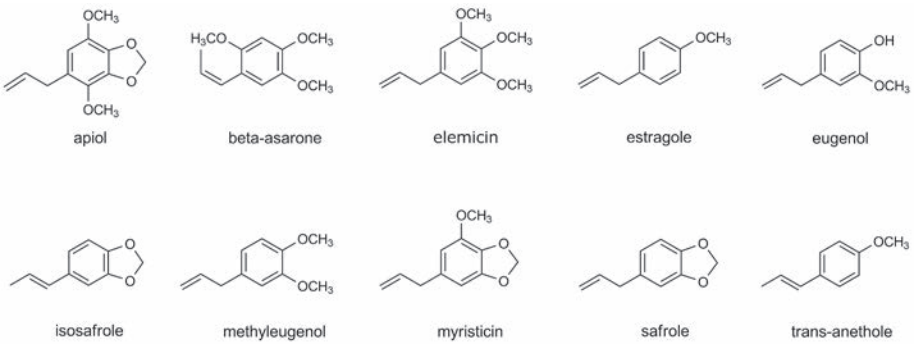

Figure 3. The structural formulas of several alkenylbenzenes.

Genotoxicity

The alkenylbenzenes estragole, methyleugenol, safrole, elemicin, myristicin, and apiol have previously been given priority by JECFA for evaluation of their risks resulting from consumption of botanicals and botanical preparations (JECFA, 2009). Estragole, methyleugenol and safrole have been tested in a number of in vitro and in vivo genotoxicity assays. However, the alkenylbenzenes elemicin, myristicin and apiol are less well studied and consequently only

limited genotoxicity studies are available. An overview of genotoxicity studies performed using estragole, methyleugenol and safrole is presented in Tables 1-3.

Based on the mode of action of the alkenylbenzenes, positive responses would be only expected in test systems containing appropriate enzymes and their cofactors required for bioactivation. Estragole (Table 1) and methyleugenol (Table 2) were found to be non-mutagenic in a number of studies using bacterial cells. This can be explained by the lack the sulfonation pathway in these test systems. In fact, a positive response for mutagenicity was recently demonstrated for methyleugenol in a reverse mutation test using *Salmonella typhimirum* TA100 expressing different SULT enzymes required for sulfonation of methyleugenol and the other alkenylbenzenes (Herrmann *et al.*, 2012). For safrole equivocal results have been found in a number of reverse mutation tests using bacterial cells (Table 3).

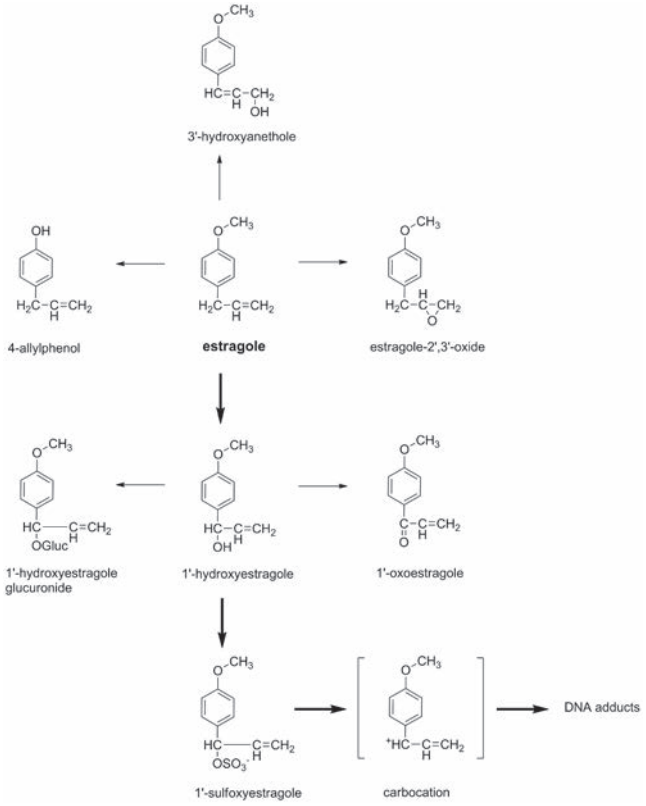

Figure 4. Metabolic pathways of estragole also relevant for the other alkenylbenzenes. Arrows in bold indicate the bioactivation to 1′-sulfoxyestragole resulting in DNA-adduct formation.

However, it should be noted that cytotoxicity was observed in several of the reverse mutation studies performed using safrole (Table 3). It is of importance to note that the majority of studies performed for the alkenylbenzenes date from the early to mid-1980s (JECFA, 2009). In these studies, relatively high concentrations were used that resulted in marked cytotoxic effects in several studies (JECFA, 2009). Moreover, during that period extreme culture conditions including hypo- and hyper osmolality and a high pH were often used (JECFA, 2009). Reportedly, such conditions can result in apoptosis and necrosis leading to DNA fragmentation and this may result in a false positive response in clastogenic assays (*e.g.* chromosomal aberration test and micronuclei test) (JECFA, 2009; Meintieres and Marzin, 2004).

Despite the fact that the alkenylbenzenes have been found to be negative in several in vitro and in vivo genotoxicity assays, the formation of DNA adducts has been observed in vitro, demonstrating DNA reactivity (Zhou *et al.*, 2007). Moreover, estragole, methyleugenol and safrole gave positive results in a variety of in vivo DNA binding assays (Boberg *et al.*, 1983; Daimon *et al.*, 1998; Phillips *et al.*, 1981a; Phillips *et al.*, 1981b; Randerath *et al.*, 1984; Wiseman *et al.*, 1985) suggesting that the alkenylbenzenes exert genotoxic characteristics. The formation of DNA adducts has also been demonstrated for the 1′-hydroxymetabolites of estragole and safrole (Wiseman *et al.*, 1985). Moreover, Randerath *et al.* (1984) demonstrated that the binding of safrole to mouse liver DNA was inhibited following pre-treatment with pentachlorophenol (PCP), a known SULT inhibitor, suggesting that the genotoxic effects are dependent on SULT-mediated metabolism. Estragole, methyleugenol and safrole were found to induce unscheduled DNA Synthesis (UDS) in several in vitro and in vivo studies (Tables 1-3). In addition, Chan and Caldwell (1992) demonstrated that the induction of UDS in rat hepatocytes by the 1′-hydroxymetabolites of estragole, methyleugenol and safrole was higher than UDS induction by the parent compounds (Chan and Caldwell, 1992). In the UDS test, freshly isolated hepatocytes from rat or mouse are generally used to detect DNA repair synthesis. These freshly isolated hepatocytes contain enzymes and cofactors required for bioactivation of alkenylbenzenes explaining the positive results obtained.

Overall, UDS tests and DNA-adduct formation in vitro and in vivo suggest that the alkenylbenzenes including estragole, methyleugenol and safrole exert genotoxic characteristics. The lack of positive results in standard genotoxicity tests and the observation that 1′-hydroxyetabolites gave positive results in UDS tests emphasize that the genotoxic effects are dependent on metabolism and suggest that the sulfonation pathway should be taken into account in standard in vitro tests as recently done by Herrmann *et al.* (2012).

Table 1. Overview of in vitro and in vivo genotoxicity studies performed with estragole, adapted from JECFA (2009).

Number of studies	End point	Type of tissue/ bacteria used in test	Concentration range	Genotoxicity	Remarks
In vitro studies					
6	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	0.05 – 2964 µg/plate	Negative (*n*=5) Positive (*n*=1) Weakly positive (*n*=1)	Studies were performed with and/or without metabolic activation. In the study in which weak positive results were obtained, estragole was found to be toxic to cell growth at several of the concentrations tested.
1	DNA repair	*Bacillus subtilis* H17Rec^{+} and M45Rec^{-}	4000 µg/disc	Negative (*n*=1)	Study was performed without metabolic activation.
1	Chromosomal aberration	V79 cells	1.48 – 468 µg/mL	Negative (*n*=1)	Study was performed with and without metabolic activation.
4	UDS	Rat hepatocytes	0.148 – 7400 µg/mL	Positive (*n*=1)	In three out of four studies marked cytotoxicity was reported at concentrations higher than 10^{-3} mol/l.
In vivo studies					
1	UDS	Rat (gavage)	500 – 2000 mg/kg bw	Positive (*n*=1)	-
1	Micronucleus induction	Mouse (gavage)	37.5 – 600 mg/kg bw	Negative (*n*=1)	-

Table 2. Overview of in vitro and in vivo genotoxicity studies performed with methyleugenol, adapted from JECFA (2009).

Number of studies	End point	Type of tissue/ bacteria used in test	Concentration range	Genotoxicity	Remarks
In vitro studies					
6	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537 and TA1538, *E. coli* WP2 trp^-	3 – 1069 µg/ plate	Negative (*n*=6)	Studies were performed with and/or without metabolic activation, one study was only performed in the absence of metabolic activation.
1	DNA repair	*Bacillus subtilis* $H17Rec^+$ and $M45Rec^-$	1000 µg/ disc	Negative (*n*=1)	Study performed without metabolic activation.
1	Mitotic recombination	*Saccharomyces cerevisae* RS9	121 – 364 µg/mL	Positive (*n*=1)	Study performed with and without metabolic activation. Marked toxicity was noted.
1	Mitotic recombination	CHO cells	5- 250 µg/mL	Positive (*n*=1) Negative (*n*=1)	Positive results were obtained with metabolic activation, negative results were obtained without metabolic activation.
1	SCE	CHO cells	50 – 500 µg/mL	Negative (*n*=1)	Study performed with and without metabolic activation.
11	UDS	Rat hepatocytes, mouse hepatocytes, human hepatocytes	0.0178 - 8900	Positive (*n*=9) Very weak positive (*n*=1) Equivocal (*n*=1)	In two studies marked cytotoxicity was reported at concentrations higher than 10^{-3} mol/l. In another two studies, the highest level of UDS was observed at 10 µmol/l and decreased with increasing concentration up to 500 µmol/l.
In vivo studies					
1	UDS	Rat (gavage)	500 mg/kg bw	Positive (*n*=1)	-
2	Micronucleus induction	Rat (gavage)	10 – 1000 mg/kg bw	Negative (*n*=2)	-

Table 3. Overview of in vitro and in vivo genotoxicity studies performed with safrole, adapted from JECFA (2009).

Number of studies	End point	Type of tissue/ bacteria used in test	Concentration range	Genotoxicity	Remarks
In vitro studies					
44	Reverse mutation	*Salmonella typhimurium* TA92, TA98, TA100, TA1535, TA1537 and TA1538, TM677,	up to 5000 µg/plate	Positive (*n*=6) Very weakly positive (*n*=2) Negative (*n*=37) Equivocal (*n*=1)	Studies were performed with and/ or without metabolic activation.
13	Differential killing	*E. coli* WP2, WP2 trp$^-$, WP2 uvrA, WP2, WP67 pol, polA$^+$ and polA$^-$	Up to 9958 µg/mL	Positive (*n*= 3) Negative (*n=7*) Weakly positive (*n= 2*) Questionable (*n*= 1)	Studies were performed with and/ or without metabolic activation.
2	SOS induction	*E. coli* PQ37	Up to 3.2 µg/assay, for one study concentrations are not provided	Negative (*n*=2)	One study was performed with and without metabolic activation.
1	Lysogenic induction	*E. coli* GY5027 envA$^-$ uvrB$^-$ and GY4015 amp^R	Up to 2000 µg/plate	Negative (*n*=1)	Study performed with metabolic activation.
4	DNA repair	*Bacillus subtilis* H17Rec$^+$ and M45Rec$^-$	Up to 21940 µg/disc, for two studies concentrations are not provided	Positive (*n*=2) Negative (*n*=2)	Two studies were performed without metabolic activation, for the other two studies information on metabolic activation was not provided.
2	mutation	*E. coli* 343/1 13/uvrB and 343/113/uvrB/leus (pKM101), *S. cerevisiae* C658-K42	0.2 – 80 µg/mL	Positive (*n*=1) Negative (*n*=1)	In one study significant toxicity to bacteria was observed at concentrations of 0.2 µg/mL and higher. Another study was perfromerd with and without metabolic activation.
19	Mitotic recombination	*S. cerevisiae* D3, D4, D61.M, D6, D7, T1, T2, JD1, PV-3,	0.1 – 2000 µg/mL	Positive (*n*=7) Negative (*n*=10) Equivocal (*n*=1)	Studies performed with and/or without metabolic activations. Marked toxicity was observed in some studies.

Table 3 (continued). Overview of in vitro and in vivo genotoxicity studies performed with safrole, adapted from JECFA (2009).

Number of studies	End point	Type of tissue/ bacteria used in test	Concentration range	Genotoxicity	Remarks
35	Forward mutation	*S. cerevisiae* PV-1, *Schizosaccharomyces pombe* P1, *Aspergus nidulans*, L5178Y mouse lymphoma cells, Chinese hamster V79 cells, human lymphoblast cells TH6, AHH-1, CHO cells	0.003 – 5480 µg/mL	Positive (*n*=13) Weakly positive (*n*=1) Negative (*n*=20) Inconclusive (*n*=1)	Studies performed with and/or without metabolic activation. In some studies cytotoxicity was observed.
15	SCE	CHO cells, Chinese hamster V79 cells, human hepatoma strain HepG2	0.01 – 810 µg/mL	Positive (*n*=6) Negative (*n*=9) Weakly positive (*n*=1)	Studies performed with and/or without metabolic activation.
3	Micronucleus induction	CHO cells, human hepatoma strain HepG2	0.162 – 143 µg/mL	Positive (*n*=1) Negative (*n*=2)	Studies were performed with and/or without metabolic activation.
12	Chromosomal aberrations	Chinese hamster lung fibroblast cells, Chinese hamster liver fibroblast CH1-L cells, CHO cells, rat liver cells, Chinese hamster V79cells, rat hepatocytes	0.16 – 486 µg/mL	Positive (*n*=3) Negative (*n*=9)	Studies were performed with and/or without metabolic activation.
23	UDS	Rat hepatocytes, human fibroblast WI-38 cells, HeLa S3 cells, mouse hepatocytes	0.000162 – 1620 µg/mL, for one study test concentrations are not provided	Positive (*n*=13) Negative (*n*=10)	Studies were performed with and/or without metabolic activation.

In vivo studies					
9	Sex-linked recessive lethal mutation	*Drosphila melanogaster*	0.1 – 6.76 mmol/l	Positive (*n*=2) Marginally positive (*n*=1) Negative (*n*=6)	-
4	Host-mediated reverse mutation	*S. typhimurium* TA1530, TA1534, TA1538, TA1950, TA1951, TA1952 in mouse, *S. cerevisiae* D3 in mouse	200 – 5500 mg/kg bw, for one study the tested concentrations are not provided	Positive (*n*=3) Negative (*n*=2)	In one study, positive results were only observed in *S. typhimurium* strain TA1950 and TA1952.
1	SCE	Mouse	0.1 – 20 mg/kg bw	Negative (*n*=1)	-
1	Chromosomal aberration	Rat	658 – 1097 mg/kg bw	Positive (*n*=1)	-
5	UDS	Rat and mouse	200 – 1000 mg/kg bw, in one study 50% of the LD50 was tested	Positive (*n*=3) Negative (*n*=2)	-
5	Micronucleus induction	Mouse	0.36 – 220 mg/kg bw, in on study 80% of the LD50 was tested	Negative (*n*=4) Equivocal (*n*=1)	-

Carcinogenicity

In literature several carcinogenicity studies are described for estragole, methyleugenol and safrole. A summary of the carcinogenicity studies performed using estragole, methyleugenol and safrole is presented below.

Estragole and its proximate carcinogenic metabolite, 1'-hydroxyestragole, have been found to be carcinogenic in CD-1 and B6C3F1 mice (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987). In these studies, mice were exposed to estragole or its 1'-hydroxymetabolite by intra peritoneal *(i.p.)* injections (Miller *et al.*, 1983; Wiseman *et al.*, 1987), subcuteneous (*s.c.)* injections (Drinkwater *et al.*, 1967; Miller *et al.*, 1983) or via the diet (Miller *et al.*, 1983). Based on the studies performed for estragole, it is suggested that male mice are more susceptible for tumor formation than female mice (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987). In addition to hepatic tumors, a few incidences of angiosarcomas and lung adenomas were observed (Miller *et al.*, 1983). Wiseman *et al.* (1976) revealed that the induction of liver tumors by estragole was inhibited by PCP suggesting that the formation of 1'-sulfoxymetabolites is an important step in the carcinogenicity of estragole. Adequate studies in which estragole was tested for carcinogenicity in rats are not available in literature. Nevertheless, Miller *et al.* (1983) showed that 1'-hydroxyestragole is carcinogenic in male Fisher rats following the subcutaneous injection of 1'-hydroxyestragole.

Methyleugenol was shown to induce hepatomas in male B6C3F1 mice following *i.p.* treatment of methyleugenol twice weekly for a period of 12 weeks (Miller *et al.*, 1983). The same effects were seen in mice treated with 1'-hydroxymethyleugenol by *i.p.* injection (Miller *et al.*, 1983). In a 2-year gavage study performed by the NTP it was demonstrated that methyleugenol is a multisite carcinogen (NTP, 2000). In fact, in male and female F344/N rats increased incidences of liver neoplasms and neuroendocrine tumors of the glandular stomach were observed (NTP, 2000). In addition, in male rats also increased incidences of kidney neoplasms, malignant mesothelioma, mammary gland fibro adenoma, and subcutaneous fibroma and fibroma or fibro sarcoma were observed (NTP, 2000). In B6C3F1 mice, target organs included the liver and glandular stomach (NTP, 2000).

Safrole was found to be carcinogenic in CD-1 and B6C3F1 mice (Borchert *et al.*, 1973; Miller *et al.*, 1983), and Fischer and CD random bred rats (Miller *et al.*, 1983; Wislocki *et al.*, 1977). In these studies, mice and rats were exposed

to safrole by *i.p.* injections (Borchert *et al.*, 1973; Miller *et al.*, 1983; Wislocki *et al.*, 1977) *s.c.* injections (Miller *et al.*, 1983; Wislocki *et al.*, 1977) or via the diet (Miller *et al.*, 1983; Wislocki *et al.*, 1977). Target organs included liver, lungs, fore stomach, kidney and the mammary glands (Wislocki *et al.*, 1977). In addition, the induction of hepatomas and lung carcinomas has been demonstrated for 1'-hydroxysafrole in mice and rats (Miller *et al.*, 1983; Wislocki *et al.*, 1977).

Overall, estragole, methyleugenol and safrole were found to be multisite carcinogens, with the liver being the most important target organ. In addition, methyleugenol and safrole were demonstrated to be multispecies carcinogens. However, for estragole only mice studies are currently available in literature suggesting that estragole is also a multispecies carcinogen as demonstrated for its structurally related analogues. In line with these findings, EPA previously indicated that mutagenic agents generally show multisite and multispecies tumor effects (EPA, 2005).

Previous risk assessments

Although genotoxic and carcinogenic characteristics have previously been indicated for estragole, methyleugenol and safrole, different safety assessments of these naturally occurring botanical ingredients present different conclusions. Previously, the Flavor and Extracts Manufacturers Association (FEMA) suggested that the present human exposure to estragole and methyleugenol from herbs and spices does not pose a significant cancer risk at the low levels of exposure resulting from flavor use (Smith *et al.*, 2002). Furthermore, the European Medicines Agency (EMA, former EMEA) concluded that there is no considerable risk for human health resulting from the short-term exposure to herbal medicinal products containing alkenylbenzenes by adults at the dosing regimen suggested (EMA, 2004, 2005). The conclusions drawn by FEMA and EMA, were based on available animal studies on the profiles of metabolism, metabolic activation, and covalent binding of estragole and methyleugenol to DNA suggesting a dose-response relationship with a non-linear mode of action. In fact, it was concluded that the relative importance of the adverse effects will be significantly reduced at low exposure levels relevant for the daily human exposure to these genotoxic carcinogens (EMA, 2004, 2005; Smith *et al.*, 2002). In contrast, the EU Scientific Committee on Food (SCF) previously indicated restrictions in the use of these compounds as pure ingredients added to food (SCF, 2001a, 2001b, 2002). These restrictions in use were suggested because of

the genotoxic and carcinogenic characteristics of these compounds. Because of these genotoxic and carcinogenic characteristics, the SCF concluded that the presence of a threshold in their mode of action cannot be assumed for these compounds thus hampering the determination of a safe exposure limit (SCF, 2001a, 2001b, 2002).

Regulatory status

In addition to differences in expert opinions on the safety assessment of the alkenylbenzenes, differences can be defined concerning the regulatory actions that have been taken at the international level. The alkenylbenzenes estragole, methyleugenol and safrole were often directly added to food as a flavoring substance in baked goods, candies and other foodstuffs (Smith *et al.*, 2002) in the US where these compounds are approved by the Food and Drug Administration (FDA) for use in foods for human consumption as a flavoring. However, the use of estragole and methyleugenol, as pure substance in food is limited to the US. The EU recently decided to prohibit the use of these compounds as flavoring agents (Regulation (EC) No 1334/2008 of the European Parliament and of the Council 16 December 2008). In addition, maximum levels of estragole and methyleugenol, naturally present in food ingredients with flavoring properties, have been defined for a number of foodstuffs (Regulation (EC) No 1334/2008 of the European Parliament and of the Council 16 December 2008). The use of safrole, as a flavoring in human nutrition, was already prohibited earlier by the Council of the European Communities (Council Directive 88/388/EEC of 22 June 1988). Safrole is also banned by the FDA (Federal Register of December 3, 1960, 25 FR 12412). Despite these prohibitions, alkenylbenzenes still occur in the regular diet because they occur naturally in herbs and spices and products made thereof. Consequently, estragole, methyleugenol and safrole can still be found in a variety of products including pesto, tomato sauces, meat-based products, such as Bologna and Vienna sausages (Siano *et al.*, 2003), and herbal teas. In contrast to the existing regulations for food within the EU, such regulations are not in place for the use of alkenylbenzene-containing PFS.

These existing differences in expert opinions and regulatory actions that have been taken at the international level related to the presence of alkenylbenzenes in food, underlines the need for uniform and well accepted procedures for risk and safety assessment of these genotoxic carcinogens.

Outline of the present thesis

The present thesis aimed at testing and validating new concepts that could be of use for the risk and safety assessment of PFS focusing on finding adequate ways to judge the risk or safety of PFS that may contain compounds that are both genotoxic and carcinogenic. The concepts tested include the MOE concept, the use of a mode of action based concept for risk assessment, and the matrix effect concept. In addition, safety assessment was also performed for botanical preparations in the form of herbal teas.

Chapter 1, the present chapter, defines the aim of the present thesis and gives a general introduction to PFS, an introduction to the concepts tested, an introduction to the model compounds tested in the present thesis, and also presents the outline of the present thesis. The general framework for safety assessment of PFS is presented in **Chapter 2.** In addition, **Chapter 2** provides some examples of types of botanical compounds of concern and their major adverse effects. **Chapter 3** describes the selection and analysis of genotoxic and carcinogenic compounds which can be found in PFS and a subsequent risk assessment using the MOE concept. The majority of selected compounds were found to belong to the group of alkenylbenzenes (*e.g.* estragole, methyleugenol, safrole and β-asarone) or the group of unsaturated pyrrolizidine alkaloids (*e.g.* riddelliine and monocrotaline). Based on the fact that unsaturated pyrrolizidine alkaloids are currently already regulated and not allowed in PFS, in subsequent chapters of the thesis studies focused on the group of alkenylbenzenes. In **Chapter 4** mode of action based PBK models were defined describing the bioactivation and detoxification of the alkenylbenzene elemicin in rat and human. The development of PBK models for elemicin allowed performing a read across from estragole and methyleugenol for which tumor data are available to elemicin for which the toxicological database is limited. Based on the results thus obtained a risk assessment could be performed for elemicin. In **Chapter 5** risk assessment was performed based on an approach that takes the matrix-derived combination effects into account. For this purpose, the risk assessment described in Chapter 3 for basil-containing PFS was updated taking the matrix-derived combination effect for the bioactivation of estragole into account. **Chapter 6** presents the analysis of estragole in dry fennel preparations and in water infusions prepared from them and the evaluation of the potential risk associated to the presence of estragole. Finally, **Chapter 7** gives a discussion of the results obtained in this thesis and presents some suggestions for steps to be taken in the near future for a further improvement of the risk and safety assessment of PFS.

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Chapter 2

Safety assessment of plant food supplements (PFS)

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Abstract

Botanicals and botanical preparations, including plant food supplements (PFS), are widely used in Western diets. The growing use of PFS is accompanied by an increasing concern because the safety of these PFS is not generally assessed before they enter the market. Regulatory bodies have become more aware of this and are increasing their efforts to ensure the safety of PFS. The present chapter describes an overview of the general framework for safety assessment of PFS focusing on different approaches currently in use to assess the safety of botanicals and/or botanical compounds including history of safe use, the tiered approach proposed by the European Food Safety Authority (EFSA), the Threshold of Toxicological Concern (TTC) and the Margin of Exposure (MOE) concept. Moreover, some examples of botanical compounds in PFS that may be of concern are discussed. Altogether it is clear that 'natural' does not equal 'safe' and that PFS may contain compounds of concern at levels far above those found in the regular diet. In addition, the traditional use of a PFS compound as an herb or tea does not guarantee its safety when used as a supplement. This points at a need for stricter regulation and control of botanical containing products, especially given their expanding market volume.

General introduction

At present, there is an increasing interest for plant ingredients and their use in drugs, for teas, and/or in food supplements. Such use may result in intake levels that exceed the normal dietary intake of these botanicals and/or their ingredients. Although plant food supplements (PFS) are widely marketed, the safety of these PFS is not generally assessed before they enter the market. Regulatory bodies have become more aware of this and are increasing their efforts to ensure the safety of botanical supplements (EFSA, 2004a; Taylor, 2004). The Scientific Committee of the European Food Safety Authority (EFSA) issued a guidance in which a general framework for safety assessment of botanicals and botanical preparations was presented (EFSA, 2007). In this opinion it was recommended to test the proposed approach for the safety assessment of botanicals and botanical preparations with a number of examples, resulting in an advice on the EFSA guidance document, based on real case studies by the EFSA Scientific Cooperation (ESCO) working group on botanicals and botanical preparations (EFSA, 2009a; Speijers *et al.*, 2010) and an updated guidance document (EFSA, 2009b).

In spite of this regulatory awareness it cannot be excluded that specific developments may still result in health concerns. Such developments include; (1) overconsumption by particular groups, sometimes stimulated by companies making misleading claims on their websites or in their literature; (2) the fact that many consumers equate 'natural' with 'safe' when considering PFS; (3) the availability of potentially harmful PFS through internet sites from countries where regulations are not in place; and (4) the fact that often there are no detailed requirements on safety and quality of PFS. The latter is especially worrying as botanicals are known to be of variable quality with high variation not only in the content of the active but also of the toxic principles, and the fact that already several cases of replacement of a harmless variety with a toxic alternative have occurred. One such example relates to Chinese star anise (*Illicium verum*) used in many cultures, mostly for preparing tea. In September 2001 in The Netherlands, more than 60 people showed nausea and vomiting after drinking an herbal tea called 'starmix tea' containing star anise, and 22 persons were hospitalized due to tonic-clonic insults (Johanns *et al.*, 2002; Oudesluys-Murphy and Oudesluys, 2002). Electroencephalograms (EEGs) showed epileptiform abnormalities indicating a diffuse cerebral disease (Biessels *et al.*, 2002). The complaints

were ascribed to a toxic star anise species comparable to Japanese star anise (*Illicium anisatum*) containing anisatin which was accidentally exchanged for the non-toxic Chinese star anise (*Illicium verum*) (Johanns *et al.*, 2002). After this incident and the detection of Japanese star anise in consignments of star anise from third countries, the EU took legislative measures to increase control and safeguard public health (Commission Decision 2002/75EC). These measures included the requirement of documentary evidence confirming that the imported products do not contain any Japanese star anise, and random sampling and analysis of these products. Sampling and control was also implemented for products already on the market. These measures were of a temporal nature as they were lifted one year later given the absence of new cases of contamination and poisoning (Commission Decision 2003/602/EC).

Another example of replacement of a harmless variety with a toxic alternative comes from the use of Chinese medical products. In 1991, a unique form of nephropathy was reported in Belgium. Over 100 young women suffered from kidney damage, developing into cancer of the kidneys and the urinary tract in several patients (Vanhaelen *et al.*, 1994; Vanherweghem *et al.*, 1993). This adverse effect was associated with the prolonged intake of a Chinese herb-based weight loss preparation in which *Stephania tetranda* was accidentally replaced by *Aristolochia fanchi*, because both plants are used under the same name 'Fangji' in Chinese folk medicine (Vanherweghem *et al.*, 1993). On the basis of this incident, risk management actions have been taken by the authorities of most EU Member States to prevent such from happening again.

The present chapter focuses on the general framework for safety assessment of PFS, presenting also some examples of types of botanical compounds of concern and their major adverse effects. Furthermore, some emphasis is given to the modulating effect of other PFS ingredients modifying the actual risk posed by a specific botanical ingredient.

General framework for risk/safety assessment of PFS

At present, a formalized framework for the safety assessment of PFS is not in place and safety assessments are performed on a national basis or by dedicated bodies like, for example, EMA (former EMEA) (European Medicines Agency). EMA, however, judges the safety of medicinal

preparations and does not refer to the safety of PFS in the field of food use.

In 2009, the Scientific Committee of EFSA published an updated guidance on the scientific data needed to carry out a safety assessment of a botanical or a botanical preparation (EFSA, 2009b). This guidance proposes a two-tiered scientific approach for the safety assessment depending on the available knowledge on a given botanical and the substance(s) it contains. The tiered approach takes into account the nature of the botanical or botanical preparation, its intended uses and levels of use including PFS and whether the botanical or botanical preparation has a long term (traditional) history of food use, showing that, at proposed exposure levels, no adverse effect on human health has been reported (Tier 1). In addition it is indicated that for botanical compounds lacking a history of food use, or for botanicals whose intended use levels will significantly exceed historical intake levels, an assessment of safety based on experimental toxicity data may be required (Tier 2). This further safety assessment can focus on a specific compound, if the compound of concern in a PFS can be well defined. In that case the safety may be judged based on existing safety values for that ingredient such as an Acceptable Daily Intake (ADI) or Tolerable Daily Intake (TDI). An example would be evaluating the intake of trans-anethole from use of bitter fennel fruits using the temporary ADI of 0-2.0 mg/kg bw for trans-anethole (EFSA, 2009a; JECFA, 1998).

The EFSA document does not give clear guidance on what to do when no health-based guidance values are available, but states that consideration of exposure to the substance of concern in relation to the Threshold of Toxicological Concern (TTC) values may be helpful. Thus, one may suggest that in cases where no health-based guidance values are available, the TTC approach could be used. The TTC concept is an approach that aims to establish a human exposure threshold value below which there is a very low probability of an appreciable risk to human health. This TTC approach compares the estimated oral intake with a TTC value derived from chronic oral toxicity data for structurally related compounds. The TTC values for the so-called Cramer structural classes (Cramer *et al.*, 1976) were established by Munro *et al.* (Munro *et al.*, 1996; Munro *et al.*, 1999) based on an analysis of data from chronic toxicity studies on 137, 28 and 448 compounds in the Cramer classes I, II and III, respectively. Based on the 5th percentile values for the No Observed Adverse Effect Level (NOAEL) distributions for each class of compounds and application of a 100-fold uncertainty factor, the corresponding human

exposure threshold values were calculated. These analyses gave thresholds of toxicological concern of 1800, 540 and 90 µg per person per day for structural classes I, II and III, respectively, equivalent to 30, 9 and 1.5 µg/kg bw/day for a 60 kg person (Kroes *et al.*, 2007). When the estimated intake of a PFS ingredient of concern would be below the TTC of its respective class, this can be used to conclude on the safety of its proposed use and use levels.

In cases where the botanical ingredient contains substances that are both genotoxic and carcinogenic, assessment of the risk for human health is complicated and an international scientific agreement concerning the best strategy for the risk assessment of genotoxic and carcinogenic compounds is lacking (EFSA, 2005). As a result, a variety of approaches is used by different regulatory and advisory bodies. While some offer qualitative advice, others present quantitative approaches with respect to the risk assessment of genotoxic carcinogens (Barlow and Schlatter, 2010). The advice that the intake of a particular substance should be as low as reasonably achievable (ALARA) is a qualitative approach that is widely used (Barlow *et al.*, 2006; O'Brien *et al.*, 2006). Nevertheless, the use of this qualitative approach can be considered to be of limited value since it does not define priorities necessary for risk management actions (EFSA, 2005; JECFA, 2005). Furthermore, this approach does not include data on carcinogenic potency nor data on human exposure (JECFA, 2005; O'Brien *et al.*, 2006). As a consequence, this approach might be applied to compounds even though their current exposure levels are of no risk to human health.

Quantitative approaches often include dose-response data, derived from epidemiological studies or rodent carcinogenicity bioassays, and exposure data to estimate the risk to human health (O'Brien *et al.*, 2006). However, risk estimates for a particular compound may show variable outcomes depending more on the selected mathematical model than on the experimental data (EFSA, 2005; O'Brien *et al.*, 2006) and the use of numerical risk estimates may be interpreted incorrectly (O'Brien *et al.*, 2006).

Considering the possible uncertainties and existing disadvantages connected to the use of qualitative and quantitative approaches such as ALARA and low-dose cancer risk extrapolation, the use of an Margin of Exposure (MOE) approach was recommended by expert groups of EFSA, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the International Life Sciences Institute (ILSI) (Barlow *et al.*, 2006; EFSA, 2005; JECFA, 2005; O'Brien *et al.*, 2006). The MOE is a dimensionless ratio based on a reference point

obtained from epidemiologic or experimental data on tumor incidence which is divided by the estimated daily intake in humans (EFSA, 2005). Thus, the MOE approach compares toxic effect levels with human exposure levels. The MOE approach is considered a useful and pragmatic option for risk assessment of substances that may be both genotoxic and carcinogenic (Barlow *et al.*, 2006; EFSA, 2005). It allows comparison between compounds and prioritization of risk management actions, especially if the calculation of the MOE is accompanied by an appropriate narrative explaining inherent uncertainties.

Alternatively, one may evaluate whether the expected exposure to the genotoxic and carcinogenic ingredient is likely to be increased, compared to the intake from other sources. We propose that another option would be to apply the TTC defined for genotoxic compounds, of 0.15 µg per person per day corresponding to 0.0025 µg/kg bw/day for a 60 kg person (Kroes *et al.*, 2004). This level reflects a low probability of a lifetime cancer risk greater than one in a million based on linear extrapolation of the TD_{50} values from rodent carcinogenicity studies on 730 structurally related compounds. Noteworthy, for the so-called high potency genotoxic chemicals (*i.e.* aflatoxin-like compounds, N-nitroso-compounds and azoxy-compounds) it is suggested that a TTC should not be considered since compound-specific risk assessment is required for this group (Kroes *et al.*, 2004). At present, there is an EFSA working group on Thresholds of Toxicological Concern (http://www.efsa.europa.eu/en/sc/scwgs.htm) and this working group may give further guidance on the use of the TTC approach.

Prioritization of botanicals of possible concern

The guidance document published by the Scientific Committee of EFSA (EFSA, 2009b) provides a set of criteria to help prioritize the safety assessment of botanical compounds which are in use. The document states that; "Priority should be given to botanicals and botanical preparations: (1) known to have an established history of food use and that have been identified to contain significant levels of substances of concern, (2) that are not allowed/recommended for food use in some European countries, but which are still in use in some other EU countries, particularly when the intended use levels in food are known or expected to be high, (3) for which some adverse health effects have been reported, either anecdotally, or on the basis of case reports of intoxication, epidemiological data or any toxicity data from livestock animals or experimental

animals, or for botanicals that closely resemble botanicals which are known to have caused toxic effects, (4) for which consumption has significantly increased during recent years in Member States, (5) for which there are both limited history of use and toxicity data available, and for which the intended use levels are expected to be relatively high (*e.g.* high interest to the food industry). Botanical ingredients that are reported to have a low toxic potential, and for which the intended intake/exposure levels are within the range of intake levels resulting from the European Member States average diet would be given a low priority."

EFSA has also compiled the available information on a large number of botanicals which have been reported to contain substances that may be of health concern when specific parts are used and/or inadequate processing procedures are used in making botanical extracts and/or botanical products. The resulting compendium can be found on Internet (EFSA, 2009c) and will be regularly updated.

Examples of PFS compounds of possible concern because of their genotoxic and carcinogenic properties

Botanicals and/or botanical preparations may contain compounds of concern because of their genotoxic and carcinogenic properties like, for example, compounds belonging to the groups of pyrrolizidine alkaloids, alkenylbenzenes or aristolochic acids.

Pyrrolizidine alkaloids (Figure 1) are converted by cytochromes P450 to pyrrolic dehydro-alkaloid metabolites that alkylate DNA and other macromolecules, causing liver cell necrosis and liver cancer (Rietjens *et al.*, 2005; WHO, 1988). The use of toxic pyrrolizidine alkaloid-containing botanicals as food or food products is restricted in several countries around the world. Nevertheless, there is no consensus in these regulations. South Africa, the UK, Belgium, Australia and New Zealand restricted the internal use of comfrey and products derived from this botanical and also completely prohibited the use of several other toxic pyrrolizidine alkaloid-containing plants in food such as *Senecio spp.*, *Symhytym spp.*, *Crotalaria spp.*, and *Heliotropium spp.* (Koninklijk besluit, 1997; The Medicines, 1994; South African department of Health, 2002; Australia New Zealand Food Standards, 2009).

The use of pyrrolizidine alkaloids in PFS is still allowed in The Netherlands, as well as in Germany, although limitations have been adopted in these countries with regard to the exposure to toxic pyrrolizidine

alkaloids resulting from the use of herbal supplements (Bundesrepublik Deutschland, 1992; Warenwet Kruidenpreparaten, 2001). Following the Dutch 'Warenwetbesluit Kruidenpreparaten' as adopted in January 2001, the total content of pyrrolizidine alkaloids present in botanical supplements may not exceed 1 µg/kg. In Germany, the exposure to pyrrolizidine alkaloids may not exceed 0.1 µg/day when pyrrolizidine alkaloids containing botanical products for oral use are consumed for a period longer than 6 weeks (Bundesrepublik Deutschland,1992). When the exposure period does not exceed this limit of 6 weeks, an exposure of 1 µg pyrrolizidine alkaloids per day is allowed resulting from the use of herbal medicines (Bundesrepublik Deutschland, 1992). Furthermore, the US Food and Drug Administration (FDA) raised serious concerns for human health with regard to the use of pyrrolizidine alkaloids, and "the agency strongly recommends that firms marketing a product containing comfrey or another source of pyrrolizidine alkaloids remove the product from the market and alert its customers to immediately stop using the product" (FDA, 2001). Within the same line of reasoning, Health Canada advised the Canadian population not to use any comfrey containing products (HealthCanada, 2003).

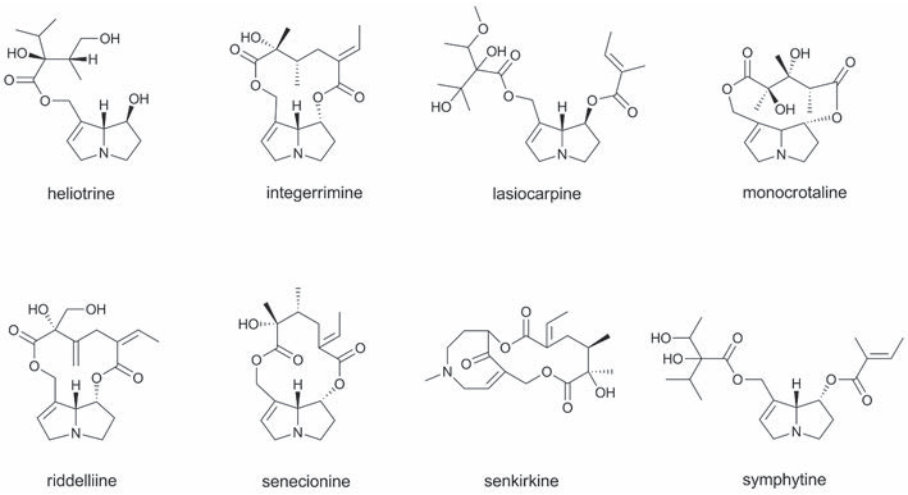

Figure 1. Structural formulas of several pyrrolizidine alkaloids.

Alkenylbenzenes including apiol, β-asarone, elemicin, estragole, methyleugenol, myristicin and safrole (Figure 2) are converted by cytochromes P450 and sulfotransferase (SULT)-mediated biotransformation to genotoxic

and carcinogenic 1'-sulfoxymetabolites that bind to DNA and cause liver cancer (Jeurissen *et al.*, 2007; Phillips *et al.*, 1981; Smith *et al.*, 2002; Wiseman *et al.*, 1985; Wiseman *et al.*, 1987). Although the use of estragole, methyleugenol (Regulation (EC) No 1334/2008 of the European Parliament and of the Council 16 December 2008), safrole and β-asarone (Council Directive 88/388/EEC of 22 June 1988) as pure compounds in food is prohibited within the EU because of their genotoxic and carcinogenic potentials, currently no harmonized restrictions have been made in the EU with regard to the use of alkenylbenzene-containing botanicals in PFS. In countries were no restrictions are applicable, PFS containing high levels of alkenylbenzenes may be on the market.

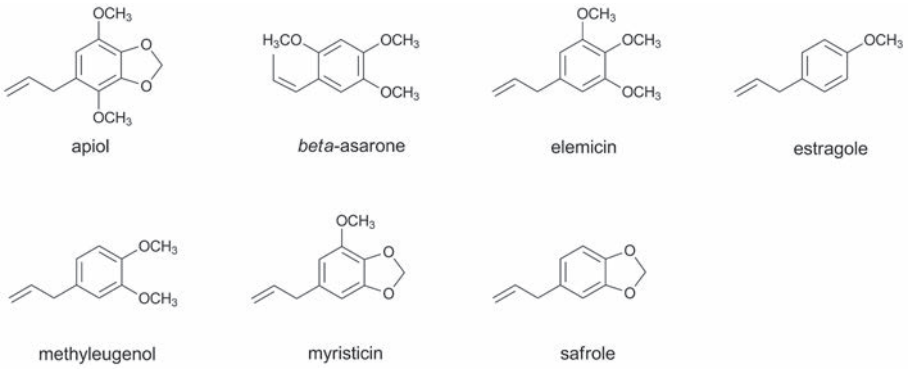

Figure 2. Structural formulas of important alkenylbenzenes

It is also important to stress that despite prohibitions, products containing compounds of concern may still be offered on the market. An example of the occurrence of prohibited compounds in botanical preparations is the presence of aristolochic acids in Chinese herbal preparations used in traditional Chinese medicine. It was previously demonstrated that several Chinese herbal preparations that are sold on the Dutch market still contain aristolochic acids which are prohibited worldwide (Martena *et al.*, 2007). Aristolochic acid I and II (Figure 3) are converted by reductive metabolic activation by cytochromes P450 and/or by other enzymes resulting in formation of reactive nitrenium ion metabolites which cause Chinese Herb Nephropathy and urothelial cancers (Gillerot *et al.*, 2001; Rietjens *et al.*, 2005). In 190 Chinese traditional herbal preparations sampled between 2002 and 2006 on the Dutch market, aristolochic acid I was found in 25 samples up to a concentration of

1676 mg/kg and aristolochic acid II was found in 13 of these samples up to 444 mg/kg (Martena *et al.*, 2007).

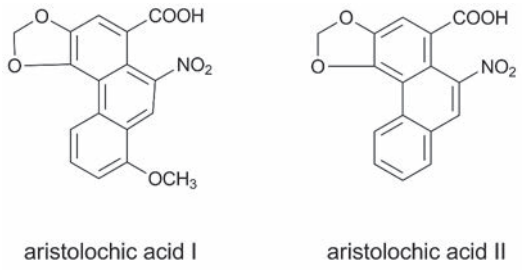

Figure 3. Structural formulas of aristolochic acid I and II.

Examples of PFS compounds of possible concern because of neurotoxicity

Another group of PFS compounds may be of concern because of their neurotoxicity. These PFS compounds include for example ephedrine analogues, anisatin and α-thujone. These compounds appear to interact with one of the neurotransmitter systems.

Ephedrine and ephedrine analogues including pseudo-ephedrine, norephedrine, methylephedrine, and norpseudo-ephedrine may act as adrenalin agonists. Figure 4 presents the structural formulas of these compounds. PFS containing botanicals like *Ephedra sinica*, *Ephedra intermedia*, and *Ephedra equisatine*, also known by their Chinese name 'Ma Huang', contain these compounds and are used for improvement of weight loss and athletic performance (Shekelle *et al.*, 2003). As adrenalin agonists, these compounds produce a sympathomimetic response, characterized by increased heart rhythm, hypertension (elevated blood pressure), and central nervous system stimulation. Also *p*-synephrine (Figure 4) acts as an adrenalin agonist. *p*-Synephrine is the main active principle found in the fruit of several Citrus species including *Citrus aurantum* and *Citrus reticulata*. In traditional Chinese medicine the fruit is also known as 'Chih-shih'. Evaluations concerning preparations containing high amounts of *p*-synephrine concluded that there may be a possible safety concern (Calapai *et al.*, 1999; Jack *et al.*, 2007), and extracts used in many dietary supplements and herbal weight-loss formulas as an alternative to *Ephedra* have concentrations of *p*-synephrine that are much higher than the *p*-synephrine concentrations reported for traditional extracts of the dried fruit or peel (Speijers

et al., 2010). This reflects another important issue to be taken into account when assessing the safety of PFS, *i.e.* that some preparations of a botanical may be marketed containing significantly higher levels of active (toxic) principles than those normally occurring in historical food uses of the same botanical.

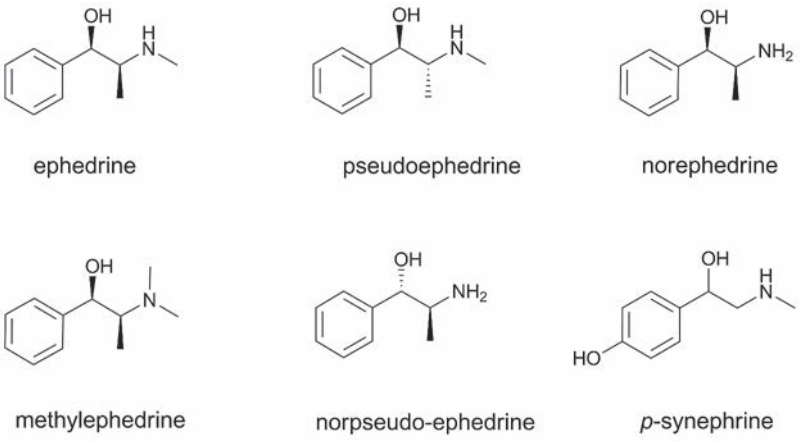

Figure 4. Structural formulas of ephedrine and its analogues pseudoephedrine, norephedrine, methylephedrine, norpseudo-ephedrine and *p*-synephrine.

Furthermore, the position isomer of synephrine found in bitter orange (*Citrus aurantium* L. *ssp. aurantium* L.) peel is *p*-synephrine, not m-synephrine. The presence of any amount of *m*-synephrine, higher amounts of the (+)-*p*-synephrine stereoisomer or higher amounts of octopamine in PFS supposedly containing only extracts of bitter orange should be considered undesirable and suspicious of adulteration, thus strongly suggesting a requirement for a more efficient quality control (Speijers *et al.*, 2010).

Anisatin (Figure 5), the toxic ingredient in Japanese star anise (*Illicium anisatum*) that was accidentally exchanged for the non-toxic Chinese star anise (*Illicium verum*) (Johanns *et al.*, 2002), acts as a non-competitive γ-amino butyric acid (GABA)-antagonist that can cause tonic-clonic insults (Kakemoto *et al.*, 1999).

The terpenoid α-thujone (Figure 5) occurs in the essential oils and parts of the plants of *Artemesia absinthum* (wormwood), *Salvia officinalis* (sage), *Salvia scarea* (clary), *Tanacetum vulgaris* (tansy) and in *Juniperus* and *Cedris spp.*, which may occur in PFS. The mechanism of neurotoxicity of α-thujone has been ascribed to the fact that it blocks the receptors for γ-aminobutyric acid (GABA) (Hold *et al.*, 2000).

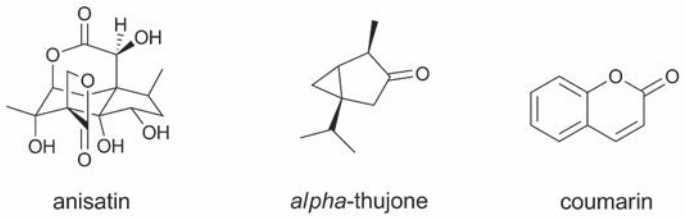

Figure 5. Structural formulas of anisatin, α-thujone and coumarin.

Examples of PFS compounds of possible concern because of other modes of action

PFS compounds of concern because of other modes of action include, for example, kavalactones, cyanogenic glycosides and coumarin.

Kavalactones originate from the rootstock of the kava (*Piper methysticum*) plant. The structural formulas of kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin and desmethoxy-yangonin, which are the main kavalactones found in *Piper methysticum*, are shown in Figure 6. Through their action on the nervous system kavalactones exert sedative, analgesic, anticonvulsant, and muscle relaxant effects. The mechanism of action for this effect may include inhibition of monoamine oxidase (MAO) activity, inhibition of noradrenalin reuptake in the presynaptic neuron, and/or action as a dopamine antagonist (Schelosky *et al.*, 1995; Spollen *et al.*, 1999). The major toxic side effects of kava kava are dermopathy and liver toxicity. The liver toxicity may be related to glutathione depletion and/or quinone formation (Johnson *et al.*, 2003; Rietjens *et al.*, 2005; Whitton *et al.*, 2003). Since 1999, cases of severe hepatic toxicity in people using kava-containing herbal products have been reported in Europe and the United States (Escher *et al.*, 2001; Centers for disease and cancer prevention, 2002), including several cases in which patients required liver transplantation following the use of kava-containing products (Centers for disease and cancer prevention, 2002). As a result, the use of kava kava is now prohibited in PFS and medicinal products in most countries.

Cyanogenic glycosides are present in a number of food plants and seeds and include compounds like amygdalin, dhurrin, linamarin, linustatin, lotaustralin, neolinustatin, prunasin, and taxiphyllin (Figure 7). Products containing high levels of amygdalin are sold via Internet as vitamin B17 or *laetrile,* for cancer treatment, although many countries, including the USA, UK, Singapore and The Netherlands have banned its sale (The Medicines, 1984;

Health Science Authority Singapore, 1985; FDA, 2009). Cyanogenic glycosides are a cause of concern because once ingested they are metabolized to cyanide. Cyanide is released from the cyanogenic glycosides by plant β-glucosidases which come into contact with the cyanogenic glycosides when fresh plant material is macerated as in chewing, or by β-glucosidases present in the gut flora. Cyanide causes toxic effects by binding to cytochrome oxidase, the terminal enzyme in the mitochondrial electron transport chain. By hampering the generation of ATP and oxygen utilization, a histotoxic anoxia is produced.

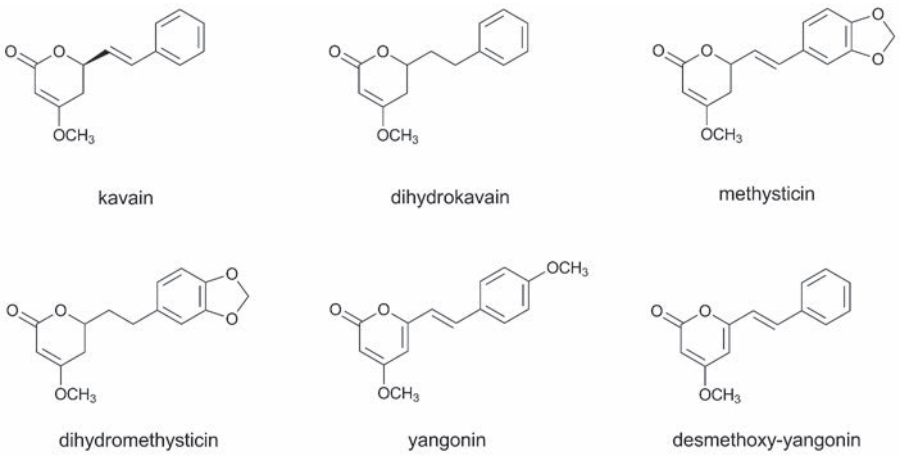

Figure 6. Structural formulas of the main kavalactones found in *Piper methysticum*.

Cinnamon-containing PFS may contain high levels of coumarin (Figure 5), which causes liver damage, and at high dose levels, liver tumors by a non-genotoxic mechanism. Coumarin induces tumors via a mechanism of action that is preceded by toxicity in the target organ, and consequently, a TDI of 0.1 mg/kg bw/day has been defined (EFSA, 2004b). This TDI can be used to judge the safety of not only coumarin-containing food but also of coumarin-containing PFS.

Examples of PFS compounds of possible concern because of their interactions with prescription drugs

PFS may contain compounds interacting with the pharmacokinetics and/or pharmacodynamics of prescription drugs when concomitantly consumed, presenting a potential safety issue. The most common botanical-drug

interactions that have been described involve botanicals like ginkgo biloba (*Ginkgo biloba* L), kava kava (*Piper methysticum*), black cohosh (*Acteae racemosa*), ginseng (*Panax ginseng*) and St. John's wort (*Hypericum perforatum*), with the latter example being studied most thoroughly.

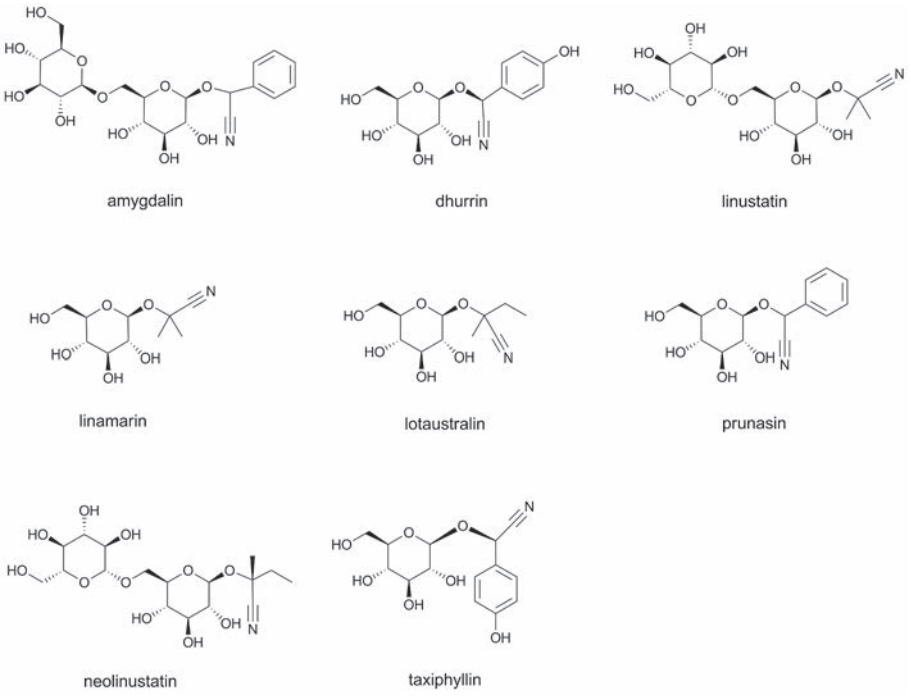

Figure 7. Structural formulas of important cyanogenic glycosides.

St. John's wort is used to treat a variety of diseases such as anxiety, mild to moderate depression, sleeping disorders and obsessive-compulsive disorder. The antidepressant effects of St. John's wort are thought to be caused by its ingredient, hyperforin, inhibiting the synaptosomal uptake of serotonin, norepinephrine and dopamine (Chatterjee *et al.*, 1998). Several interactions with prescribed drugs have been reported for St. John's wort and a number of studies indicated that changes in the activity of specific cytochromes P450 may underlie these botanical-drug interactions (Gurley *et al.*, 2005; Markowitz *et al.*, 2003; Mathijssen *et al.*, 2002; Piscitelli *et al.*, 2000). As a result of the potent inhibition of these metabolizing enzymes, plasma levels of prescribed drugs including alprazolam, irinotecan and indinavir might be decreased

after concurrent use with St. John's wort (Markowitz *et al.*, 2003; Mathijssen *et al.*, 2002; Piscitelli *et al.*, 2000). In 2000, the FDA published a health advisory instructing health care professionals to report potential adverse effects related to the use of St. John's wort in combination with prescription drugs (FDA, 2000).

Most of the current evidence for botanical-drug interactions is based on case reports while experimental data are limited. To date, possible botanical-drug interactions are often not indicated on the label of PFS nor the drug, making it difficult for consumers to make an informed decision. Moreover, interactions are not frequently ascribed to the effects of natural products as these products are often regarded as 'safe'. Therefore, management of botanical-drug interactions should take an important position in promoting the safe use of PFS underlining awareness for possible interactions between PFS and prescribed drugs. Currently, a database is being prepared providing an overview of the existing data, but also producing new data, including the interactions between botanicals and prescribed drugs (www.plantlibra.eu/web) to assist in monitoring the safety of PFS and facilitating information to consumers and health care providers to create more awareness on possible botanical-drug interactions.

Matrix effects modulating toxicity

When evaluating the safety of PFS compounds, it is important to consider that the kinetics and/or the toxicity of a PFS ingredient could be modified by the matrix in which it is present. This could result in the toxicity being unchanged, reduced or even increased.

An example of increased toxicity can be found in the fact that epigallocatechin gallate (EGCG) given in a green tea extract to rats appears to be eliminated less readily from the body and to have a higher toxicity than when given as a pure compound (Chen *et al.*, 1997; Johnson *et al.*, 1999). An example of decreased toxicity is presented by the reduction of the level of DNA binding of the proximate carcinogenic metabolite 1′-hydroxyestragole by a methanolic basil extract (Alhusainy *et al.*, 2010; Jeurissen *et al.*, 2008). This inhibition by the basil extract was shown to occur at the level of the SULT-mediated bioactivation of 1′-hydroxyestragole to 1′-sulfoxyestragole and to be mediated by the flavonoid nevadensin present in basil at a high level (Alhusainy *et al.*, 2010; Jeurissen *et al.*, 2008).

Whenever a matrix effect is advocated to support the safety of specific levels of PFS compounds (*e.g.* that data from a pure compound may overestimate effects of the compound in the botanical matrix), data should be provided to demonstrate the occurrence of the matrix effect of the preparation and its magnitude. It is also important to realize that when a matrix effect is demonstrated for an intact botanical, the matrix effect for the PFS may be different. Thus, the matrix effect should be taken into account in the safety assessment of PFS compounds on a case-by-case basis.

Conclusions

PFS on the market may contain active compounds that are of concern. For several compounds regulatory authorities are aware of the problems encountered and have taken or are considering appropriate regulatory actions to protect the public. These regulatory actions may vary from setting TDIs (such as, for example, for coumarin and trans-anethole), setting restrictions in use (such as for estragole, methyleugenol, safrole and β-asarone), informing the public to be cautious and aware of possible adverse side effects (as for kava kava derived kavalactones), or taking specific plant varieties and/or their compounds from the market (such as plants containing aristolochic acids, toxic pyrrolizidine alkaloids and kavalactones).

However, at present a general framework for the safety assessment of PFS is not in place and safety assessments are performed on a national and *ad-hoc* basis, often following incidents. Besides, even when regulatory measures are in place, PFS containing compounds of concern may still be offered for sale on Internet, such as in case of the cyanogenic glycoside amygdalin (vitamin B17) or PFS containing high levels of alkenylbenzenes. Altogether it is clear that 'natural' does not equal 'safe' and PFS may contain compounds of concern at levels far above those found in the regular diet. In addition, the traditional use of a PFS compound as an herb or tea does not guarantee its safety when used as a supplement. This points at a need for stricter regulation and control of botanical-containing products, especially given their expanding market volume.

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Chapter 3

Levels of genotoxic and carcinogenic compounds in plant food supplements and associated risk assessment

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Abstract

The present study describes the selection, analysis and risk assessment of genotoxic and carcinogenic compounds of botanicals and botanical preparations which can be found in plant food supplements (PFS). First an inventory was made of botanical compounds that are of possible concern for human health because of their genotoxic and/or carcinogenic properties. In total, 30 botanical compounds were selected and subsequently judged for their actual genotoxic and/or carcinogenic potential. Among the 30 compounds considered, 18 compounds were judged to be both genotoxic and carcinogenic. Interestingly, the majority of these compounds belong to the group of alkenylbenzenes or unsaturated pyrrolizidine alkaloids. Subsequently, based on available carcinogenicity data and estimated daily human exposure that was determined focusing on the intake from PFS, the Margin of Exposure (MOE) was calculated for the alkenylbenzenes estragole, methyleugenol, safrole and β-asarone. Calculating the MOE values for intake estimates of these alkenylbenzenes from PFS resulted in MOE values that were generally lower than 10,000 and often lower than 100. In some cases the MOE was even below 10 meaning that the estimated daily intake is in the range of dose levels causing malignant tumors in experimental animals. This result indicates that the use of PFS containing the genotoxic carcinogens estragole, methyleugenol, safrole or β-asarone might raise a potential concern for human health and would be of high priority for risk management.

Introduction

Herbal products and ingredients such as herbal teas, food flavors and food supplements have been, and still are, widely used in Western diets. Currently, there is a growing interest in plant food supplements (PFS), which are suggested to have the ability to maintain and promote health or reduce the risk factors for diseases. In Europe, PFS are easily accessible since they can be bought in supermarkets, health-food shops or pharmacies where they are sold over the counter at relatively low costs. Moreover, PFS generally have a high acceptance by consumers who often consider that 'natural' equals 'safe'. However, this reasoning should be considered with care since several botanicals are known to contain toxic or even genotoxic and carcinogenic compounds (Rietjens *et al.*, 2008). Examples of genotoxic and carcinogenic botanical ingredients are the alkenylbenzenes estragole, methyleugenol and safrole, which are present in a wide range of botanicals including basil, nutmeg, tarragon and fennel (SCF, 2001a, 2001b, 2002a). In the past, the Scientific Committee on Food has suggested restrictions in the use of these alkenylbenzenes because of their genotoxic and carcinogenic properties (SCF, 2001a, 2001b, 2002a).

In 2004, the Scientific Committee of the European Food Safety Authority (EFSA) presented a discussion paper describing general concerns relating to the quality and safety of botanicals and botanical preparations such as PFS and related products (EFSA, 2004a). In line with the existing concerns with respect to the use of botanicals, a European collaborative project under the Seventh Framework Programme was recently started. This project, known as PlantLIBRA (PLANT food supplements: Levels of Intake, Benefits and Risk Assessment), aims to encourage the safe use of food supplements containing botanicals or botanical ingredients by increasing science-based decision making by regulators as well as food chain operators. Within PlantLIBRA, different methodologies for risk assessment of botanicals and botanical ingredients used as PFS will be defined, validated and disseminated in order to enlarge the knowledge on PFS and ensure a safe use of PFS.

In general, assessment of the risk for human health from genotoxic and carcinogenic compounds occurring in food, the presence of which cannot be easily avoided, is accompanied with difficulties (EFSA, 2005). Currently, an international scientific agreement is lacking regarding the best strategy for the risk assessment of genotoxic and carcinogenic compounds

and a variety of approaches is used by different regulatory and advisory bodies (EFSA, 2005). While some offer qualitative advice, others present quantitative approaches with respect to the risk assessment of genotoxic carcinogens (Barlow and Schlatter, 2010; Van den Berg *et al.*, 2011).

Amongst the different available qualitative and quantitative approaches, the use of a Margin of Exposure (MOE) approach was recommended by expert groups of EFSA, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the International Life Sciences Institute (ILSI) (JECFA, 2005; Barlow *et al.*, 2006; EFSA, 2005; O'Brien *et al.*, 2006). The MOE is a dimensionless ratio based on a reference point obtained from epidemiologic or experimental data on tumor incidence which is divided by the estimated daily intake in humans (EFSA, 2005). The selected reference point corresponds to a dose level causing a low though quantifiable incidence of tumors (EFSA, 2005). Different approaches have been proposed to select an appropriate reference point from the dose-response-curve, of which the use of BenchMark Dose (BMD) modeling is the preferred approach (Barlow *et al.*, 2006). The BMD approach makes use of different mathematical models for the analysis of the observed carcinogenicity data (US EPA, 1995). Making use of this approach, a dose can be estimated that causes a predefined cancer response, known as the BenchMark Response (BMR) (US EPA, 1995). Generally, a dose that gives 1%, 5% or 10% extra tumor incidence compared to the background level is chosen (US EPA, 1995). However, it is indicated that the use of a dose giving 10% extra cancer risk above the background level (BMD_{10}) is accompanied with the least uncertainties and is therefore preferred (Barlow *et al.*, 2006). The $BMDL_{10}$, the 95% lower confidence bound of the BMD_{10}, is frequently used as a reference point to calculate the MOE. This reference point takes into account uncertainties in the experimental data (EFSA, 2005). Moreover, the use of the BMDL instead of the BMD will assure with 95% confidence that the value of the BMR will not go beyond the predefined value of, for example, 10% (EFSA, 2005).

An advantage of the use of the MOE approach is that it sets a basis for priority setting that can be used by risk managers. An MOE >10,000 is considered as a low priority for risk management actions and would be of low concern from a public health point of view (EFSA, 2005). The value of 10,000 was defined based on considering various factors that cause uncertainties in the MOE including (1) a factor 100 for species differences and human variability in toxico-kinetics and toxico-dynamics, (2) a factor 10 for inter-

individual human variability in cell cycle control and DNA repair, and (3) a factor 10 for the reason that the $BMDL_{10}$ when used as a reference point is not identical to a No Observed Adverse Effect Level (NOAEL) (EFSA, 2005).

In the presented chapter, the MOE approach was applied to a series of compounds known to be present in botanicals and botanical preparations including especially PFS. To this end, first an inventory was made of botanical ingredients that are of possible concern for human health because of their genotoxic and/or carcinogenic properties. In a next step, cancer data for a selected number of these compounds were used to derive a reference point preferably a $BMDL_{10}$. Finally, the MOE values for these ingredients were calculated based on the intake estimates resulting from chemical analysis of selected PFS as well as their proposed uses. The outcomes generally pointed at a high priority for risk management of PFS containing the alkenylbenzenes estragole, methyleugenol, safrole and β-asarone.

Materials and methods

Selection of botanical ingredients

An inventory was made of botanical ingredients that are of possible concern for human health because of their genotoxic and/or carcinogenic properties. Selection of these botanical ingredients included three strategies. First, compounds indicated to be genotoxic or carcinogenic in the EFSA compendium (EFSA, 2009a) were selected as potential candidates for risk assessment using the MOE concept. The EFSA compendium summarizes a large number of botanicals and botanical ingredients containing substances which might be of possible concern for human health. Second, the selection of botanical ingredients relevant for risk assessment using the MOE concept was based on data obtained from 2-year studies performed by the National Toxicology Program (NTP). To be eligible for selection, evidence of genotoxic and/or carcinogenic activities from these NTP studies was considered as a requirement. Furthermore, compounds tested positive for genotoxicity or carcinogenicity by the NTP were only selected if they were listed in the EFSA compendium, because that ascertained their occurrence in botanicals or botanical preparations, and they were selected even when the EFSA compendium did not list them as genotoxic or carcinogenic. Finally, as a third strategy, this list of selected compounds was completed by

including compounds of known concerns because of their genotoxic and/or carcinogenic characteristics, but not (yet) included in the EFSA compendium or not (yet) tested by NTP. These included aristolochic acids as well as a number of botanical ingredients classified as alkenylbenzenes or unsaturated pyrrolizidine alkaloids. For alkenylbenzenes and unsaturated pyrrolizidine alkaloids, priority for risk assessment has been indicated by the partners collaborating within the PlantLIBRA project. Moreover, concerns for human health were raised by the EU with respect to the occurrence of unsaturated pyrrolizidine alkaloids in plants (CCCF, 2010), and the alkenylbenzenes including estragole, methyleugenol, safrole, elemicin, myristicin, and apiol have been given priority by JECFA for evaluation of their risks resulting from consumption of botanicals and botanical preparations (JECFA, 2009).

Selection of carcinogenicity data relevant for benchmark modeling

In general, the calculation of $BMD(L)_{10}$ values was based on rodent carcinogenicity bioassays preferably accomplished under current guidelines. For this purpose, several studies implemented by the NTP were used for modeling. These studies often included three exposure concentrations administered to rats and mice of both sexes (50 animals per group) during a lifetime (*i.e.* 2 years). However, not for all selected botanical compounds such studies were available. Therefore, studies were included that were not performed according to current standards. Selection of studies suitable for $BMDL_{10}$ modeling included the requirement that studies were conducted using a control group and two or more exposure levels. When studies were completed in a period shorter than the standard lifespan of 2 years, an adjustment for the length of treatment and the observation period was made. This was done following the method reported by the European Chemical Agency (ECHA, 2008) and previously applied in the MOE approach as performed by an Expert Panel of ILSI (Benford *et al.*, 2010). This adjustment allows a correction for the underestimation in tumor incidence that would occur when an experiment is discontinued before the standard lifespan (Benford *et al.*, 2010). For this purpose, a correction of ($w1$/104) x ($w2$/104) was applied to the dose in which $w1$ represents the duration of dosing in weeks and $w2$ reflects the period of observation in weeks. For example, if rodents were treated for 52 weeks in an experiment proceeding for 80 weeks, the dose (d) was corrected following (52/104) x (80/104) x d. In addition, the dose levels were also corrected for the number of doses per week by adjusting the dose to equal a daily administration

of the test compound keeping the total weekly dose constant. Thus, if the test compound was administered for 5 times a week, a correction factor of 5/7 was applied to the dose. When studies reported the dose administered in mg/kg diet, a daily food consumption of 130 g diet/kg bw/day, 120 g diet/kg bw/day and 40 g/kg bw/day for female and male mice and male rats, respectively, was used to calculate the dose in mg/kg bw/day (Gold *et al.*, 1984). Only organ-specific carcinogenicity data were included for modeling with the emphasis on malignant neoplasms. Data on benign tumors were precluded since these neoplastic lesions do not express the same characteristics as malignant tumors that are capable of spreading by invading surrounding tissues or by metastasizing. Although malignant tumors are able of metastasizing, only primary tumors were taken into consideration for modeling. When different malignant tumors were observed in a cancer bioassay, the tumor-site with the highest incidence at comparable dose levels was chosen. Data for males and females were not combined. Other criteria for selecting carcinogenicity data were: the occurrence of a treatment-related dose-response relationship, oral administration of the compound (*i.e.* through the diet, drinking water or by gavage) and a tumor type that is of relevance for humans. The number of animals in a group is also needed for adequate BMD modeling and equaled the number of animals that were examined microscopically.

Benchmark dose modeling

$BMD(L)_{10}$ values were obtained by fitting the carcinogenicity data to a number of different mathematical models using EPA BMDS software, version 2.1.2. The following models were used: Gamma, Logistic, LogLogistic Probit, LogProbit, Multistage, Weibull and the Quantal-linear model. BMDS software was applied using default settings for model restrictions, risk type (extra), confidence level (95%), and BMR (10%). The $BMD(L)_{10}$ values derived from the different fitted models were only accepted if the fit of the selected model was not of poorer quality than that of the so-called full model representing a perfect fit to the dose-response data. For this purpose, the log-likelihood ratio test was applied in order to measure the goodness-of-fit of the chosen models. The acceptance of a model was based on comparing the critical differences in the log-likelihood values between the full and the fitted model as reported by Slob (2002). The evaluation of these critical differences was based on the discrepancy in the amount of parameters used by the full and the selected model (*i.e.* the degrees

of freedom) (Slob, 2002). Exceeding the critical difference implied that the model with the highest number of parameters (*i.e.* the full model) had the best fit (Slob, 2002) and consequently the selected model was not accepted for BMD(L) calculation. Moreover, the p-value representing the goodness-of-fit of the selected dose was taken into account with a value below 0.05 resulting in model rejection.

Method for determination of intake levels

Theoretical estimations

Intake estimates resulting from use of PFS were not available in the literature and were made in the present study. PFS were selected that are offered for sale on the market via Internet and/or health shops and that consist of botanicals known to contain different quantities of one or more of the compounds of interest. Exposure estimates were made taking into consideration the content of the compound of interest in the plant as reported in literature. When such data were not available, the amount of essential oil that is reported to be present in the plant and the amount of the compound in the essential oil were used to estimate the ingredient level in the plant. When the amount of compound (in mg) present in a particular botanical was determined, the daily exposure to the compound of interest (in mg/kg bw/day) was calculated based on the daily intake of that botanical resulting from the use of PFS at the dose recommended by the respective manufacturer. In general, data of PFS prepared from one botanical were used. When a PFS consisted of two or more botanicals, the relative amount of the botanical of interest present in the PFS as indicated by the manufacturer was used to correct the daily dose of the PFS. Since manufacturing methods for preparing the PFS from the original plant were not available, it was assumed that the PFS equals the botanical as such.

Quantitative detection of alkenylbenzenes in PFS

To determine if the theoretical levels represent the actual levels of the genotoxic carcinogens of interest in commercially available PFS, a quantitative analysis was performed. To this end, the content of the alkenylbenzenes of interest in 28 different PFS containing basil, cinnamon, sassafras, nutmeg, fennel or calamus was measured. Exposure estimates were made based on the content of the compound of interest in the PFS as measured together with the weight of the capsules and the recommended daily dose as indicated on the label.

PFS and chemicals

PFS were selected that are widely offered for sale on the market via Internet and/or health shops and consist of botanicals known to contain different quantities of one or more of the compounds of interest. 28 PFS were purchased via Internet, local Dutch and Italian health shops and a Dutch pharmacy. Methyleugenol (purity 99%), safrole (purity >97%), β-asarone (purity 70%), methanol (HPLC supra gradient) and acetonitrile (ULC/MS gradient) were supplied by Sigma-Aldrich (Steinheim, Germany). Estragole (purity 98%) was purchased from Acros Organics (Geel, Belgium). Nanopure water was obtained from a Barnstead Nanopure Type I ultrapure water system.

Sample preparation

All 28 alkenylbenzene-containing PFS were extracted using methanol based on the method described by Gursale *et al.* (2010) with minor modifications. To prepare these methanolic extracts, 1 g of powdered PFS material was suspended in 25 mL methanol followed by ultrasonic extraction for 15 min at room temperature. An aliquot of each extract solution was filtered through a 0.45 µm cellulose acetate membrane filter (VWR International) and stored at -20˚C until Ultra Performance Liquid Chromatography (UPLC)-analysis.

UPLC analysis

Before UPLC analysis, aliquots of the extract solutions were diluted in nanopure water (1:100 v/v). However, when the level of the analyte was found to be outside the acceptable level for quantification, the final dilution factor was adjusted. Accordingly, extract solutions were diluted in a range of 2 to 1000 fold depending on the quantity of estragole, methyleugenol, safrole or β-asarone present in the sample. After centrifugation at 16,000*g* for 5 min, 3.5 µL of each sample was subjected to UPLC analysis (*n*=3).

UPLC analysis was performed on a Waters ACQUITY UPLC H-Class system connected to an ACQUITY UPLC photodiode array detector and a quaternary solvent manager. Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 1.7 µm column, 2.1 x 50 mm. The column was thermostated at 30˚C and the sample manager was set at 10˚C. The gradient was made with nanopure water and acetonitrile. The mobile phase started with 23% acetonitrile for 10 min after which the acetonitrile percentage was increased to 58% in 3 min and further increased to 80% in 1 min at which it was kept for 30 seconds. Starting conditions

were reached within the next 30 seconds and retained for another 30 seconds. During the whole run, the flow rate was set at 0.6 mL/min. Under these conditions, the retention times for methyleugenol, β-asarone, safrole and estragole were 9.0, 9.6, 12.3, and 12.7 min, respectively. The levels of the alkenylbenzenes of interest were quantified by comparing the peak areas to the calibration curves of these compounds derived from commercially available standards.

For nutmeg-containing PFS, a different method was used to separate the structurally related alkenylbenzenes elemicin and methyleugenol which both occur in nutmeg (EFSA, 2009a). The gradient was made with nanopure water and acetonitrile. The acetonitrile percentage was increased from 32% to 35% in 3.5 min and further increased to 60% in 1.5 min and to 80% in 30 seconds at which it was kept for 30 seconds. Starting conditions were obtained in 30 seconds and the system was equilibrated for another 30 seconds. Elution was at a flow rate of 0.6 mL/min and the column temperature and sample temperature were set at 22˚C and 10˚C respectively. Under these conditions, the retention times were 2.9, 3.1 and 4.8 min for elemicin, methyleugenol and safrole in the order given.

Accuracy

The accuracy of the developed method was assessed by means of a recovery study. Pure standards of commercially available estragole, methyleugenol, safrole or β-asarone were added in different quantities (final concentrations of 250 µM, 500 µM and 750 µM) to 1 g powdered PFS consisting of fennel, nutmeg or calamus dissolved in a final volume of 25 mL methanol. Samples were prepared and analyzed by UPLC as described above (n=6). The average percentage recoveries were found to equal 87.6 ± 4.9% for estragole, 89.7 ± 8.1% for methyleugenol, 98.9 ± 15.0% for safrole and for β-asarone this was 81.1 ± 7.7%. Based on these outcomes the levels of the alkenylbenzenes detected in the different PFS were corrected for sample recovery.

Calculation of the Margin of Exposure

The full range of $BMDL_{10}$ values (*i.e.* lowest to highest $BMDL_{10}$ value) was compared with the estimated daily intakes of the alkenylbenzenes of interest resulting from the use of PFS to derive an MOE range. MOE values are rounded to a single significant figure.

Results

Selection of compounds

First an inventory was made of botanical ingredients that are of possible concern for human health because of their genotoxic and/or carcinogenic properties. As described in the Materials and Method section, the selection of these botanical ingredients was performed based on (1) the EFSA compendium, (2) the available NTP studies on botanical ingredients and (3) knowledge from partners within the PlantLIBRA consortium. Table 1 provides an overview of the 30 compounds thus selected that might be of concern because of their genotoxic and/or carcinogenic potentials. Most of these compounds occur in a variety of botanicals several of which, such as fennel, are commonly used in PFS. Table 1 also lists the overall judgment on the genotoxicity and carcinogenicity of the selected compounds as based on available literature data. This reveals that not all 30 selected compounds appear to be both genotoxic and carcinogenic. For the majority of the selected botanical ingredients carcinogenicity has been reported or suspected. However, for several compounds, such as for coumarin and trans-anethole, a genotoxic mode of action is not indicated. 18 Of the 30 selected compounds, appeared to be both genotoxic and carcinogenic. These compounds include apiol, aristolochic acid (I and II), elemicin, estragole, heliotrine, lasiocarpine, lucidin, methyleugenol, monocrotaline, myristicin, ptaquiloside, reserpine, riddelliine, safrole, senecionine and symphytine. Although for β-asarone the genotoxicity data are equivocal, EFSA previously concluded that when the carcinogenic mode of action of a certain compound is not identified, a genotoxic mode of action will usually be assumed (EFSA, 2005). However, EFSA mentioned that this presumption of genotoxicity is based on a lack of other information and should not be interpreted as the actual mode of action. Interestingly, most of the selected botanical ingredients that are both genotoxic and carcinogenic belong to the groups of the alkenylbenzenes or the unsaturated pyrrolizidine alkaloids, with the exception of aristolochic acid (I and II), lucidin, reserpine and ptaquiloside. For 8 of the 18 compounds that appeared to be both genotoxic and carcinogenic, data on carcinogenicity are available from which a reference point (*e.g.* $BMDL_{10}$ or T25) could be obtained, allowing the use of the MOE concept. These compounds included reserpine, the alkenylbenzenes estragole, methyleugenol, safrole and β-asarone and the unsaturated pyrrolizidine alkaloids riddelliine, lasiocarpine and monocrotaline.

Given that the use of unsaturated pyrrolizidine alkaloids in food, including PFS, is already regulated and not allowed, in the present study further emphasis was placed on the risk assessment of PFS containing the alkenylbenzenes estragole, methyleugenol, safrole and/or β-asarone.

Carcinogenicity data and $BMDL_{10}$

Table 2 gives an overview of the carcinogenicity data describing the incidences of hepatocellular carcinomas in rodents exposed to estragole, methyleugenol or safrole. In the same table, the results of an unpublished study referred to by JECFA (1981) is given on the incidences of leiomyosarcomas of the small intestines of male rats exposed to increasing concentrations of β-asarone. Table 3 presents the results of BMD analysis of these data (for details see the Supplementary Materials Tables S1-S4). For estragole, the $BMDL_{10}$ value varies between 3.3 and 6.5 mg/kg bw/day. For methyleugenol, $BMDL_{10}$ values ranging between 15.3 and 34.0 mg/kg bw/day were found for male rats whereas $BMDL_{10}$ values of 48.8-73.6 mg/kg bw/day were found for female rats. The $BMDL_{10}$ values range between 1.9 and 5.1 mg/kg bw/day for safrole and between 9.6 and 21.5 mg/kg bw/day for β-asarone.

Exposure data

Intake estimates of alkenylbenzenes from consumption of PFS

Characteristics of the PFS for which intake estimates were made are presented in Table 4. Table 5 presents estimated daily exposure to estragole, methyleugenol, safrole or β-asarone from the use of PFS. These estimates were based on PFS available on the market and known to contain different quantities of botanicals that contain one or more of the alkenylbenzenes of interest. Using the suggested dose of PFS intake as recommended by the manufacturer of the PFS and the estimated alkenylbenzene level in the PFS, daily intake estimates of the alkenylbenzenes resulting from use of the various PFS were obtained. The alkenylbenzene level in the PFS was calculated based on the percentage of the alkenylbenzene present in the essential oil, the percentage of the essential oil in the plant and the amount of the plant in the PFS. Thus, assuming a dose of 230-690 mg basil supplement, 0.11-85% of estragole present in the essential oil of sweet basil (EFSA, 2009a; Zheljazkov *et al.*, 2008) and 0.11-1.9% of essential oil in this plant (Zheljazkov *et al.*, 2008), the estimated daily intake of estragole would amount to 0.005-186 μg/kg bw/day for a 60 kg person considering the full range of data.

Table 1. Overview of botanical ingredients with genotoxic and/or carcinogenic characteristics that have a possible risk from the public health point of view.

Compound	Group	Carcinogenic	Genotoxic	Natural Occurrence	Remarks/ tumor type	Reference
trans-Anethole	Alkenylbenzene	Yes	No	*Illicium verum* Hook.f., *Myrrhis odorata* (L.) Sc.	t-Anethole was found to be carcinogenic according to the Expert Panel of FEMA. This conclusion is based on a 2-years study in which a significant increase in the incidence of hepatocellular carcinomas, secondary to hepatotoxicity, was found in female rats. Based on several in vitro and in vivo experiments, the Expert Panel concluded that t-anethole is not genotoxic.	(Newberne *et al.*, 1999)
Apiol	Alkenylbenzene	Probably*	Yes	*Petroselinum crispum* (Mill.) A.W.Hill	Both dill apiol and parsley apiol did not initiate a significant increase of hepatic tumors. DNA-adducts were detected in HepG2 cells and in vivo but these effects were smaller than was seen for the structurally related compounds estragole and safrole.	(Miller *et al.*, 1983; Philips *et al.*, 1984; Randerath *et al.*, 1985; Zhou *et al.*, 2007)
β-Asarone	Alkenylbenzene	Yes	Equivocal	*Acorus calamus* L., *Asarum europaeum* L.	Leiomyosarcomas were formed in the small intestine of male rats upon administration of β-asarone during 2-years. Moreover, hepatomas were observed after injections of β-asarone to pre-weanling mice. In vitro genotoxicity tests gave equivocal results. However, the EU-SCF concluded that these in vitro genotoxic potentials of β-asarone should not be ignored.	(SCF, 2002b)
Aristolochic acid I and II	Nitro-phenanthrene carboxylic acid	Yes	Yes	*Aristolochia* spp., *Sinomenium acutum* (Thunb.) Rehder & E.H.Wilson and some plants of the species *Diploclisia*	A mixture of aristolochic acids I and II was indicated to be carcinogenic in rats, mice and rabbits. Moreover, urothelial cancers have been observed in patients suffering from aristolochic acid nephropathy. Aristolochic acid I and II are genotoxic mutagens that form DNA-adducts.	(Arlt *et al.*, 2002)

Table 1 (continued). Overview of botanical ingredients with genotoxic and/or carcinogenic characteristics that have a possible risk from the public health point of view.

Compound	Group	Carcinogenic	Genotoxic	Natural Occurrence	Remarks/ tumor type	Reference
Citral	Monoterpene aldehyde	Equivocal	No	*Ocimum gratissimum* L.	Based on a 2-year NTP study, no evidence was found for carcinogenic activity of citral in rats and male mice. However, treatment-related increased incidences of malignant lymphomas were observed in female mice. Several in vitro as well as in vivo tests for genotoxicity gave negative results for citral. Nevertheless, positive results were found in the sister chromatid exchange assay. However, EFSA concluded that the genotoxicity is not of concern.	(NTP, 2003a; EFSA, 2009b)
Coumarin	Benzopyrone	Yes	No	Coumarin occurs in a variety of plants *e.g. Cinnamomum cassia* (Nees) Blume, *Galium odoratum* (L.), *Melittis melissophyllum* L.	Coumarin is a carcinogen in rats causing adenomas and carcinomas in the liver and bile ducts as well as adenomas of the kidneys. In mice, exposure to coumarin resulted in adenomas of the liver and both adenomas and carcinomas of the lung. Recently, EFSA concluded that coumarin is not genotoxic in vivo.	(EFSA, 2004; NTP, 1993a; SCF, 1994; SCF, 1999)
Curcumin	Polyphenol	No	Equivocal	*Curcuma longa* L.	A significant increase of benign neoplastic lesions was observed for rats and mice in a long-term NTP study. However, malignant tumors were not significantly increased compared to the control groups. Moreover, there was a lack of dose-dependent effects as well as consistency across sexes and/or species. Previously, it was concluded by JECFA that curcumin was not genotoxic. However, new in vitro and in vivo studies gave positive results (*e.g.* DNA adducts and chromosomal aberrations). According to EFSA 'the available in vivo genotoxicity studies were insufficient to eliminate the concerns regarding genotoxicity'. Nevertheless, there is no concern over carcinogenicity.	(NTP, 1993b; EFSA, 2010)

Elemicin	Alkenylbenzene	Probably*	Yes	Elemicin is present in several plants *e.g. Petroselinum crispum* (Mill.) A.W.Hill, Sassafras *albidum* (Nutt.) Nees.	No significant increase in the formation of hepatic tumors was found upon administration of elemicin or its 1'-hydroxymetabolite to male mice. However, 1'-hydroxyelemicin had hepatocarcinogenic activities at high doses tested. Elemicin was found positive in a DNA-binding assay as well as an UDS assay. However, DNA-binding was not as potent as was found for structurally related carcinogenic compounds *e.g.* estragole.	(Hasheminejad and Caldwell, 1994; Miller *et al.*, 1983; Philips *et al.*, 1984; Randerath *et al.*, 1985; Wiseman *et al.*, 1987)
Estragole	Alkenylbenzene	Yes	Yes	Estragole occurs in a variety of plants *e.g. Foeniculum vulgare* Mill., *Illicium verum* Hook.f., *Ocimum basilicum* L.	Indicated in the EFSA compendium as 'genotoxic and carcinogenic in rodents'.	(EFSA, 2009a)
Eugenol	Alkenylbenzene	No	Equivocal	*Hoslundia opposita* Vahl, *Laurus nobilis* L, *Lippia junelliana* (Moldenke) Tronc., *Syzygium aromaticum* (L.) Merr.& L.M.Perry	A 2-year NTP-study showed no significantly increased incidences of treatment-related malignant liver tumors compared to background incidences in rats while equivocal results were obtained for mice. In a study of Miller *et al.* (1983) no hepatocarcinogenic activity was shown in male mice. A number of in vitro assays indicated the genotoxicity of eugenol. However, according to EFSA, this genotoxicity might be the result of oxidative DNA damage with modest evidence for genotoxic effects in the in vivo situation.	(EFSA, 2009c; NTP, 1983)

Table 1 (continued). Overview of botanical ingredients with genotoxic and/or carcinogenic characteristics that have a possible risk from the public health point of view.

Compound	Group	Carcinogenic	Genotoxic	Natural Occurrence	Remarks/ tumor type	Reference
Heliotrine	Pyrrolizidine alkaloid	Yes	Yes	*Heliotropium* spp.	In a limited study in male rats, increases of pancreatic islet-cell tumors, transitory cell papilomas of the urinary bladder and intestinal testicular tumors were found following administration of heliotrine in the absence or presence of nicotinamide. One rat, treated with heliotrine only, developed an adenoma of the pancreatic islet cells. Several in vitro and in vivo tests indicated the genotoxic potentials of heliotrine (*e.g.* DNA-adduct formation and chromosomal aberrations).	(Chen *et al.*, 2010; Fu *et al.*, 2004; Schoental, 1975)
Integerrimine	Pyrrolizidine alkaloid	Unknown	Yes	*Cineraria maritime* L.	Carcinogenicity studies of integerrimine were not found in the available literature. However, several studies indicated genotoxic potentials.	(Chen *et al.*, 2010; Fu *et al.*, 2004)
Isosafrole	Alkenylbenzene	Yes	No	*Angelica sinensis* (Oliv.) Diels, *Sassafras* spp.	Isosafrole is a weak hepatocarcinogen in both rats and mice. The increased incidences of tumors are likely to be mediated by a non-genotoxic mechanism based on negative outcomes of genotoxicity tests.	(SCF, 2003)
Lasiocarpine	Pyrrolizidine alkaloid	Yes	Yes	*Heliotropium lasiocarpum* Fisch. & C.A.Mey, *Heliotropium europaeum* L.	Angiosarcomas of the liver were observed in a 2-year NTP study. A treatment related trend was observed in both male and female rats except for female rats treated with the highest dose. Several in vitro and an in vivo test indicated the genotoxic potential of lasiocarpine.	(Chen *et al.*, 2010; Fu *et al.*, 2004; NTP, 1978)
Lucidin	Anthraquinone	Probably	Yes	*Rubia tinctorum* L.	Indicated in EFSA compendium as 'Presence of lucidin (1,2,3-hydroxy anthraquinone) with genotoxic and most likely carcinogenic activity'.	(EFSA, 2009a)

8-Methoxy-psoralen	Furocoumarin	Equivocal	Yes	*Citrus limon* (L.) Burm.f, *Citrus reticulata* Blanco, *Petroselinum crispum* (Mill.) A.W.Hill, *Levisticum officinale* W.J.D.Koch	Data from a 2-years NTP gavage study suggest clear evidence for carcinogenicity in male rats based on several neoplastic lesions including adenomas, and adenocarcinomas of the kidney and carcinomas of the Zymbal gland. However, the incidence of adenocarcinomas was not significantly increased compared to the control. Moreover, several of the observed effects were not dose-dependent. Results were not consistent across sexes: there was no evidence of carcinogenic activity for female rats. 8-Methoxypsoralen has shown to be genotoxic in several in vitro and in vivo genotoxicity assays.	(NTP, 1989)
Methyleugenol	Alkenylbenzene	Yes	Yes	Methyleugenol occurs in a wide variety of plants *e.g. Illicium anisatum* L., *Laurus nobilis* L., *Zingiber officinale* Roscoe.	Indicated in EFSA compendium as 'methyleugenol along with its 1-hydroxy-metabolite is mutagenic in many systems and able to induce DNA adducts and liver tumors in mice'.	(EFSA, 2009a)
Monocrotaline	Pyrrolizidine alkaloid	Yes	Yes	*Crotalaria sessiflora* L., *Crotalaria spectabilis* Roth	In male rats given monocrotaline by gavage, liver cell carcinomas were formed. Genotoxicity was indicated in a number of in vitro as well as in vivo tests.	(Chen *et al.*, 2010; COC, 2008; Fu *et al.*, 2004; Mori *et al.*, 1985a; Newberne, 1973)
β-Myrcene	Monoterpene	Yes	No	*Commiphora mukul* Engl., *Fortunella japonica* (Thunb.) Swingle	Administration of β-myrcene induced neoplastic lesions in the renal tubules (male rats) and liver (male mice) as observed during a 2-year NTP gavage study. No evidence of genotoxicity was found based on in vitro and in vivo genotoxicity assays.	(EFSA, 2009d; NTP, 2009a)
Myristicin	Alkenylbenzene	Yes	Yes	Myristicin can be found in a variety of plants *e.g. Myristica fragrans* Houtt., *Sassafras albidum* (Nutt.) Nees.	Indicated in the EFSA compendium as 'myristicin, present in the oleoresin and having mutagenic activities and capable of inducing the formation of DNA adducts and its metabolite (1' -hydroxy-myristicin) is considered carcinogenic'.	(EFSA, 2009a)

Table 1 (continued). Overview of botanical ingredients with genotoxic and/or carcinogenic characteristics that have a possible risk from the public health point of view.

Compound	Group	Carcinogenic	Genotoxic	Natural Occurrence	Remarks/ tumor type	Reference
Ptaquiloside	Nor-sesquiterpene glycoside	Yes	Yes	*Pteridium aquilinum* (L.) Kuhn.	Indicated in the EFSA compendium as 'Ptaquiloside is a carcinogenic compound (formation of an alkylant conjugated dienone) and its transformation in ptaquiloside B leads to a neurotoxic compound.' The genotoxicity of ptaquiloside was previously demonstrated using the umu test. Moreover, it was seen that ptaquiloside was able to cause chromatid exchanges in Chinese hamster lung cells and unscheduled DNA synthesis in rat hepatocytes.	(Hirono *et al.*, 1984; Matsuoka *et al.*, 1989; Mori *et al.*, 1985a; Schmidt *et al.*, 2005)
Pulegone	Monoterpene	Equivocal	No	Pulegone can be found in a variety of plants *e.g. Agastache* spp., *Hedeoma pulegioides* (L.) Pers., *Mentha canadensis* L.	From a 2-year NTP study it was concluded that there was no evidence of carcinogenicity for rats but there was clear evidence of carcinogenic activity of pulegone in male and female mice based on increased incidences of liver tumors. However, most of the formed tumors were benign. Malignant tumors were not dose-dependent nor significantly different from the control. Bacterial mutagenicity assays as well as in vivo tests gave negative results.	(NTP, 2009b)
Quercetin	Flavonoid	No	Yes	Quercetin occurs in a variety of plants *e.g. Cupressus sempervirens* L. and *Camellia sinensis* (L.) Kuntze	Based on a long term carcinogenicity bioassay, there was no evidence of carcinogenic activity in female rats and some evidence in male rats, the latter being based on increased incidences of benign neoplasms. Only in one male rat, exposed to the highest tested dose, an adenocarcinoma was formed. NTP in vitro assays indicated genotoxic activities for quercetin.	(Harwood *et al.*, 2007; NTP, 1992)

Reserpine	Indolomonoterpenic alkaloid	Yes	Yes	*Rauvolfia* spp.	In a 2-year NTP study, positive effects for carcinogenic activity of reserpine were found in male rats and female and male mice: pheochromocytomas of the adrenal, malignant tumors of the mammary gland in females and carcinomas of the seminal vesicles in males. Reserpine did not show genotoxicity in several in vitro and in vivo tests except for 2 studies in which chromosome aberrations in vivo and cell transformations were found.	(Brambilla and Martelli, 2006; NTP, 1982)
Riddelliine	Pyrrolizidine alkaloid	Yes	Yes	*Senecio* spp.	Indicated by EFSA as 'Riddelliine, a pyrrolizidine alkaloid, is found in *Senecio riddellii* and other *Senecio* species, including *S. longibus*, which is used as an herbal remedy in the south-western USA. Riddelliine was evaluated by IARC (2002). IARC found no data on the carcinogenicity of riddelliine in humans but found sufficient evidence in experimental animals for the carcinogenicity of riddelliine. Riddelliine was classified in Group 2B (riddelliine is possibly carcinogenic to humans).' Riddelliine gave positive results in several in vitro and in vivo genotoxicity assays.	(Chen *et al.*, 2010; COC, 2008; Fu *et al.*, 2004; EFSA, 2009a NTP, 2003b)
Safrole	Alkenylbenzene	Yes	Yes	Safrole occurs in a wide variety of plants *e.g. Illicium verum* Hook.f., *Myristica fragrans* Houtt., *Ocimum basilicum* L., *Sassafras* spp.	Indicated in the EFSA compendium as 'safrole, a weak carcinogen in rats and mice, trans-placental carcinogen in mice and mutagenic in a variety of assays. Safrole is a genotoxic carcinogen.'	(EFSA, 2009a)

Table 1(continued). Overview of botanical ingredients with genotoxic and/or carcinogenic characteristics that have a possible risk from the public health point of view.

Compound	Group	Carcinogenic	Genotoxic	Natural Occurrence	Remarks/ tumor type	Reference
Senecionine	Pyrrolizidine alkaloid	Probably	Yes	*Brachyglottis* spp., *Cineraria maritima* L., *Erechtites* spp., *Leucanthemum vulgare* Lam., *Petasites officinalis* Moench, *Senecio* spp.	Senecionine was positive in several genotoxicity assays including UDS assays and a Wing spot test. Moreover, DNA-adduct formation was found in a limited study. However, also negative results were reported for senecionine in the Ames test. Carcinogenicity of pure senecionine is not known, however, according to Green *et al.*, 1981 it is most likely that senecionine is carcinogenic.	(Chen *et al.*, 2010; Fu *et al.*, 2004; Green *et al.*, 1981; Mori *et al.*, 1985a)
Senkirkine	Pyrrolizidine alkaloid	Equivocal	Yes	*Tussilago farfara* L.	In a limited study, an increased incidence of hepatocellular adenomas was found in male rats upon senkirkine administration. However, the Committee On Carcinogenicity (COC) concluded that the present data are not sufficient to ascertain the carcinogenicity of senkirkine. Nevertheless, the IARC concluded that there is limited evidence for the carcinogenicity of this compound based on an experimental study. Several in vitro genotoxicity studies gave (weak) positive results.	(Chen *et al.*, 2010; COC, 2008a; Fu *et al.*, 2004; IARC, 1983)
Symphytine	Pyrrolizidine alkaloid	Yes	Yes	*Symphytum officinale* L.	Increased incidences of liver haemangioendothelial sarcomas and liver cell adenomas were found in a study using male rats. Considering the results of this restricted study and the structure of symphytine the COC mentioned that a carcinogenic activity is likely. Genotoxic potentials were identified in a Wing spot test.	(Chen *et al.*, 2010; COC, 2008; Fu *et al.*, 2004)

* Dependent on dose tested, structurally related compounds are carcinogenic.

Table 2. Summary of malignant tumors formed after administration to estragole, methyleugenol, safrole or β-asarone.

Compound	Gender/ Species	Experimental dose (mg/kg diet)#	Time-adjusted dose (mg/kg bw/day)	No. of animals	Cancer incidence	Reference
Estragole	Female mice	0	0	50	0	(Miller *et al.*, 1983)
		2300	54	48	27	
		4600	107	49	35	
Methyleugenol	Male rats	0	0	50	2	(NTP, 2000)
		37	26	50	3	
		75	54	50	14	
		150	107	50	25	
Methyleugenol	Female rats	0	0	50	0	(NTP, 2000)
		37	26	50	0	
		75	54	49	4	
		150	107	49	8	
Safrole	Female mice	0	0	50	0	(Miller *et al.*, 1983)
		2500	58	47	34	
		5000	117	49	39	
β-asarone	Male rats	0	0	25	0	(JECFA, 1981)
		400	16	25	1	
		800	32	25	6	
		2000	52	25	9	

Dose levels of methyleugenol are presented in mg/kg bw/day.

For methyleugenol, the daily intake resulting from the use of the same PFS amounts to 0.02-200 µg/kg bw/day taking into account that the essential oil contains 0.46-91.1% methyleugenol (Zheljazkov *et al.*, 2008). In general, intake estimates for estragole, methyleugenol, safrole and β-asarone, resulting from the use of PFS, range between 0.004 to 2.96 mg/kg bw/day, depending on the botanical of interest, the variable amounts of the genotoxic carcinogens within the plants, and the different recommended daily intakes for the PFS under consideration. When interpreting these intake estimates, it should be kept in mind that all calculations were performed under the assumption that PFS contained the botanical as such and corrections for the manufacturing procedure were not applied. However, when PFS consist of powdered herb material prepared by freeze drying for example, the actual level of the compound of interest may be higher compared to the intake estimates that have been made for the use of the botanical as such leading to lower MOE values. On the contrary, levels may be lower when the PFS is prepared by decoction. Therefore, in a next step the levels of these alkenylbenzenes in commercial available PFS and the resulting estimated human intakes were quantified based on chemical analysis.

Table 3. Results of a BMD analysis of the carcinogenicity data on the incidence of malignant tumors in rodents exposed to estragole, methyleugenol, safrole, or β-asarone. The data used as input for the BMD analysis are presented in Table 2.

Compound	Species and sex	Tumors	$BMDL_{10}$ (mg/kg bw/day)
Estragole	Female mice	hepatocellular carcinomas	3.3-6.5
Methyleugenol	Male rats	hepatocellular carcinomas	15.3-34.0
Methyleugenol	Female rats	hepatocellular carcinomas	48.8-73.6
Safrole	Female mice	hepatocellular carcinomas	1.9-5.1
β-asarone	Male rats	leiomyosarcomas of the small intestine	9.6-21.5

Intake estimates of alkenylbenzenes from consumption of PFS based on analytical quantification

Table 6 shows the results of the quantitative measurement of estragole, methyleugenol, safrole and β-asarone present in different commercially available PFS for which also theoretical estimations were made. Among the different PFS, estragole was found in a range of 0.07-241.56 mg/g PFS corresponding to an estimated daily intake of 0.001-4.78 mg/kg bw/day based on the recommended daily intake of the PFS as indicated on the label of the respective supplements.

The levels of methyleugenol were equal to 0.34-1.04 mg/g PFS in the tested nutmeg-containing PFS. Based on the recommended daily intake of the corresponding nutmeg PFS, the daily methyleugenol intake would equal 0.005-0.065 mg/kg bw/day. For calamus-containing PFS, the amount of β-asarone was found to be in the range of 0.12-44.28 mg/g PFS, resulting in a daily intake of β-asarone equal to 0.003-0.635 mg/kg bw/day. These daily intake estimates based on chemical analysis of the alkenylbenzene levels in the different PFS are in line with the estimated intake levels of the alkenylbenzenes obtained on the basis of the theoretical estimates for the levels of the alkenylbenzenes in these PFS.

Margins of Exposure for alkenylbenzenes

The MOE values obtained by comparison of the $BMDL_{10}$ values with the estimated daily intake of estragole, methyleugenol, safrole or β-asarone resulting from the use of different PFS, appear to be relatively low (Table 5). For example, upon use of a particular basil-containing PFS consisting of the essential oil, the MOE amounts to 1-2,000 for estragole and a value ranging between 5 and 5,000 was found for methyleugenol. For fennel-containing PFS, the MOE values range between 3 and 20,000 for estragole. Taking into account the consumption of a β-asarone-containing calamus PFS, the MOE was found equal to 60-7,000. Also when considering the actual levels of these compounds in commercially available PFS as quantitatively determined, relatively low MOE values were derived (Table 6). In some cases the MOE was even found to be below 10 which means that the daily intake of the compounds of interest are within the range of the dose levels causing tumors in experimental animals.

Discussion

In the presented chapter, the MOE approach was used for the risk assessment of a series of compounds that are both genotoxic and carcinogenic and known to be present in botanicals and PFS. In total, 30 botanical ingredients were identified as a possible risk for human health based on their genotoxic and/or carcinogenic characteristics. Out of these selected botanical ingredients 18 compounds appeared to be both genotoxic and carcinogenic. Interestingly, most of these compounds are a member of the

group of alkenylbenzenes or unsaturated pyrrolizidine alkaloids. Based on this thorough selection of genotoxic and carcinogenic botanical compounds of interest and the availability of carcinogenicity data allowing the use of the MOE concept, this concept was applied to the alkenylbenzenes estragole, methyleugenol, safrole and β-asarone. The results presented show that the consumption of PFS containing estragole, methyleugenol, safrole or β-asarone may represent a high priority for risk management actions.

Performing the exposure assessment, intake estimates were made based on available literature data. However, the prediction of the content of the botanical compounds of interest in PFS was hampered by uncertainties in the processing techniques used to prepare the respective PFS and varying amounts of these compounds found in plants belonging to the same species depending on the plant maturity at harvest and climatic conditions among others (Smith *et al.*, 2002). To determine if the estimates of human dietary exposure represent the actual levels of the genotoxic carcinogens of interest in commercially available PFS, the content of alkenylbenzenes in different PFS was analyzed. The theoretical estimates made for the levels of estragole, methyleugenol, safrole and β-asarone in the different PFS were generally in agreement with the levels of these compounds actually detected in chemical analysis. In general, a wide range of estimated intake levels was obtained for each PFS of interest because of the different amounts of the respective compounds in the plants and their corresponding PFS, resulting in a wide range of MOE values. Moreover, making use of the levels of the alkenylbenzenes of interest in a particular PFS, as determined by chemical analysis, resulted also in a wide range of MOE values. It was seen that the wide variations in MOE values for a specific PFS is not due to a large variation in the $BMDL_{10}$ values, because these values vary only 2 to 3 fold. On the contrary, intake estimates may vary greatly. For example, for a basil-containing PFS, the recommended daily intake equals 1-4 tablets (*i.e.* 270-1080 mg/day) and while the daily methyleugenol exposure resulting from the daily use of 1 tablet does not indicate a potential risk for human health, the daily intake of 4 tablets does. As a consequence, it is difficult to express these relatively wide ranging MOE values in terms of concern for human health and this involves an increased risk of misinterpretation, demonstrating the importance of a narrative describing the context of uncertainties in the exposure assessment. The importance of an explanatory narrative was previously indicated (Barlow *et al.*, 2006; Benford *et al.*, 2010) and will allow a better interpretation of the potential risk for human health.

Based on the analytical measurements, it was shown that also between the PFS consisting of the same botanical species, differences occur in the levels of the alkenylbenzenes of interest. The variability in these alkenylbenzene concentrations in the different products can have several causes. The theoretical approach presented in this chapter and tested towards a number of PFS available on the market, made heavily clear some points of concern. Alkenylbenzenes are present in the essential oil of the plant. The quantity of the essential oil and the amount of alkenylbenzenes is dependent on genetic factors and geographical influences in cultivation. Further compositional changes are seen depending on the moment of harvesting, the way of drying, processing and manufacturing conditions. Therefore, when manufacturing PFS a strict analytical procedure is necessary in order to determine and follow up the amount of the alkenylbenzenes. In herbal trade, the use of the binomial plant name including the author, the chemotype, variety, hybrid or cultivar should be used and when possible, the geographical origin. In case of using botanicals known to contain genotoxic and carcinogenic compounds, this should be a legal requirement and in fact also be a requirement for all botanicals used in food and food supplements. Basil is an example. 'Basil' is an undefined vernacular name. The sweet basil is *Ocimum basilicum* L. containing often less than 0.2% methyleugenol. However, when cultivated in Egypt the amount increases to an average of 5.6%. *O. basilicum* var. *basilicum* contains on average 1.6% methyleugenol and *O. basilicum* L. var. *minimum* up to 55% methyleugenol. Both are sold as 'basil' or also as *Ocimum basilicum* L. Even for a morphological well defined species, also different chemotypes exist: 'linalool' type, 'linalool and estragole' and 'linalool and eugenol' all having huge differences in the amount of the alkenylbenzenes. The amount of methyleugenol and estragole also depends on the size of the herb with small plants having a higher amount of methyleugenol and less estragole whereas taller plants showing a higher amount of estragole and lower methyleugenol levels. The moment of harvesting is therefore important as well as the drying methods which heavily influence the content of the volatile compounds of basil (di Cesare *et al.*, 2003; Miele *et al.*, 2001).

Table 4. Characteristics of PFS used to derive (estimated) daily intakes of estragole, methyleugenol, safrole or β-asarone resulting from te use of the respective PFS

Botanical in supplement	Characteristics of PFS
Basil #	*Ocimum basilicum* L.
1	Mixture of 100% essential oil including 12.5 g *Ocimum basilicum* essential oil per 100 g supplement. Recommended daily dose equals 6 capsules, consistent with 1560 mg total PFS.
2	Mixture of 100% essential oil including 10 mg *Ocimum basilicum* essential oil per capsule. Recommended daily dose equals 1-4 capsules, consistent with 270-1080 mg total PFS.
3	Supplements consist of powdered plant material, preparation method unknown. Recommended daily dose equals 1-3 capsules, consistent with 230-690 mg PFS.
4	Mixture of 100% essential oil including 30 mg *Ocimum basilicum* essential oil per capsule. Recommended daily dose equals 2-3 capsules, consistent with 565-848 mg total PFS.
5	Supplements consist of powdered plant material, preparation method unknown. Recommended daily dose equals 4-6 capsules, consistent with 1580-2370 mg total PFS.
Fennel	*Foeniculum vulgare* Mill.
1	Supplements are standardized for 1% essential oil and contain 390 mg *Foeniculum vulgare* Mill. Recommended daily dose equals 3 capsules, consistent with 1170 mg total PFS per day.
2	Supplements are standardized for 2% essential oil and indicated to contain 300 mg *Foeniculum vulgare* Mill. Recommended daily dose equals 3-6 capsules, consistent with 855-1710 mg total PFS.
3	Supplements consist of 330 mg powdered plant material, preparation method unknown. Recommended daily dose equals 2 capsules, consistent with 660 mg total PFS per day.
4	Supplements consist of 480 mg powdered plant material, preparation method unknown. Recommended daily dose equals 3 capsules. Complete capsules were found to be 550 mg each, consistent with 1650 mg total PFS.
5	Supplements consist of a mixture of powdered plant material, with 55 mg fennel per capsule. Recommended daily dose equals 1-2 capsules, consistent with 300-600 mg total PFS.
6	Supplements consist of 225 mg powdered plant material, preparation method unknown. Recommended daily intake equals 4 capsules. Complete capsules were found to be 300 mg each, consistent with 1200 mg total PFS.
7	Supplements are standardized for 2% essential oil and contain 500 mg per capsule. Recommended daily intake equals 2 capsules. Complete capsules were found to be 1000 mg each, consistent with 2000 mg total PFS.
Sassafras	*Sassafras albidum* (Nutt.) Nees.
1	Supplements consist of powdered plant material, preparation method unknown. Recommended daily dose equals 1-3 capsules, consistent with 200-600 mg PFS per day.

2	Supplements consist of 480 mg powdered root bark, preparation method unknown. Recommended daily dose equals 3 capsules. Complete capsules were found to be 120 mg each, consistent with 360 mg total PFS.
3	Supplements consist of powdered root bark, preparation method unknown. Recommended daily dose equals 2 capsules, consistent with 860 mg PFS, consistent with 860 mg PFS per day.
4	Supplements consist of powdered root bark, preparation method unknown. Recommended daily dose equals 1-2 capsules, consistent with 390-780 mg PFS, consistent with 390 - 780 mg PFS per day.
Nutmeg	*Myristica fragrans* Houtt.
1	Supplements consist of powdered plant material, preparation method unknown. Recommended daily dose equals 1-3 capsules, consistent with 360-1080 mg PFS.
2	Supplements consist of 750 mg powdered plant material, preparation method unknown. Recommended daily dose equals 1-5 capsules, consistent with 750-3750 mg PFS.
3	Supplements consist of 330 mg powdered plant material per capsule, preparation method unknown. Recommended daily dose equals 2 capsules. Complete capsules were found to be 310 mg each, consistent with 620 mg total PFS.
4	Supplements consist of powdered plant material, preparation method unknown. Recommended daily dose equals 2 capsules, consistent with 1330 mg PFS.
Cinnamon	*Cinnamomum verum* J.Presl.
1	PFS contain a mixture including *Cinnamomum verum* in unspecified quantities. Recommended daily intake equals 2 capsules, consistent with 240 mg total PFS per day.
2	Supplements consist of 500 mg powdered plant material, preparation method unknown. Recommended daily dose equals 2 capsules. Complete capsules were found to be 465 mg each, consistent with 930 mg total PFS.
3	Supplements are standardized for 4% essential oil and contain 125 mg *Cinnamomum verum* essential oil per capsule. Recommended daily dose equals 1 capsule. Complete capsules were found to be 860 mg each, consistent with 860 mg total PFS per day.
4	Supplements consist of 450 mg powdered plant material, preparation method unknown. Recommended daily dose equals 1-3 capsules. Complete capsules were found to be 420 mg each, consistent with 420-1260 mg total PFS per day.
Calamus	*Acorus calamus* L.
1	Supplements consist of powdered plant material, preparation method unknown. Recommended daily dose equals 1-3 capsules, consistent with 220-660 mg total PFS.
2	PFS contains 108 mg calamus per capsule. Recommended daily dose equals 6-16 capsules. Complete capsules were found to be 245 mg each, consistent with 1470-3920 mg total PFS.
3	Supplements consist of 450 mg powdered plant material, preparation method unknown. Recommended daily intake equals 3 capsules, consistent with 860 mg total PFS per day.
4	Supplements consist of a mixture of various plants: *Emblica officinalis, Terminalia chebula, Glycyrrhila glabra* and 50 mg *Acorus calamus*. Recommended daily intake equals 2 capsules, consistent with 630 mg total PFS per day.

when the botanical contains high levels of estragole, methyleugenol levels are often low and vice versa (Zheljazkov *et al.*, 2008).

Table 5. Estimated daily exposure to estragole, methyleugenol, safrole or β-asarone present in a variety of PFS. E estragole; ME methyleugenol; S safrole; BA β-asarone.

Botanical in supplement	% essential oil in plant	% compound in the essential oil	Recommended daily intake of botanical from PFS (mg)	Daily intake compound of interest (mg/kg bw/day)	MOE using data from Table 3.
Basil #					
1	100	E: 0.11-85 (EFSA, 2009a; Zheljazkov *et al.*, 2008) ME: 0.46-91.1 (Zheljazkov *et al.*, 2008)	195	E: 0.0036-2.76 ME: 0.015-2.96	E: 1-2,000 ME: 5-5,000
2	100	E: 0.11-85 (EFSA, 2009a; Zheljazkov *et al.*, 2008) ME: 0.46-91.1 (Zheljazkov *et al.*, 2008)	10-40	E: 0.0002-0.57 ME: 0.0008–0.61	E: 6-30,000 ME: 30-90,000
3	0.11-1.9 (Zheljazkov *et al.*, 2008)	E: 0.11-85 (EFSA, 2009a; Zheljazkov *et al.*, 2008) ME: 0.46-91.1 (Zheljazkov *et al.*, 2008)	230-690	E: 0.000005-0.186 ME: 0.00002-0.20	E: 20-1,000,000 ME: 80-4,000,000
4	100	E: 0.11-85 (EFSA, 2009a; Zheljazkov *et al.*, 2008) ME: 0.46-91.1 (Zheljazkov *et al.*, 2008)	60-90	E: 0.001-1.28 ME: 0.005-1.37	E: 3-7,000 ME: 10-10,000
5	0.11-1.9 (Zheljazkov *et al.*, 2008)	E: 0.11-85 (EFSA, 2009a; Zheljazkov *et al.*, 2008) ME: 0.46-91.1 (Zheljazkov *et al.*, 2008)	1580-2370	E: 0.00003-0.64 ME: 0.0001-0.68	E: 5-200,000 ME: 20-700,000
Fennel					
1	1	E: 0.8-80 (EFSA, 2009a)	1170	E: 0.0016-0.16	E: 20-4,000
2	2	E: 0.8-80 (EFSA, 2009a)	900-1800	E: 0.0024-0.48	E: 7-3,000
3	6 (Smith *et al.*, 2002)	E: 0.8-80 (EFSA, 2009a)	660	E: 0.005-0.53	E: 6-1,000
4	6 (Smith *et al.*, 2002)	E: 0.8-80 (EFSA, 2009a)	1440	E: 0.012-1.15	E: 3-500

5	6 (Smith *et al.*, 2002)	E: 0.8-80 (EFSA, 2009a)	55-110	E: 0.0004-0.09	E: 40-20,000
6	6 (Smith *et al.*, 2002)	E: 0.8-80 (EFSA, 2009a)	900	E: 0.0072-0.72	E: 5-900
7	2	E: 0.8-80 (EFSA, 2009a)	1000	E: 0.0027-0.27	E: 10-2,000
Sassafras					
1	6-9 (EFSA, 2009a)	ME: 3.03 (SimiÇ *et al.*, 2004) S: 82.04 (SimiÇ *et al.*, 2004)	200-600	ME: 0.006-0.027 S: 0.16-0.74	ME: 600-10,000 S: 3-30
2	6-9 (EFSA, 2009a)	ME: 3.03 (SimiÇ *et al.*, 2004) S: 82.04 (SimiÇ *et al.*, 2004)	1440	ME: 0.043-0.065 S: 1.18-1.77	ME: 200-2,000 S: 1-4
3	6-9 (EFSA, 2009a) addictive, psychotropic	ME: 3.03 (SimiÇ *et al.*, 2004) S: 82.04 (SimiÇ *et al.*, 2004)	860	ME: 0.026-0.039 S: 0.71-1.06	ME: 400-3,000 S: 2-7
4	6-9 (EFSA, 2009a)	ME: 3.03 (SimiÇ *et al.*, 2004) S: 82.04 (SimiÇ *et al.*, 2004)	390-780	ME: 0.012-0.035 S: 0.32-0.96	ME: 400-6,000 S: 2-20
Nutmeg					
1	3.9-16.5 (Maya *et al.*, 2004)	ME: 0.1-17.9 (De Vincenzi *et al.*, 2000) S: 0.1-22.1 (Maya *et al.*, 2004)	360-1080	ME: 0.0002-0.53 S: 0.0002-0.65	ME: 30-400,000 S: 3-30,000
2	3.9-16.5 (Maya *et al.*, 2004)	ME: 0.1-17.9 (De Vincenzi *et al.*, 2000) S: 0.1-22.1 (Maya *et al.*, 2004)	750-3750	ME: 0.0005-1.85 S: 0.0005-2.28	ME: 8-100,000 S: 1-10,000
3	3.9-16.5 (Maya *et al.*, 2004)	ME: 0.1-17.9 (De Vincenzi *et al.*, 2000) S: 0.1-22.1 (Maya *et al.*, 2004)	660	ME: 0.0004-0.32 S: 0.0004-0.32	ME: 50-200,000 S: 6-10,000
4	3.9-16.5 (Maya *et al.*, 2004)	ME: 0.1-17.9 (De Vincenzi *et al.*, 2000) S: 0.1-22.1 (Maya *et al.*, 2004)	1330	ME: 0.0009-0.68 S: 0.0009-0.68	ME: 20-80,000 S: 3-6,000

Table 5 (continued). Estimated daily exposure to estragole, methyleugenol, safrole or β-asarone present in a variety of PFS. E estragole; ME methyleugenol; S safrole; BA β-asarone.

Botanical in supplement	% essential oil in plant	% compound in the essential oil	Recommended daily intake of botanical from PFS (mg)	Daily intake compound of interest (mg/kg bw/day)	MOE using data from Table 3.
Cinnamon					
1	1.54 (Wang *et al.*, 2009)	ME: 0.01 (EFSA, 2009a)	140	ME: 0.000004 S: 0.00018	ME: 4,000,000-20,000,000 S: 10,000-30,000
2	1.54 (Wang *et al.*, 2009)	ME: 0.01 (EFSA, 2009a)	1000	ME: 0.00003 S: 0.00128	ME: 500,000-2,000,000 S: 1,000-4,000
3	4	ME: 0.01 (EFSA, 2009a)	125	ME: 0.000008 S: 0.0004	ME: 2,000,000-9,000,000 S: 5,000-10,000
4	1.54 (Wang *et al.*, 2009)	ME: 0.01 (EFSA, 2009a)	450-1350	ME: 0.000012-0.000035 S: 0.0006 - 0.0017	ME: 400,000-6,000,000 S: 1,000-9,000
Calamus					
1	1-3	BA: 19 (Rost and Bas, 1979)	220-660	BA: 0.007-0.063	BA: 200-3,000
2	1-3	BA: 19 (Rost and Bas, 1979)	648-1728	BA: 0.021-0.164	BA: 60-1,000
3	1-3	BA: 19 (Rost and Bas, 1979)	1350	BA: 0.043 - 0.128	BA: 80-500
4	1-3	BA: 19 (Rost and Bas, 1979)	100	BA: 0.003-0.010	BA: 1,000-7,000

NOTE: PFS have the same characteristics as those presented in Table 4 corresponding to the number of the respective PFS.

when the botanical contains high levels of estragole, methyleugenol levels are often low and vice versa (Zheljazkov *et al.*, 2008)

Table 6. Chemically determined levels of estragole, methyleugenol, safrole or β-asarone present in a variety of PFS, corresponding daily exposures and MOE values.

Botanical in supplement	Average ± StDEV (mg / g supplement)	Recommended daily intake of supplement (mg)	Daily Intake compound of interest (mg/kg bw/day)	MOE using data from Table 3
Basil				
1	E: 183.85 ± 2.24 ME: 0.85 ± 0.02	1560	E: 4.78 ME: 0.022	E: 1 ME: 700-3,000
2	E: 32.71 ± 1.46 S: 0.29 ± 0.02 ME: 0.16 ± 0.01	270-1080	E: 0.147 – 0.589 S: 0.001-0.005 ME: 0.001 – 0.003	E: 6-40 S: 400-5,000 ME: 5,000-70,000
3	E: 1.21 ± 0.11 ME: 0.07 ± 0.003	230-690	E: 0.005 – 0.014 ME: 0.0003-0.0008	E: 200-1,000 ME: 20,000-200,000
4	E: 241.56 ± 62.02 ME: 1.60 ± 0.04	565-848	E: 2.275-3.414 ME: 0.015-0.023	E: 1-3 ME: 700-5,000
5	E: 0.20 ± 0.01 ME: 0.28 ± 0.03	1580-2370	E: 0.005-0.008 ME: 0.007-0.011	E: 400-1,000 ME: 1,000-10,000
Fennel				
1	E: 0.97 ± 0.07	1170	E: 0.019	E: 200-300
2	E: 0.62 ± 0.08	855-1710	E: 0.009-0.018	E: 200-700
3	E: 7.60 ± 0.39	660	E: 0.084	E: 40-80
4	E: 1.40 ± 0.08	1650	E: 0.038	E: 90-200
5	E: 0.16 ± 0.02	300-600	E: 0.001-0.002	E: 2,000-7,000
6	E: 0.07 ± 0.005	1200	E: 0.001	E: 3,000-7,000
7	E: 0.16 ± 0.01	2000	E: 0.005	E: 700-1,000
Sassafras				
1	S: 0.13 ± 0.01	200-600	S: 0.0004-0.001	S: 2,000-10,000
2	S: 3.17 ± 0.15 ME: 0.21 ± 0.02	360	S: 0.019 ME: 0.001	S: 100-300 ME: 20,000-70,000
3	S: 0.21 ± 0.02	860	S: 0.003	S: 600-2,000
4	S: 0.20 ± 0.01	390-780	S: 0.001-0.003	S: 600-5,000

Table 6 (continued). Chemically determined levels of estragole, methyleugenol, safrole or β-asarone present in a variety of PFS, corresponding daily exposures and MOE values.

Botanical in supplement	Average ± StDEV (mg / g supplement)	Recommended daily intake of supplement (mg)	Daily Intake compound of interest (mg/kg bw/day)	MOE using data from Table 3
Nutmeg				
1	S: 2.17 ± 0.13 ME: 0.84 ± 0.02	360 – 1080	S: 0.013-0.039 ME: 0.005-0.015	S: 50-400 ME: 1,000-10,000
2	S: 3.46 ± 0.22 ME: 1.04 ± 0.10	750-3750	S: 0.043-0.217 ME: 0.013-0.065	S: 9-100 ME: 200-6,000
3	S: 1.16 ± 0.05 ME: 0.90 ± 0.03	620	S: 0.012 ME: 0.009	S: 200-400 ME: 2,000-8,000
4	ME: 0.34 ± 0.02	1330	ME: 0.008	ME: 2,000-9,000
Cinnamon				
1	ND	280	NC	NC
2	ND	930	NC	NC
3	ND	860	NC	NC
4	ND	420-1260	NC	NC
Calamus				
1	BA: 1.33 ± 0.04	220-660	BA: 0.005-0.015	BA: 600-4,000
2	BA: 0.12 ± 0.01	1470-3920	BA: 0.003-0.008	BA: 1,000-7,000
3	BA: 44.28 ± 8.02	860	BA: 0.635	BA: 20-30
4	BA: 1.51 ± 0.14	630	BA: 0.016	BA: 600-1,000

ND Not detected (detection limit 0.02 mg/g supplement); NC Not Calculated: E Estragole; ME Methyleugenol; S Safrole; BA β-asarone.
NOTE: PFS have the same characteristics as those presented in Table 4 corresponding to the number of the respective PFS.

The potential risks resulting from the use of alkenylbenzene-containing PFS are especially of concern because multiple supplements might be consumed in addition to the regular daily diet. Previous studies already performed a risk assessment based on the MOE concept for the botanical ingredients estragole (Rietjens *et al.*, 2010) and methyleugenol (Benford *et al.*, 2010; Rietjens *et al.*, 2008) indicating that the use of a regular diet containing estragole or methyleugenol is associated with concerns from the human health point of view since low MOE values ranging between 129 and 3300 were found for estragole taking into account the total estimated daily intake (Rietjens *et al.*, 2010). Also for methyleugenol low MOE values were obtained amounting to 42-416 (Rietjens *et al.*, 2008) and 100-800 (Benford *et al.*, 2010). Moreover, it is important to recognize that often various structurally related compounds, causing the same adverse effects, occur together in one botanical such as the alkenylbenzenes estragole and methyleugenol which can be found in sweet basil. However, one should keep in mind that whenever basil (*Ocimum basilicum* L.) expresses for example high levels of estragole, methyleugenol levels are often low and vice versa (Zheljazkov *et al.*, 2008). Although the addition of estragole, methyleugenol (Regulation (EC) No 1334/2008 of the European Parliament and of the Council 16 December 2008), safrole and β-asarone (Council Directive 88/388/EEC of 22 June 1988) as pure compounds in food is prohibited within the EU because of their genotoxic and carcinogenic potentials, currently no restrictions have been made with regard to the use of alkenylbenzene-containing PFS.

Methyleugenol and structurally related alkenylbenzenes are not carcinogenic by nature but metabolic conversion into a biological active form is required to give expression to the carcinogenic capacities in rodent bioassays. It is reported that the metabolic pathway responsible for the formation of a DNA reactive carbonium ion occurs at relatively high intake levels when metabolic conversion through a standard metabolic pathway is saturated (Smith *et al.*, 2002). Accordingly, Benford *et al.* (2010) stated that "derivation of a BMD/BMDL based on modeling of high-dose animal study data does not allow threshold effects in metabolism to be accounted for and thus alters the interpretation of the calculated MOE". However, a recent study showed only a minor increase (<1-fold) in the relative formation of the ultimate carcinogenic metabolite of methyleugenol at high dose levels as compared to low dose levels (Al-Subeihi *et al.*, 2011). Using a physiologically based kinetic (PBK) model, it was predicted that the formation of the ultimate carcinogen (*i.e.* 1'-sulfoxymethyleugenol) in

rats would correspond to 0.043% of the initial dose at 0.05 mg/kg bw followed by an increase to 0.06% of the dose at a dose of 300 mg/kg bw (Al-Subeihi *et al.*, 2011). These data are also available for the bioactivation of estragole and safrole. For estragole, the formation of the ultimate carcinogenic metabolite was predicted to show a 2-fold relative increase when comparing a low dose (0.05 mg/kg bw) with a high dose (300 mg/kg bw) of estragole (Punt *et al.*, 2008). Whereas for safrole, the PBK model predicted that the increase in the formation of the ultimate carcinogen with the dose is negligible (Martati *et al.*, 2011).

Conclusion

In conclusion, a number of genotoxic and/or carcinogenic compounds can be found in botanicals and botanical preparations. Especially compounds belonging to the group of alkenylbenzenes and unsaturated pyrrolizidine alkaloids are of possible concern for human health resulting from their genotoxic and carcinogenic potentials. In fact, PFS that are on the market may contain alkenylbenzenes at levels that, at the recommended daily dose of the supplement, may result in a daily intake that is in the range of dose levels causing malignant tumors in experimental animals. While in several countries the use of the alkenylbenzenes estragole, methyleugenol, safrole and β-asarone as flavorings in food products is regulated and prohibited, the use of PFS containing these ingredients in high levels is not regulated. This is remarkable since the use of PFS might result in high exposures to these compounds and consequently low MOE values suggesting a high priority for risk management. It must be emphasized that in the present chapter the MOE values are calculated based on $BMDL_{10}$ values obtained in studies administering high doses of the pure alkenylbenzene compounds to rodents, instead of dosing the botanical as such or in a form of a multicomponent extract. Ideally the studies in rodents should be performed with the botanical or the standardized traditional extract(s) of that botanical. This because studies are already available suggesting that results obtained with the botanical or its extract, containing the genotoxic compound in its food matrix, may be different from those obtained when using the purified genotoxic carcinogenic compounds (de Paula *et al.*, 2007; Jeurissen *et al.*, 2008; Smith *et al.*, 2002). Such differences may occur for example when additional compounds present in the botanical or its extract may modulate the bioactivation and/or

detoxification pathways of the genotoxic carcinogen of interest. For example, Jeurissen *et al.* (2008) demonstrated that a methanolic basil extract was able to adequately inhibit the sulfotransferase catalyzed bioactivation and DNA-adduct formation in HepG2 human hepatoma cells exposed to the proximate carcinogen of estragole. The basil compound responsible for the inhibition was subsequently identified as nevadensin (Alhusainy *et al.*, 2010). These results indicate that the bioactivation of estragole is likely to be lower when estragole is consumed in the presence of other herbal ingredients compared to the exposure of estragole as a pure compound. If such a matrix effect would occur also upon intake of the alkenylbenzenes from PFS, the use of the $BMDL_{10}$ data obtained in studies with pure estragole, methyleugenol, safrole and β-asarone to calculate the MOE values, would be a worst-case approach and may result in an overestimation of the potential risk for human health. In spite of this, it is still concluded that the use of PFS containing the genotoxic carcinogens estragole, methyleugenol, safrole or β-asarone might raise a potential concern for human health and would be of high priority for risk management.

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Supplementary Materials Chapter 3

Table S1. Results from a BMD analysis for induction of hepatocellular carcinomas in mice administered 0, 2300 or 4600 mg/kg diet estragole 3 days a week for 12 months (Miller *et al.*, 1983) using BMDS software version 2.1.2, a BMD of 10% and default settings. The data used as input for the BMD analysis are presented in Table 2.

Model	No. of parameters	Log Likelihood	p-value	Accepted	BMD_{10} (mg/kg bw/day)	$BMDL_{10}$ (mg/kg bw/day)
Null	1	-100.09				
Full	3	-62.21				
Gamma	1	-62.71	0.61	Yes	8.0	6.5
Logistic	2	-70.84	0.00	No		
LogLogistic	1	-62.21	1.00	Yes	4.7	3.3
Probit	2	-70.14	0.00	No		
LogProbit	2	-62.21	1.00	Yes	4.9	ND*
Multistage	1	-62.71	0.61	Yes	8.0	6.5
Weibull	1	-62.71	0.61	Yes	8.0	6.5
Quantal-linear	1	-62.71	0.61	Yes	8.0	6.5

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

Table S2. Overview of BMD analysis for hepatocellular carcinomas in male and female rats administered 0, 37, 75 or 150 mg/kg bw methyleugenol 5 days per week for 105 weeks (NTP, 2000). BMDS software version 2.1.2 was used for BMD analysis using a BMD of 10% and default settings. The data used as input for the BMD analysis are presented in Table 2.

Gender	Model	No. of parameters	Log Likelihood	p-value	Accepted	BMD_{10} (mg/kg bw/day)	$BMDL_{10}$ (mg/kg bw/day)
Male	Null	1	-105.38				
	Full	4	-84.05				
	Gamma	3	-84.77	0.23	Yes	35.2	21.1
	Logistic	2	-85.67	0.20	Yes	40.8	34.0
	LogLogistic	3	-84.70	0.25	Yes	35.0	21.9
	Probit	2	-85.38	0.26	Yes	37.8	31.6
	LogProbit	3	-84.50	0.35	Yes	35.7	23.3
	Multistage	3	-85.06	0.16	Yes	35.3	19.3
	Weibull	3	-84.91	0.19	Yes	34.3	20.3
	Quantal-linear	2	-86.54	0.08	Yes	20.1	15.3
Female	Null	1	-45.27				
	Full	4	-35.67				
	Logistic	2	-37.81	0.12	Yes	87.6	73.6
	LogLogistic	2	-36.70	0.35	Yes	76.2	55.6
	Probit	2	-37.51	0.16	Yes	84.4	69.9
	LogProbit	2	-36.50	0.43	Yes	74.2	54.9
	Multistage	1	-36.75	0.53	Yes	77.5	57.3
	Weibull	2	-36.74	0.34	Yes	76.9	56.0
	Quantal-linear	1	-37.62	0.27	Yes	75.9	48.8

Table S3. Results from a BMD analysis for induction of hepatocellular carcinomas in mice administered 0, 2500 or 5000 mg/kg diet safrole 3 days a week for 12 months (Miller *et al.*, 1983) using BMDS software version 2.1.2, a BMD of 10% and default settings. The data used as input for the BMD analysis are presented in Table 2.

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	-52.51				
Gamma	1	-54.32	0.16	Yes	6.2	5.1
Logistic	2	-65.17	0.00	No		
LogLogistic	1	-52.71	0.82	Yes	2.8	1.9
Probit	2	-65.11	0.00	No		
LogProbit	2	-52.51	1.00	Yes	0.2	ND*
Multistage	1	-54.32	0.16	Yes	6.2	5.1
Weibull	1	-54.32	0.16	Yes	6.2	5.1
Quantal-linear	1	-54.32	0.16	Yes	6.2	5.1

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

Table S4. Overview of BMD analysis performed using BMDS software version 2.1.2, a BMD of 10% and default settings. BMD10 and BMDL10 values are derived from the incidence of leiomyosarcomas of the small intestine in male rats following a 2 year period of treatment with 0, 400, 800, or 2000 mg/kg diet β-asarone (JECFA, 1981). The data used as input for the BMD analysis are presented in Table 2.

Model	No. of parameters	Log Likelihood	p-value	Accepted	BMD_{10} (mg/kg bw/day)	$BMDL_{10}$ (mg/kg bw/day)
Null	1	-43.97				
Full	4	-34.31				
Gamma	2	-34.60	0.75	Yes	21.6	10.7
Logistic	2	-35.68	0.25	Yes	27.6	21.5
LogLogistic	2	-34.59	0.76	Yes	21.5	10.1
Probit	2	-35.36	0.35	Yes	26.2	20.2
LogProbit	2	-34.50	0.83	Yes	21.6	10.4
Multistage	2	-34.70	0.68	Yes	22.0	10.5
Weibull	2	-34.65	0.71	Yes	21.4	10.6
Quantal-linear	1	-35.30	0.58	Yes	14.1	9.6

Chapter 4

Physiologically based kinetic models for the alkenylbenzene elemicin in rat and human and possible implications for risk assessment

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Abstract

The present study describes physiologically based kinetic (PBK) models for the alkenylbenzene elemicin (3,4,5-trimethoxyallylbenzene) in rat and human, based on the PBK models previously developed for the structurally related alkenylbenzenes estragole, methyleugenol, and safrole. Using the newly developed models, the level of metabolic activation of elemicin in rat and human was predicted to obtain insight in species differences in the bioactivation of elemicin and read across to the other methoxy allylbenzenes, estragole and methyleugenol. Results reveal that the differences between rat and human in the formation of the proximate carcinogenic metabolite 1'-hydroxyelemicin and the ultimate carcinogenic metabolite 1'-sulfoxyelemicin are limited (<3.8-fold). In addition, a comparison was made between the relative importance of bioactivation for elemicin and that of estragole and methyleugenol. Model predictions indicate that compound differences in the formation of the 1'-sulfoxymetabolites are limited (<11-fold) in rat and human liver. The insights thus obtained were used to perform a risk assessment for elemicin using the Margin of Exposure (MOE) approach and read across to the other methoxy allylbenzene derivatives for which in vivo animal tumor data are available. This reveals that elemicin poses a lower priority for risk management as compared to its structurally related analogues estragole and methyleugenol. Altogether, the results obtained indicate that PBK modeling provides an important insight in the occurrence of species differences in the metabolic activation of elemicin. Moreover, they provide an example of how PBK modeling can facilitate a read across in risk assessment from compounds for which in vivo toxicity studies are available to a compound for which only limited toxicity data have been described, thus contributing to the development of alternatives for animal testing.

Introduction

Elemicin (3,4,5-trimethoxyallylbenzene) is an aromatic compound that naturally occurs in a variety of herbs and spices such as nutmeg (*Myristica fragrans* Houtt.), parsley [*Petroselinum crispum* (Mill.) A. W. Hill], sassafras [*Sassafras albidum* (Nutt.) Nees.] (EFSA, 2009) and products made thereof including plant food supplements (PFS). Belonging to the group of alkenylbenzenes, elemicin is structurally related to estragole, methyleugenol, safrole, apiol, and myristicin (Figure 1). A number of rodent carcinogenicity studies revealed hepatocarcinogenic effects of estragole, methyleugenol, and safrole, and a genotoxic mode of action has been suggested for these compounds (SCF 2001a, 2001b, 2002). However, elemicin is less well studied as compared to its structurally related analogues and only limited toxicological data are available.

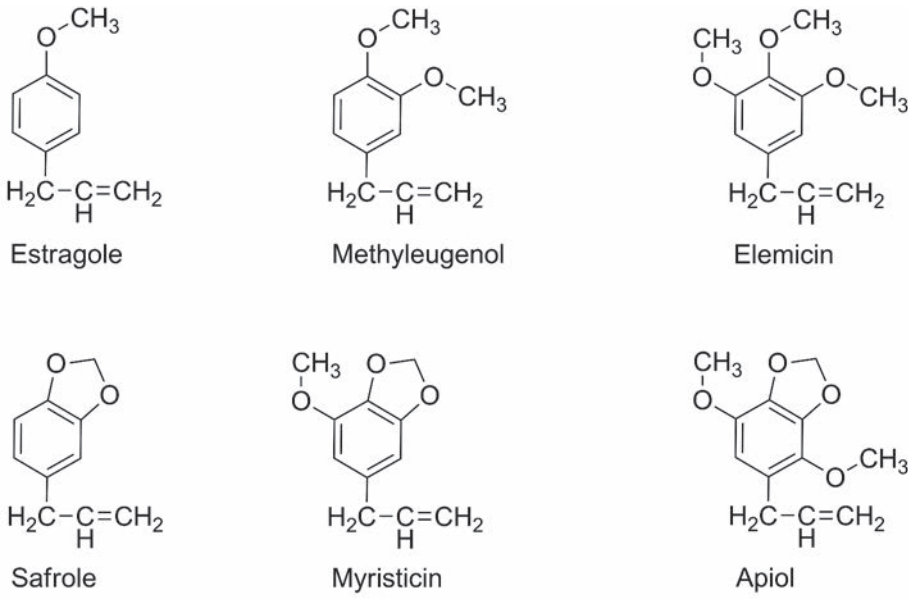

Figure 1. The structural formulas of estragole, methyleugenol, elemicin, safrole, myristicin and apiol.

On the basis of the studies available, elemicin is indicated to be DNA reactive; however, evidence for hepatocarcinogenic activity in rodents is equivocal (Hasheminejad and Caldwell, 1994; Miller *et al.*, 1983; Phillips *et*

al., 1984; Randerath *et al.*, 1984; Wiseman *et al.*, 1987). Elemicin gave positive results in several genotoxicity assays, that is, an unscheduled DNA synthesis (UDS) assay in adult rat hepatocytes and two in vivo DNA-binding assays using mice, indicating DNA reactivity (Hasheminejad and Caldwell, 1994; Phillips *et al.*, 1984; Randerath *et al.*, 1984). Nevertheless, DNA binding was up to 27-fold lower at comparable experimental conditions as compared to that of structurally related compounds including estragole, methyleugenol, and safrole (Phillips *et al.*, 1984; Randerath *et al.*, 1984). Miller *et al.* (1983) revealed that there was no significant hepatocarcinogenic response in mice exposed to elemicin or its metabolite 1′-hydroxyelemicin via *i.p.* injections with a total dose corresponding to 4.75 µmol/animal calculated to be equivalent to 49.5 mg/kg bw elemicin or 53.3 mg/kg bw 1′-hydroxyelemicin assuming a bw of 20 g. In line with these results, Wiseman *et al.* (1987) showed that 1′-hydroxyelemicin had no hepatocarcinogenic activities in male mice after *i.p.* injections of a single dose of 0.1 or 0.25 µmol/g bw 1′-hydroxyelemicin corresponding to 22.4 or 56.3 mg/kg bw 1′-hydroxyelemicin. However, a significant increased incidence in the formation of treatment-related liver tumors was observed upon administration of 1′-hydroxyelemicin to male mice at the highest dose tested (*i.e.* four injections with a total dose of 9.5 µmol which equals 106.5 mg/kg bw) (Wiseman *et al.*, 1987).

The DNA reactive and carcinogenic activities of estragole, methyleugenol, and safrole have been ascribed to their metabolic activation to an unstable DNA reactive 1′-sulfoxymetabolite (Boberg *et al.*, 1983; Phillips *et al.*, 1981; Randerath *et al.*, 1984; Wiseman *et al.*, 1985). On the basis of the related structural characteristics of estragole, methyleugenol, safrole, elemicin, myristicin, and apiol (*i.e.* all containing alkylated and no free hydroxyl substituents and an allyl side chain with a C2=C3 double bond), similar metabolic pathways can be expected for these six alkenylbenzenes. Figure 2 gives an overview of the different metabolic pathways of elemicin based on the metabolites formed from the related alkenylbenzenes and also the metabolites identified in rat and human urine after elemicin exposure (Beyer *et al.*, 2006). *O*-demethylation, 1′-hydroxylation, 3′-hydroxylation and epoxidation are the most important cytochrome P450-catalyzed conversions. *O*-demethylation of the alkoxy ring substituents represents a metabolic detoxification because the resulting (di)phenolic derivate can be conjugated and excreted in the urine as the sulfate or glucuronic acid metabolite (Anthony *et al.*, 1987; Benedetti *et al.*, 1977; Beyer *et al.*, 2006; Lee *et al.*, 1998; Solheim and Scheline, 1973). Another

detoxification route is the formation of a 2',3'-epoxide arising after epoxidation of the alkene side chain. Although epoxides of related alkenylbenzenes have been shown to be able to form DNA adducts in vitro, these epoxides when formed in vivo are rapidly detoxified by glutathione S-transferases and epoxide hydrolases (Luo and Guenthner, 1996; Luo *et al.*, 1992). In addition, the formation of 3'-hydroxymetabolites is considered to be an important metabolic pathway. Although it is stated in the literature that 1'-hydroxymetabolites can be isomerized yielding 3'-hydroxymetabolites (Borchert *et al.*, 1973b; Solheim and Scheline, 1976, 1980; Stillwell *et al.*, 1974), others pointed toward a direct formation of 3'-hydroxymetabolites from the parent alkenylbenzene (Boberg *et al.*, 1986; Martati *et al.*, 2011; Punt *et al.*, 2008). 3'-Hydroxymetabolites can be oxidized to cinnamic acid derivatives and can ultimately be excreted as glycine conjugates (Anthony *et al.*, 1987; Boberg *et al.*, 1986; Solheim and Scheline, 1973, 1976, 1980). 1'-Hydroxylation of the alkene side chain yields the proximate carcinogenic metabolite and is considered as the primary bioactivation pathway (Borchert *et al.*, 1973a; Borchert *et al.*, 1973b; Gardner *et al.*, 1997; Zangouras *et al.*, 1981). It is reported in the literature that a notable increase in the formation of the 1'-hydroxymetabolite occurs after an increase in dose of the parent compound which is accompanied by a shift in metabolic pathways (Anthony *et al.*, 1987; Benedetti *et al.*, 1977; Zangouras *et al.*, 1981). 1'-Hydroxylation of the alkene side chain appears to be more prominent at relatively high exposure levels when enzymes involved in the *O*-demethylation pathway become saturated (Zangouras *et al.*, 1981). However, going from dose levels at which for different alkenylbenzenes tumor incidence is already observed (3-50 mg/kg bw/day) down to dose levels representing realistic human exposure the metabolic shift and changes in the relative amount of bioactivation to the 1'-hydroxymetabolites might be limited. Sulfonation of the 1'-hydroxymetabolite results in the formation of the ultimate carcinogenic 1'-sulfoxymetabolite. Because of the unstable nature of this metabolite in an aqueous environment, a reactive electrophilic metabolite is formed that can bind covalently to proteins, RNA, and DNA (Boberg *et al.*, 1983; Phillips *et al.*, 1981; Randerath *et al.*, 1984; Wiseman *et al.*, 1985). In addition to being converted in the sulfonation pathway, the 1'-hydroxymetabolite can be converted and detoxified by glucuronidation or oxidation, the latter possibly followed by glutathione (GSH) conjugation (Fennell *et al.*, 1984; Punt *et al.*, 2009).

In 2008, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that for the alkenylbenzenes estragole, methyleugenol, safrole, elemicin, myristicin, and apiol "further research is needed to assess the potential risk to human health from low-level dietary exposure to alkoxy-substituted allylbenzenes present in foods and essential oils and used as flavoring agents" (JECFA, 2008). However, for an assessment of the potential risk to human health at low-level dietary exposure scenarios, it is of importance to take non-linear effects as well as species differences in kinetics into consideration when extrapolating data obtained in animal experiments performed with high dose levels to low dose levels corresponding to relevant dietary human exposures. On the basis of the mode of action, physiologically based kinetic (PBK) models for rat and human for estragole (Punt *et al.*, 2008, 2009), methyleugenol (Al-Subeihi *et al.*, 2011, 2012), and safrole (Martati *et al.*, 2011, 2012) were recently developed. Mode of action based PBK modeling enables quantification of the relative importance of detoxification and bioactivation, taking non-linear effects in kinetics into account, allowing a read across from one compound to another and between species, such as from rat to human. The aim of the present study was to extend the rat and human PBK models of estragole, methyleugenol, and safrole to rat and human PBK models for elemicin, to predict the bioactivation and detoxification of elemicin in rat and human at realistic low exposure levels. This will facilitate read across from data on the two structurally related methoxy allylbenzenes estragole and methyleugenol for which in vivo toxicity studies are available, to elemicin for which the toxicological database is far more limited.

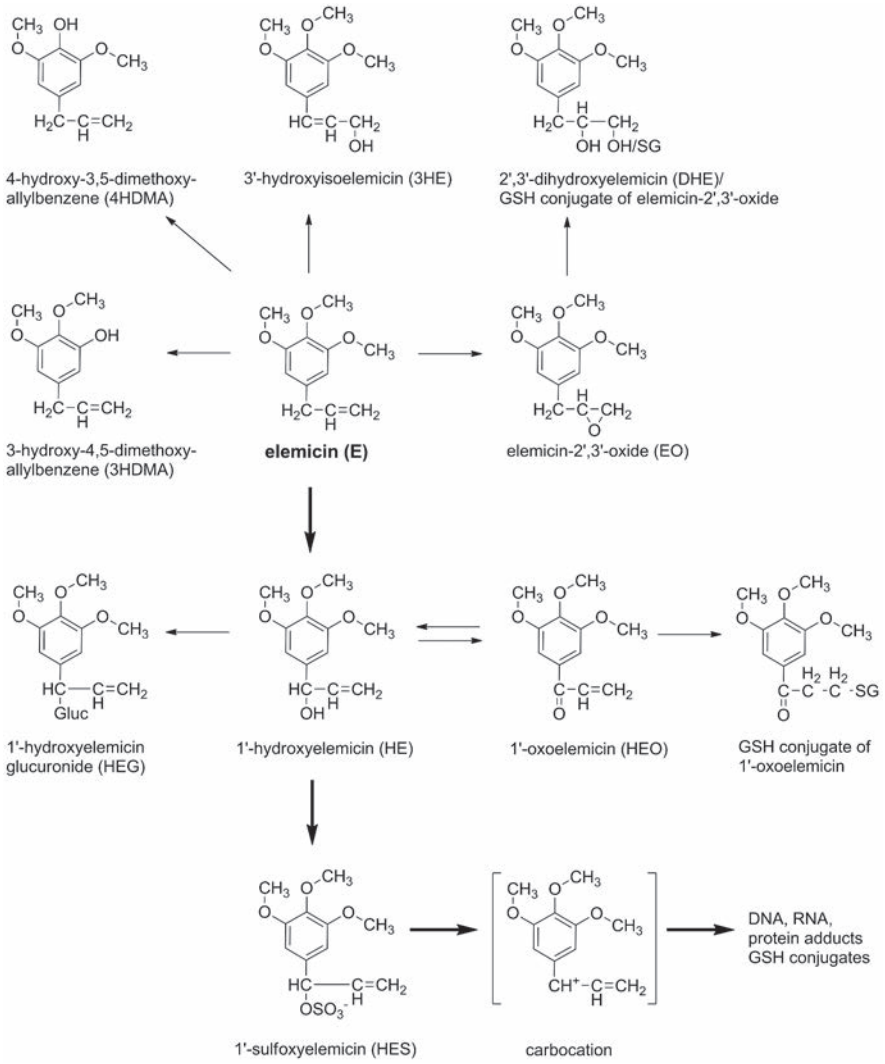

Figure 2. Proposed metabolic pathways of the alkenylbenzene elemicin.

Materials and methods

Caution: elemicin and 1'-hydroxyelemicin are possible carcinogens and should be handled with care.

Chemicals and reagents

Elemicin was purchased from Synchem OHG (Felsberg, Germany). Tris(hydroxymethyl)aminomethane, uridine 5'-diphosphoglucuronic acid (UDPGA), reduced L-glutathione (GSH), alamethicin (from *Trichoderma viride*), 3'-phosphoadenosine 5'-phosphosulfate (PAPS), L-cysteine, N-acetyl-L-cysteine and 4-allyl-2,6-dimethoxyphenol (also known as 4-hydroxy-3,5-dimethoxyallylbenzene) were purchased from Sigma-Aldrich (Steinheim, Germany). β-Nicotinamide adenine dinucleotide (NAD^+), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) and reduced β-nicotinamide adenine dinucleotide (NADH) were from Roche Diagnostics (Mannheim, Germany). β-glucuronidase (from *Escherichia Coli*) was purchased from Boehringer (Mannheim, Germany) and dimethylsulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium).Potassium dihydrogen phosphate, dipotassium hydrogen phosphate trihydrate, acetic acid and magnesium chloride were supplied by VWR International (Darmstadt, Germany). Acetonitrile (ULC/MS grade) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Pooled male rat liver microsomes and S9 from Sprague Dawley and mixed gender pooled human liver microsomes and S9 were from BD Gentest (Woburn, USA). Pooled male Sprague Dawley rat lung, kidney and small intestinal microsomes and pooled gender human lung, kidney and intestinal microsomes were purchased from BioPredic International (Rennes, France).

Synthesis of metabolites

A synthetic standard of 1'-hydroxyelemicin was prepared as described previously for 1'-hydroxysafrole using 1,2,3-trimethoxybenzaldehyde as starting material (Jeurissen *et al.*, 2004). GC-MS analysis was performed as described by Jeurissen *et al.* (2004) for the analysis of 1'-hydroxysafrole. Based on GC-MS and HPLC analyses, the purity of 1'-hydroxyelemicin was found to be more than 95%. Synthesis of 3'-hydroxyisoelemicin was based on the method described previously using 1'-hydroxyelemicin as starting material (Borchert

et al., 1973b; Solheim and Scheline, 1980). Based on HPLC analysis, the purity of 3′-hydroxyisoelemicin was found to be more than 65%. The synthesis of 1′-oxoelemicin from 1′-hydroxyelemicin was carried out as described previously for the synthesis of 1′-oxosafrole from 1′-hydroxysafrole (Wislocki *et al.*, 1976). HPLC analysis revealed that the purity of 1′-oxoelemicin was more than 95%. Elemicin-2′,3′-oxide was prepared based on the procedure reported by Luo *et al.* (1992) using elemicin as starting material. Based on HPLC analysis the purity was found to equal 10% with the remaining 90% consisting of the starting material elemicin, but this was sufficient for identification.

The identities of elemicin, 1′-hydroxyelemicin, 3′-hydroxyisoelemicin, 1′-oxoelemicin and elemicin-2′,3′-oxide were confirmed by NMR analysis. Characteristic NMR chemical shifts are presented in Table S1 of the Supplementary Materials of Chapter 4.

In vitro Incubations

Microsomal metabolism of elemicin by relevant rat and human tissue fractions

In a first step, it was determined which organs are involved in the biotransformation of elemicin in rat and human. For this purpose, liver, kidney, lung and small intestine microsomes from male Sprague Dawley rat and human were used. Incubations were performed by adding 1 mg/mL of the microsomal protein preparations to an incubation mixture containing (final concentrations) 3 mM NADPH in 0.2 M Tris-HCl (pH 7.4). After a 1 min pre-incubation at 37°C, elemicin (final concentration 100 µM) was added from a 100 times concentrated stock solution in DMSO so that the final DMSO content was 1% (v/v). Incubations were carried out for 30 min after which the reactions were terminated by adding 25 µL ice-cold acetonitrile. All samples were centrifuged for 5 min at 16,000*g* and the supernatant was stored at -20˚C until High Performance Liquid Chromatography (HPLC) analysis.

For male rats, metabolism of elemicin was observed in incubations with liver and lung microsomes and hence kinetic constants for the formation of microsomal metabolites were determined in the respective tissue fractions (see result section). For humans these constants were determined for elemicin microsomal metabolism occurring in incubations with liver microsomes only since microsomal samples from no other human organs investigated were found capable of metabolizing this alkenylbenzene (see results section). Incubations to determine kinetic constants were performed following the conditions

described above using final concentrations of elemicin from 25 to 1000 µM for rat and human liver, and from 50 to 2000 µM for rat lung. In all incubations, the final concentration of DMSO, in which elemicin was dissolved, was kept at a final concentration of 1% (v/v). Formation of different microsomal metabolites was linear with time and microsomal protein concentrations under the conditions described. Blank incubations were performed in the absence of the cofactor NADPH. All incubations were performed in duplicate.

Stereochemistry of 1′-hydroxyelemicin

1′-Hydroxyelemicin is a chiral molecule. To determine if the synthetic and metabolically formed 1′-hydroxyelemicin existed of two enantiomers, the chirality of both the synthetized 1′-hydroxyelemicin as well as of the 1′-hydroxyelemicin formed in microsomal incubations was analyzed using the chiral HPLC method described by Punt *et al.* (2007) to quantify the enantiomers of 1′-hydroxyestragole with minor modifications. In short, an isocratic elution using 10 mM sodium acetate (pH 4.5) was applied at a flow rate of 0.4 mL/min to separate both enantiomers.

Glucuronidation of 1′-hydroxyelemicin

The kinetic constants for the metabolic conversion of 1′-hydroxyelemicin to 1′-hydroxyelemicin glucuronide in both male rat and human liver fractions were determined as described previously for related 1′-hydroxyalkenylbenzenes (Al-Subeihi *et al.*, 2011 and 2012; Martati *et al.*, 2011 and 2012; Punt *et al.*, 2008 and 2009) with minor modifications. In short, incubations were performed using (final concentrations) 10 mM UDPGA and 0.5 mg/mL male Sprague Dawley S9 protein in 0.2 M Tris-HCl (pH 7.4) with 10 mM $MgCl_2$. To overcome enzyme latency and to obtain maximal glucuronidation activity, incubations containing S9 were pre-treated on ice with 0.025 mg/mL alamethicin added from a 200 times concentrated stock dissolved in methanol (Fisher *et al.*, 2000; Lin and Wong, 2002). After 15 min of alamethicin treatment, samples were pre-incubated at 37˚C for 1 min, and reactions were subsequently started by adding 1′-hydroxyelemicin in final concentrations of 50 to 4000 µM. 1′-Hydroxyelemicin was added from a 200 times concentrated stock dissolved in DMSO. The reaction was incubated for 30 min and terminated by adding 25 µL of ice-cold acetonitrile. Blank incubations were carried out in the absence of the cofactor UDPGA. Experiments were performed in duplicate. Similar incubations were

performed using mixed gender human pooled liver S9 proteins. However, because of a lower rate of glucuronidation by human S9 proteins, samples were incubated for a longer time, that is, 120 min. The formation of 1′-hydroxyelemicin glucuronide was linear with time and S9 protein concentration under the experimental conditions described.

The formation of 1′-hydroxyelemicin glucuronide was verified by incubating a sample made as described above, for which the reaction was not terminated using acetonitrile, with β-glucuronidase. For this purpose, 90 µL sample was added to 10 µL of 1 M potassium phosphate (pH 6.2) and 2 µL of β-glucuronidase solution (final concentration 0.3 U) and subsequently incubated for 1 h at 37˚C. All samples were centrifuged for 5 min at 16,000*g* and the supernatant was stored at -20˚C until HPLC analysis.

Oxidation of 1′-hydroxyelemicin

The kinetic constants for the enzymatic conversion of 1′-hydroxyelemicin to 1′-oxoelemicin were determined using incubation mixtures containing (final concentrations) 3 mM NAD^+, 2 mM GSH and 1 mg/mL rat liver microsomes or human liver S9 in 0.2 M Tris-HCl (pH 7.4). GSH was added to the incubation mixtures to trap the reactive 1′-oxometabolite formed after oxidation of 1′-hydroxyelemicin (Punt *et al.*, 2009). Although the formation of the GSH adduct of 1′-oxoelemicin was also observed in incubations using human liver homogenates with $NADP^+$ as a cofactor, NADP is mainly present in reduced form with a $NADP^+$/NADPH ratio of about 0.005 in human liver. Incubations using different ratios of $NADP^+$/NADPH (*i.e.* 0.005, 0.01, 0.05, 0.1, 0.5, and 1) revealed that the rate of conversion of 1′-hydroxyelemicin to 1′-oxoelemicin was not significant when in addition to $NADP^+$ also NADPH was present at a physiologically relevant ratio (data not shown). Therefore, kinetic constants for this reaction in rat and human liver were derived by performing incubations with NAD^+ as a cofactor, given that in rat and human liver, NAD is mainly present in an oxidized form with levels of NAD^+ being much higher than that of NADH. Prior to the addition of 1′-hydroxyelemicin at final concentrations ranging between 40 and 4000 µM to the incubation mixture, samples were pre-incubated for 1 min at 37˚C. Reactions were terminated after 5 min by adding 25 µL of ice-cold acetonitrile. The formation of the GSH conjugate of 1′-oxoelemicin, GS-1′-oxoelemicin, was linear with time and microsomal or S9 protein concentrations under the experimental conditions used. Blank incubations were performed

without the cofactor NAD^+. Incubations were performed in duplicate. All samples were centrifuged for 5 min at 16,000*g*, and the supernatant was stored at -20˚C until HPLC analysis.

Sulfonation of 1'-hydroxyelemicin

The formation of 1'-sulfoxyelemicin was determined using incubations containing male rat liver Sprague Dawley S9 or mixed gender pooled human liver S9 proteins, PAPS as a cofactor and GSH, which acts as a scavenger of the reactive carbocation formed due to the unstable nature of the 1'-sulfoxymetabolite in an aqueous environment (Al-Subeihi *et al.*, 2011; Martati *et al.*, 2011). Incubation mixtures containing (final concentrations) 10 mM GSH, 0.2 mM PAPS and 4 mg/mL male rat liver S9 or mixed gender pooled human liver S9 proteins in 0.1 M potassium phosphate (pH 8.2) were pre-incubated for 1 min at 37˚C. After this pre-incubation, 1'-hydroxyelemicin dissolved in DMSO was added in final concentrations ranging between 0.5 and 4000 µM, keeping the final percentage of DMSO at 1%. The reaction was terminated after 180 min by adding 25 µL of ice-cold acetonitrile. The formation of the GSH conjugate of 1'-sulfoxyelemicin was linear with time and S9 protein concentrations under the experimental conditions used. The scavenging may be either chemically or catalyzed by the glutathione S-transferases present in the S9 incubations in which the sulfonation of 1'-hydroxyelemicin was measured. The mechanism underlying the scavenging of 1'-sulfoxyelemicin by GSH was investigated to a further extent performing additional incubations with cysteine or N-acetylcysteine as trapping agent using incubation conditions as described above. Blank incubations were performed in the absence of PAPS. Incubations were performed in duplicate. All samples were centrifuged for 5 min at 16,000*g* and the supernatant was stored at -20˚C until Ultra Performance Liquid Chromatography (UPLC) analysis.

Identification and quantification of elemicin metabolites

For identification and quantification of microsomal elemicin metabolites, aliquots of 50 µL of each sample were subjected to HPLC analysis as described by Punt *et al.* (2008). Identification was achieved by comparing the retention times and UV spectra of the formed metabolites to the retention times and UV spectra of the commercially available or synthesized reference standards. Quantification of the formed metabolites was done by comparing the peak areas to those of calibration curves of the corresponding reference compounds

at a wavelength of 208 nm. The synthetized elemicin-2′,3′-oxide still contained a substantial amount of the starting compound elemicin and could thus be used for identification but not for quantification. Therefore quantification of elemicin-2′,3′-oxide and 2′,3′-dihydroxyelemicin, reflecting the formation of elemicin-2′,3′-oxide, was achieved using the calibration curve for 1′-hydroxyelemicin which was shown to have the same UV spectrum. Quantification of 3′-hydroxyisoelemicin was done at a wavelength of 265 nm.

Chromatographic analysis of 1′-hydroxyelemicin glucuronide and 1′-oxoelemicin in both male rat and human liver incubations was performed as described previously by Punt *et al.* (2008) and Punt *et al.* (2009), respectively. Because a synthetic reference compound was not available for 1′-hydroxyelemicin glucuronide, quantification was based on comparison of the peak area of the formed metabolite to the calibration curve of 1′-hydroxyelemicin. This could be done because 1′-hydroxyelemicin glucuronide was found to have the same UV spectrum as 1′-hydroxyelemicin. Quantification of GS-1′-oxoelemicin was based on a calibration curve for GS-1′-oxoelemicin made as previously described (Al-Subeihi *et al.*, 2011; Martati *et al.*, 2011; Punt *et al.*, 2009). In short, 60 µM of the synthetic standard of 1′-oxoelemicin, dissolved in acetonitrile, was incubated with different concentrations of GSH (*i.e.* 0 to 20 µM) in 0.2 M Tris-HCl (pH 7.4) for 6 h at 37 °C resulting in full conversion of the GSH to GS-1′-oxoelemicin. Quantification was done comparing the peak areas of the formed GS-1′-oxometabolite in the incubation mixtures with peak areas of the GS-1′-oxoelemicin calibration curve thus obtained at a wavelength of 283 nm.

Quantification of 1′-sulfoxyelemicin was done using UPLC analysis as described for the quantification of 1′-sulfoxysafrole (Martati *et al.*, 2011). The UV spectrum of the GSH adduct of 1′-sulfoxyelemicin was found to be similar to the UV spectrum of 3′-hydroxyisoelemicin and quantification of the GSH adduct of 1′-sulfoxyelemicin was thus accomplished by comparing the peak area of this metabolite to the calibration curve of 3′-hydroxyisoelemicin.

Determination of kinetic constants

Kinetic constants for the metabolic conversions of elemicin and 1′-hydroxyelemicin were derived by fitting the data to a standard Michaelis-Menten equation; $v = V_{max} \times [S] / K_m + [S]$, in which [S] represents the substrate concentration, V_{max} the maximum velocity and K_m the Michaelis-Menten constant for the formation of the different metabolites of elemicin or 1′-hydroxyelemicin. Data analysis was accomplished using GraphPad Prism, version 5.04 (GraphPad Software, San Diego, California, USA).

PBK models

Two PBK models were developed describing the relative importance of bioactivation and detoxification of elemicin in, respectively, rat and human at different oral dose levels. The models developed in this study were essentially based on the PBK models previously defined for the metabolism of estragole (Punt *et al.*, 2008 and 2009), methyleugenol (Al-Subeihi *et al.*, 2011 and 2012), and safrole (Martati *et al.*, 2011 and 2012) in rat and human. A schematic overview of the developed PBK models for elemicin metabolism in rat and human is shown in Figure 3. The models consist of several compartments representing different organs and tissues (*i.e.* liver, lungs, fat tissue, richly perfused tissues and slowly perfused tissues) that are mutually connected through the systemic circulation. First order kinetics was used to describe the uptake of elemicin from the gastrointestinal tract assuming a direct uptake by the liver with an absorption rate constant (k_a) of 1.0 h^{-1} which is based on the fast and complete absorption of the structurally related alkenylbenzene estragole from the gastrointestinal tract (Punt *et al.*, 2008).

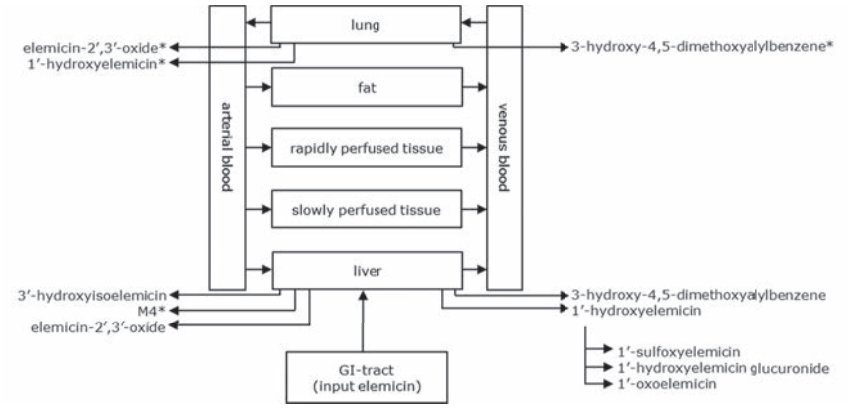

Figure 3. Schematic overview of the physiologically based biokinetic (PBK) model for elemicin in rat and human. Metabolites marked with an asterisk (*) were only found in rat tissue fractions.

For rat, the liver and lungs were able to convert elemicin to different microsomal metabolites (see result section). 2',3'-Dihydroxyelemicin, 1'-hydroxyelemicin, 3'-hydroxyisoelemicin, an unidentified minor metabolite referred to as M4, elemicin-2',3'-oxide, 4-hydroxy-3,5-dimethoxyallylbenzene and 3-hydroxy-4,5-dimethoxyallylbenzene were formed in rat liver whereas

in rat lung minor amounts of 2′,3′-dihydroxyelemicin, elemicin-2′,3′-oxide, 1′-hydroxyelemicin and 3-hydroxy-4,5-dimethoxyallylbenzene were formed (see result section). Since 2′,3′-dihydroxyelemicin is formed after hydrolysis of elemicin-2′,3′-oxide, data for both metabolites that result from epoxidation of elemicin were combined and referred to as elemicin-2′,3′-oxide. 4-Hydroxy-3,5-dimethoxyallylbenzene was only formed in minor quantities at the highest concentrations tested and therefore excluded from further analysis. Accordingly, mass balance equations for elemicin in rat liver and lung were as follows:

$$\text{Liver: } dAL_E/dt = dUptake_E/dt + QL*(CA_E - CL_E/PL_E) - V_{max,L\_HE}*CL_E/PL_E/(K_{m,L\_HE} + CL_E/PL_E) - V_{max,L\_3HE}*CL_E/PL_E/(K_{m,L\_3HE} + CL_E/PL_E) - V_{max,L\_EO}*CL_E/PL_E/(K_{m,L\_EO} + CL_E/PL_E) - V_{max,L\_M4}*CL_E/PL_E/(K_{m,L\_M4} + CL_E/PL_E) - V_{max,L\_3HDMA}*CL_E/PL_E/(K_{m,L\_3HDMA} + CL_E/PL_E)$$

$$dUptake_E/dt = -dAGI_E/dt = K_a*AGI_E,\ AGI_E\ (0) = \text{oral dose}$$

$$CL_E = AL_E/VL$$

$$\text{Lung: } dALu_E/dt = QC * (CV_E - Clu_E/PLu_E) - V_{max,Lu\_EO}*CLu_E/PLu_E/(K_{m,Lu\_EO} + CLu_E/PLu_E) - V_{max,Lu\_HE}*CLu_E/PLu_E/(K_{m,Lu\_HE} + CLu_E/PLu_E) - V_{max,Lu\_3HDMA}*CLu_E/PLu_E/(K_{m,Lu\_3HDMA} + CLu_E/PLu_E)$$

$$CLu_E = ALu_E/VLu$$

where $uptake_E$ (µmol) is the amount of elemicin taken up from the gastrointestinal tract, AGI_E (µmol) is the amount of elemicin remaining in the gastrointestinal tract, ATi_E (µmol) is the amount of elemicin in a tissue (Ti = L (liver) or Lu (lung)). CTi_E is the elemicin concentration in a tissue (µmol/L). CA_E and CV_E are the elemicin concentrations in the arterial and venous blood (both in µmol/L), QTi is the blood flow rate to a tissue (L/h), QC is the cardiac output (L/h), VTi is the volume of a tissue (L), PTi_E is the tissue/blood partition coefficient of elemicin, and V_{max,Ti_M} and K_{m,Ti_M} are the values representing the maximum rate of formation and Michaelis-Menten constant, respectively, for the formation of 1′-hydroxyelemicin (HE), 3′-hydroxyisoelemicin (3HE), elemicin-2′,3′-oxide (EO), M4 (M4), and 3-hydroxy-4,5-dimethoxyallylbenzene (3HDMA).

In contrast to rat lung tissue fractions, elemicin was not converted in incubations with human lung tissue fractions and metabolism was only observed in incubations with human liver fractions (see results section). Moreover, M4 was not formed in a significant quantity in incubations with human liver tissue and was therefore not included in the human PBK model. As a result, the mass balance equation for elemicin metabolism in human liver tissue is as follows:

$$\begin{aligned}\text{Liver: } dAL_E/dt = {} & dUptake_E/dt \\ & + QL*(CA_E - CL_E/PL_E) \\ & - V_{max,\,L\_HE}*CL_E/PL_E/(K_{m,L\_HE} + CL_E/PL_E) \\ & - V_{max,\,L\_3HE}*CL_E/PL_E/(K_{m,L\_3HE} + CL_E/PL_E) \\ & - V_{max,\,L\_EO}*CL_E/PL_E/(K_{m,L\_EO} + CL_E/PL_E) \\ & - V_{max,\,L\_3HDMA}*CL_E/PL_E/(K_{m,L\_3HDMA} + CL_E/PL_E)\end{aligned}$$

$$dUptake_E/dt = - dAGI_E/dt = K_a * AGI_E,\ AGI_E\,(0) = \text{oral dose}$$
$$CL_E = AL_E/VL$$

In both models, the conversion of elemicin to 3'-hydroxyisoelemicin, elemicin-2',3'-oxide, and 3-hydroxy-4,5-dimethoxyallylbenzene are described, but further reactions with these metabolites were not included in the model. For the rat model, also no further conversion of M4 was included. It was assumed that these metabolites and/or their possible conjugates were completely excreted into the urine. Since the liver is the major target organ for alkenylbenzene-induced carcinogenicity in rats and mice, the present study focused primarily on metabolic conversion of 1'-hydroxyelemicin by glucuronidation, oxidation and sulfonation in the liver only. The mass balance equation for 1'-hydroxyelemicin in rat and human liver is as follows:

$$\begin{aligned}\text{Liver: } dAL_{1HE}/dt = {} & V_{max,\,L\_HE}*CL_E/PL_E/(K_{m,\,L\_HE} + CL_E/PL_E) \\ & V_{max,\,L\_HEG}*CL_{HE}/PL_{HE}/(K_{m,\,L\_HEG} + CL_{HE}/PL_{HE}) \\ & V_{max,\,L\_HEO}*CL_{HE}/PL_{HE}/(K_{m,\,L\_HEO} + CL_{HE}/PL_{HE}) \\ & V_{max,\,L\_HES}*CL_{HE}/PL_{HE}/(K_{m,\,L\_HES} + CL_{HE}/PL_{HE})\end{aligned}$$
$$CL_{HE} = AL_{HE}/VL$$

where AL_{HE} is the amount of 1'-hydroxyelemicin in the liver (µmol), CL_{HE} is the 1'-hydroxyelemicin concentration in the liver (µmol/L), PL_{HE} is the liver/

blood partition coefficient of 1′-hydroxyelemicin, and V_{max,L_M} and K_{m,L_M} are the maximum rate of formation and Michaelis-Menten constant, respectively, for the formation of the different 1′-hydroxyelemicin metabolites in the liver, including 1′-hydroxyelemicin glucuronide (HEG), 1′-oxoelemicin (HEO), and 1′-sulfoxyelemicin (HES).

V_{max} and K_m values for the different metabolic pathways of elemicin and 1′-hydroxyelemicin were derived in vitro in the present study. V_{max} values that were derived in vitro expressed as nmol min^{-1} (mg liver microsomal or S9 protein)$^{-1}$ were scaled to values representing the V_{max} per µmol h^{-1} (g liver)$^{-1}$ using microsomal and S9 protein yields of 35 and 143 mg/g liver respectively as defined by Punt *et al.* (2008, 2009) based on Medinsky *et al.* (1994). For lung V_{max} values expressed as nmol min^{-1} (mg lung microsomal protein)$^{-1}$ were scaled using a microsomal protein yield of 20 mg/g lung (Punt *et al.*, 2009).

Tables 1 and 2 summarize the physiological parameters (*i.e.* tissue volumes, cardiac output and tissue blood flows) for rat and human, respectively, which were derived from literature (Brown *et al.*, 1997). Partition coefficients were derived *in sillico* based on a method described by DeJongh *et al.* (1997) using the log K_{ow}. Log K_{ow} values for elemicin (ClogP 2.51) and 1′-hydroxyelemicin (ClogP 0.958) were estimated using ChemBio3D 2010 (CambrigeSoft, USA). Mass balance equations were coded and numerically integrated in Berkely Madonna 8.3.18 (Macey and Oster, UC Berkeley, CA, USA) using the Rosenbrock's algorithm for stiff systems.

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

Physiological parameters (Brown et al., 1997)		Tissue: blood partition coefficients (DeJongh et al., 1997)	
Body weight (kg)	0.25		
Percentage of body weight		Elemicin	
liver	3.4	liver	1.68
lung	0.5	lung	1.68
fat	7.0	fat	37.73
rapidly perfused	5.1	rapidly perfused	1.68
slowly perfused	60.2	slowly perfused	0.67
arterial blood	1.85		
venous blood	5.55	1′-hydroxyelemicin	
Cardiac output (L/h)	5.4	liver	0.94
Percentage of cardiac output			
liver	25.0		
fat	7.0		
rapidly perfused	51.0		
slowly perfused	17.0		

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

Physiological parameters (Brown et al., 1997)		Tissue: blood partition coefficients (DeJongh et al., 1997)	
Body weight (kg)	60		
Percentage of body weight		Elemicin	
liver	2.6	liver	4.22
fat	21.4	fat	83.45
rapidly perfused	5.0	rapidly perfused	4.22
slowly perfused	51.7	slowly perfused	2.81
blood	7.9		
		1′-hydroxyelemicin	
Cardiac output (L/h)	310	liver	0.93
Percentage of cardiac output			
liver	22.7		
fat	5.2		
rapidly perfused	47.3		
slowly perfused	24.8		

Sensitivity analysis

To determine which parameters have the greatest influence on model predictions, a sensitivity analysis was performed as described previously (Al-Subeihi *et al.*, 2011 and 2012; Martati *et al.*, 2011 and 2012; Punt *et al.*, 2008 and 2009). For this purpose normalized sensitivity coefficients (SC) were determined using the following equation: SC = (C′ – C)/(P′ – P) * (P/C), with C being the initial

value of model output, C' the modified value of the model output resulting from an increase in parameter value, P is the initial parameter value and P' represents the modified parameter value. An increase of 5% in parameter values was used to analyze the effect of a change in parameter on the formation of 1'-hydroxyelemicin and 1'-sulfoxyelemicin (expressed as a percentage of the dose over 24 h). Each parameter was analyzed individually while the other parameters were kept at their initial value.

Comparison of the PBK model based prediction of bioactivation of elemicin by rat and human to the PBK model predictions for bioactivation of the structurally related compounds estragole and methyleugenol

The predicted model outcomes for the formation of 1'-hydroxyelemicin and 1'-sulfoxyelemicin were compared with the predicted dose-dependent formation of the 1'-hydroxy and 1'-sulfoxymetabolites of the structurally related alkenylbenzenes. For this purpose the previously defined PBK models for estragole (Punt *et al.*, 2008 and 2009), and methyleugenol (Al-Subeihi *et al.*, 2011 and 2012) were run as described by Martati *et al.* (2011) and Martati *et al.* (2012) for rat and human models, respectively, with minor modifications. The models describing the metabolism of elemicin, estragole and methyleugenol were run for a period of 24 h.

Important to note is that in spite of the fact that safrole shares structural characteristics with elemicin (*i.e.* alkylated and no free hydroxyl substituents and an allyl side chain with a C2=C3 double bond), it contains a methylenedioxy substituent of the allylbenzene nucleus while elemicin contains methoxy substituents similar to estragole and methyleugenol. Hence, the read across from data of estragole and methyleugenol, for which in vivo data are available, may provide a better representation of the carcinogenic potency of elemicin for which the toxicological database is far more limited and gives a better basis for the risk assessment of elemicin. For this reason, a comparison of the PBK model based prediction of bioactivation of elemicin by rat and human to the PBK model prediction for bioactivation of safrole was not made.

Results

Stereochemistry of 1′-hydroxyelemicin

Chiral HPLC analysis of synthetic 1′-hydroxyelemicin and metabolically formed 1′-hydroxyelemicin in male rat and human microsomal liver fractions showed two enantiomers eluting at 14.8 and 16.2 min. Chromatographic analysis revealed that synthetic 1′-hydroxyelemicin consisted of both enantiomers in a ratio of approximately 45:55 and for metabolically formed 1′-hydroxyelemicin ratios of 70:30 and 55:45 were found for rat and human microsomal liver fractions respectively. Since the 1′-hydroxyelemicin formed by rat and human microsomal liver fractions and the one used for the studies on its glucuronidation, oxidation, and sulfonation were shown to consist of a racemic mixture, the synthetic 1′-hydroxyelemicin could be used as a substrate for determining kinetic constants of the respective metabolic conversions of 1′-hydroxyelemicin.

Microsomal conversion of elemicin

To identify which organs are involved in the metabolism of elemicin in both male rat and human, incubations were performed using microsomal protein preparations from liver, kidney, lung and small intestine. Chromatographic analysis of these incubations revealed that for rat no metabolism occurred in incubations with small intestine microsomes or kidney microsomes, while elemicin metabolism was observed in incubations with rat liver and lung tissue fractions at varying rates. For human, microsomal conversion of elemicin to different metabolites was observed in incubations with mixed gender pooled human liver microsomes only, whereas human lung, kidney, and small intestine microsomes were incapable of converting elemicin (data not shown).

Figure 4 shows an example of a chromatogram of an incubation of elemicin with male rat liver microsomes and NADPH as a cofactor. In incubations with rat liver microsomes 2′,3′-dihydroxyelemicin (RT = 7.5 min), 1′-hydroxyelemicin (RT = 13.2 min), 3′-hydroxyisoelemicin (RT = 13.9 min), a minor unidentified metabolite referred to as M4 (RT = 14.8 min), elemicin-2′,3′-oxide (RT = 19.6 min), 4-hydroxy-3,5-dimethoxyallylbenzene (RT = 35.5 min) and 3-hydroxy-4,5-dimethoxyallylbenzene (RT = 36.7 min) were formed. In incubations with human liver microsomes the same seven metabolites were found (for details see Supplementary Materials Figure S1A). In incubations with rat lung microsomes 2′,3′-dihydroxyelemicin, elemicin-2′,3′-oxide, 1′-hydroxyelemicin and 3-hydroxy-4,5-dimethoxyallylbenzene were identified (for details see

Supplementary Materials Figure S1B). Identification was done based on comparison of the UV spectra and retention times of the formed metabolites with those of the specific synthesized or commercially available reference compounds. However, tentative identification of 2',3'-dihydroxyelemicin was done based on analogy of HPLC chromatograms of metabolic conversions of estragole, methyleugenol and safrole and the fact that elemicin-2',3'-oxide, isolated following incubation with liver microsomes, NADPH and elemicin, was found to be hydrolyzed by epoxide hydrolase to 2',3'-dihydroxyelemicin as was previously also demonstrated for the structurally related alkenylbenzenes (Al-Subeihi *et al.*, 2011; Guenthner and Luo, 2001; Luo and Guenthner, 1996; Luo *et al.*, 1992; Martati *et al.*, 2011; Punt *et al.*, 2008). Furthermore, no reference compound for the metabolite eluting at 36.7 min was available. Therefore, tentative identification was based on the metabolism of elemicin proposed by Beyer *et al.* (2006) describing the formation of two *O*-demethylated products, that is, 4-hydroxy-3,5-dimethoxyallylbenzene and 3-hydroxy-4,5-dimethoxyallylbenzene. Moreover, comparison to chromatographic details obtained for microsomal metabolites of methyleugenol was used for tentative identification (Al-Subeihi *et al.*, 2011). Al-Subeihi *et al.* (2011) demonstrated the formation of two *O*-demethylated metabolites of methyleugenol comprising highly similar structures to those formed after metabolism of elemicin namely 4-hydroxy-3-methoxyallylbenzene and 3-hydroxy-4-methoxyallylbenzene. The difference between these metabolites of methyleugenol and the ones formed upon elemicin metabolism is that the latter ones have an extra methoxy moiety attached to their allylbenzene nucleus. In the study of Al-Subeihi *et al.* (2011) it was shown that both compounds eluted closely before the parent compound methyleugenol with 4-hydroxy-3-methoxyallylbenzene eluting first followed by 3-hydroxy-4-methoxyallylbenzene. These results are in line with what was found for the chromatographic profile of elemicin microsomal incubations with two metabolites eluting closely before elemicin of which the metabolite eluting at 35.5 min was identified as 4-hydroxy-3,5-dimethoxyallylbenzene based on the UV spectrum and retention time of its commercially available reference compound. Based on these considerations, the metabolite eluting at 36.7 min was tentatively identified as 3-hydroxy-4,5-dimethoxyallylbenzene. The metabolite indicated as M4 was only formed to a small extent and hence its identification was not deemed necessary. However, it should be noted that in rat liver, the kinetic constants and the formation of M4 are in the same range

as the formation of 3-hydroxy-4,5-dimethoxyphenol (see Table 4, Figure 7). Data revealed that 3′-hydroxyisoelemicin was formed directly from elemicin rather than from isomerization of 1′-hydroxyelemicin since the formation of 3′-hydroxyelemicin was not observed in incubations of 1′-hydroxyelemicin with liver microsomes and NADPH (data not shown).

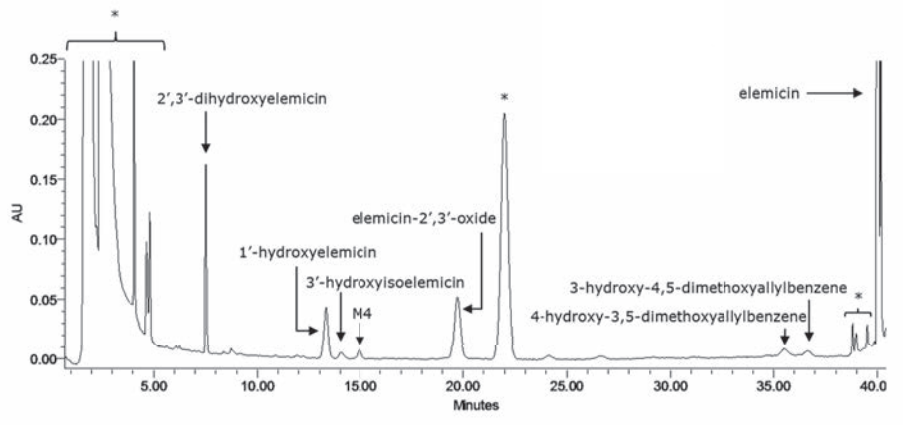

Figure 4. Chromatogram showing the different metabolites of elemicin formed in incubations with male rat liver microsomes. Incubations were performed using a final concentration of 100 µM elemicin and NADPH as a cofactor for 30 min at 37 ˚C. The peaks marked with an asterisk (*) were also present in blank incubations performed in the absence of the cofactor NADPH.

Figure S2 in the Supplementary Materials of Chapter 4 presents the rates of formation of different microsomal metabolites in incubations with male rat liver microsomes (A), male rat lung microsomes (B), and human liver microsomes (C) with increasing elemicin concentrations ranging from 25 to 2000 µM. Table 3 shows the kinetics constants (*i.e.* V_{max} and K_m) derived from these plots and the catalytic efficiencies, calculated as V_{max}/K_m. Analysis of incubations that were performed with male rat liver microsomal preparations revealed that the metabolite arising from epoxidation of elemicin, namely elemicin-2′,3′-oxide, had the highest V_{max} value. Moreover, 1′-hydroxyelemicin and 3′-hydroxyisoelemicin were abundantly formed in incubations with male rat microsomal liver preparations with a high affinity. In incubations performed with male rat liver microsomes, 3-hydroxy-4,5-dimethoxyallylbenzene and M4 were the least important metabolites formed upon conversion of elemicin. In general, the catalytic efficiency for the formation of 1′-hydroxyelemicin by male rat liver microsomes had the highest value, followed by the catalytic efficiently

for formation of 3′-hydroxyisoelemicin, elemicin-2′,3′-oxide, 3-hydroxy-4,5-dimethoxyallylbenzene and M4. The catalytic efficiency for the formation of 1′-hydroxyelemicin was found to be approximately 30-fold higher compared to that for formation of M4. In male rat lung microsomes, elemicin-2′,3′-oxide, 1′-hydroxyelemicin and 3-hydroxy-4,5-dimethoxyallylbenzene were formed with relatively low in vitro catalytic efficiencies indicating that the formation of microsomal elemicin metabolites is more efficient when using rat liver microsomes compared to rat lung microsomes, demonstrating that extra hepatic metabolism of elemicin in rat contributes to only a minor extent to the overall microsomal metabolism as was previously also shown for safrole (Martati *et al.*, 2011).

In incubations with human liver fractions, 1′-hydroxyelemicin was found to be the most abundant metabolite formed followed by 3′-hydroxyisoelemicin and elemicin-2′,3′-oxide. Analysis of the catalytic efficiencies for the formation of different microsomal metabolites of elemicin, obtained with pooled human liver microsomes, showed that the formation of 3-hydroxy-4,5-dimethoxyallylbenzene, 3′-hydroxyisoelemicin and 1′-hydroxyelemicin were the least important routes of elemicin metabolism, whereas the formation of elemicin-2′,3′-oxide represents the major pathway for the conversion of elemicin. M4 and 4-hydroxy-3,5-dimethoxyallylbenzene were only detected in incubations performed with the highest substrate concentrations tested in minor quantities and were therefore not included in further analysis.

Glucuronidation of 1′-hydroxyelemicin

Chromatographic analysis of incubations with male rat liver S9 and mixed gender pooled human liver S9, UDPGA as cofactor and 1′-hydroxyelemicin as substrate revealed a peak at 17.2 min (chromatogram not shown) tentatively identified as 1′-hydroxyelemicin glucuronide since treatment of the sample with β-glucuronidase resulted in full elimination of the respective peak accompanied by a concomitant increase of the peak of 1′-hydroxyelemicin. Moreover, chromatographic analysis of incubations performed in the absence of the cofactor UDPGA did not show a peak at a retention time of 17.2 min. Together these data indicate that the compound eluting at 17.2 min corresponds to 1′-hydroxyelemicin glucuronide.

The rate of the metabolic conversion of 1′-hydroxyelemicin to

1′-hydroxyelemicin glucuronide in incubations with both male rat and human liver fractions with increasing concentrations of 1′-hydroxyelemicin is presented in Figure 5A. Table 3 presents the kinetic constants derived from these plots.

Oxidation of 1′-hydroxyelemicin

Figure 5B shows the rate of oxidation of 1′-hydroxyelemicin in incubations with male rat liver microsomes and pooled human liver S9 with increasing concentrations of 1′-hydroxyelemicin. The kinetic constants derived from these plots are presented in Table 3.

Table 3. Kinetic constants for metabolism of elemicin and 1′-hydroxyelemicin in incubations with Sprague Dawley male rat liver and lung microsomes and mixed gender pooled human liver microsomes.

Metabolite	Organ	Rat			Human		
		V_{max} [a,b]	K_m [a,c]	in vitro catalytic efficiency[d]	V_{max} [a,b]	K_m [a,c]	in vitro catalytic efficiency[d]
Conversion of elemicin							
1′-hydroxyelemicin	Liver	0.5	19	26.3	1.1	415	2.7
	Lung	0.06	189	0.3	ND	ND	-
3′-hydroxyisoelemicin	Liver	0.4	16	25.0	0.6	83	7.27
M4	Liver	0.2	213	0.9	ND	ND	-
elemicin-2′,3′-oxide	Liver	1.8	139	12.9	0.5	27	18.5
	Lung	0.02	37	0.5	ND	ND	-
3-hydroxy-4,5-dimethoxyallylbenzene	Liver	0.1	34	2.9	0.1	54	1.9
	Lung	0.02	194	0.1	ND	ND	-
Conversion of 1′-hydroxyelemicin							
1'-hydroxyelemicin glucuronide	Liver	2.4[e]	1543[e]	1.6	0.2[e]	337[e]	0.6
1'-oxoelemicin	Liver	26.3	15232	1.7	2.1[e]	6895[e]	0.3
1'-sulfoxyelemicin	Liver	$7*10^{-4}$ [e]	120[e]	0.006	$1*10^{-4}$ [e]	39[e]	0.003

[a] Mean values of two independent measurements
[b] nmol min^{-1} (mg microsomal or S9 protein)$^{-1}$
[c] µM
[d] µL min^{-1} (mg microsomal or S9 protein)$^{-1}$, (V_{max}/K_m x 1000 µL/mL)
[e] experiments performed with S9 tissue fractions
ND not detected

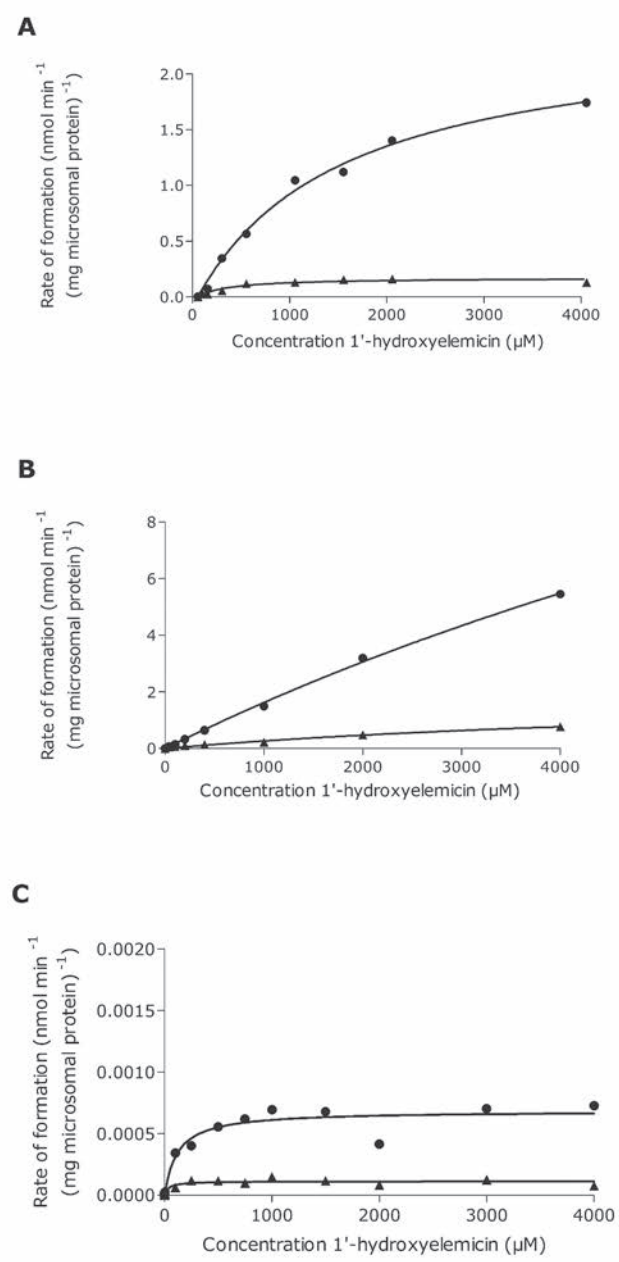

Figure 5. Concentration dependent rate of A) glucuronidation of 1′-hydroxyelemicin in pooled male rat liver S9 (•) or pooled human mixed gender liver S9 (▲), B) oxidation of 1′-hydroxyelemicin in pooled male rat liver microsomes (•) or pooled human mixed gender liver S9 (▲) and C) sulfonation of 1′-hydroxyelemicin in pooled male rat liver S9 (•) or pooled human mixed gender liver S9 (▲). Data points represent mean values of two individual experiments.

Sulfonation of 1′-hydroxyelemicin

In the present study, GSH was used to trap the reactive 1′-sulfoxyelemicin formed upon sulfonation of the proximate carcinogenic metabolite of elemicin. The scavenging may be either chemically or catalyzed by the glutathione S-transferases present in the S9 incubations in which the sulfonation of 1′-hydroxyelemicin was measured. However, the level of 1′-sulfoxyelemicin conjugates formed in incubations using cysteine or N-acetylcysteine, representing enzymatic-independent trapping methods, was comparable to the 1′-sulfoxyelemicin adduct formation detected using GSH as a trapping agent, indicating that the mechanism underlying the scavenging of 1′-sulfoxyelemicin by GSH is mainly chemical (data not shown). In addition, it was found that the amount of the GSH conjugate of 1′-sulfoxyelemicin detected was not increased when the concentration of GSH was doubled from 10 to 20 mM (data not shown). Together these data indicate that under the conditions applied, the scavenging of 1′-sulfoxyelemicin by GSH proceeds by an efficient chemical reaction and is not enzyme mediated.

Chromatographic analysis of incubations with male rat liver S9 and mixed gender pooled human liver S9, 1′-hydroxyelemicin, PAPS and GSH revealed a peak at 1.1 min (chromatogram not shown), which was tentatively identified as the GSH-adduct of the carbocation of 1′-sulfoxyelemicin. Tentative identification was achieved based on the chromatographic analysis of incubations performed in the absence of GSH and the presence of PAPS and liver S9 proteins since in these incubations no peak was found at 1.1 min. Figure 5C presents the rate of formation of 1′-sulfoxyelemicin in incubations with male rat liver S9 and mixed gender pooled human liver S9. The kinetic constants for the conversion of 1′-hydroxyelemicin to 1′-sulfoxyelemicin in rat and human liver are presented in Table 3.

Comparison of the kinetic constants for conversion of elemicin and 1′-hydroxyelemicin by male rat and mixed gender pooled human tissue fractions

To allow a comparison between the kinetic constants for metabolism of elemicin and 1′-hydroxyelemicin by male rat and mixed gender pooled human tissue fractions, V_{max} values that were derived in vitro expressed as nmol min^{-1} (mg microsomal or S9 protein)$^{-1}$ were scaled to values representing the V_{max} per µmol h^{-1} (g tissue)$^{-1}$ using microsomal and S9 protein yields as described in literature (Medinsky *et al.*, 1994) and previously used by Punt *et al.* (2008,

2009), Al-Subeihi *et al.* (2011, 2012), and Martati *et al.* (2011, 2012). Making use of the in vivo V_{max} values derived accordingly, a scaled catalytic efficiency (scaled V_{max} in vivo/K_m) for the formation of elemicin metabolites could be calculated (Table 4). These values show that the catalytic efficiency for formation of the proximate carcinogenic metabolite of elemicin, 1′-hydroxyelemicin, was found to be 10-fold higher in male rat liver compared to human liver. This difference in catalytic efficiency for the formation of 1′-hydroxyelemicin is predominantly caused by the high affinity (expressed as K_m) for its formation from elemicin in male rat liver since the K_m value in rat liver incubations was 22-fold lower than that in human liver incubations.

The detoxification of 1′-hydroxyelemicin by formation of 1′-hydroxyelemicin glucuronide was found to be the main metabolic reaction with 1′-hydroxyelemicin in rat and human liver. However, this pathway was more efficient in rat than in human liver fractions. Although glucuronidation of 1′-hydroxyelemicin occurs in male rat with a relative low affinity (*i.e.* K_m 1.5 mM whereas in human this was 337 μM), analysis of the data revealed a high V_{max} value resulting in a catalytic efficiency that was 2.6-fold higher in male rats compared to human.

Oxidation of 1′-hydroxyelemicin was found to be 1.4 times more efficient in male rat liver compared to human liver resulting from a 3.1-fold higher V_{max} and a 2.2-fold higher K_m.

Sulfonation was found to be the least efficient metabolic pathway for 1′-hydroxyelemicin in both rats and human. For rat, the in vivo scaled catalytic efficiency was 0.05 mL h^{-1} (g S9 protein)$^{-1}$ and for human this was 0.03 mL h^{-1} (g S9 protein)$^{-1}$, indicating that sulfonation of 1′-hydroxyelemicin is more efficient in male rat liver than human liver.

Altogether, it can be concluded that glucuronidation of 1′-hydroxyelemicin, representing a detoxification pathway, is the most important pathway in rat and in human. Moreover, based on the kinetic data obtained, bioactivation of 1′-hydroxyelemicin following sulfonation was found to represent only a minor pathway in both rat and human.

Table 4. Scaled kinetic constants for metabolic conversion of elemicin and 1′-hydroxyelemicin by male rat and human tissue fractions.

Metabolite	Organ	Rat[a]			Human[a]		
		Scaled *V*max, in vivo (µmol/h/g tissue)[b]	*K*m (µM)	In vivo catalytic efficiency (mL/h/g tissue)[c]	Scaled *V*max, in vivo (µmol/h/g tissue)[b]	*K*m (µM)	In vivo catalytic efficiency (mL/h/g tissue)[c]
Conversion of elemicin							
1′-hydroxyelemicin	Liver	1.1	19	55.3	2.3	415	5.6
	Lung	0.07	189	0.4	ND	ND	-
3′-hydroxyisoelemicin	Liver	0.8	16	52.5	1.3	83	15.2
M4	Liver	0.4	213	2.0	ND	ND	-
elemicin-2′,3′-oxide	Liver	3.8	139	27.2	1.1	27	38.9
	Lung	0.02	37	0.6	ND	ND	-
3-hydroxy-4,5-dimethoxyallyl-benzene	Liver	0.2	34	6.2	0.2	54	3.9
	Lung	0.02	194	0.1	ND	ND	-
Conversion of 1'-hydroxyelemicin							
1'-hydroxyelemicin glucuronide	Liver	20.6	1543	13.3	1.7	337	5.1
1'-oxoelemicin	Liver	55.2	15232	3.6	18.0	6895	2.6
1'-sulfoxyelemicin	Liver	0.006	120	0.05	0.001	39	0.03

[a] Mean values of two independent measurements

[b] Scaled V_{max} were converted from in vitro V_{max} based on microsomal protein yield of 35 and 20 mg/g tissue for liver and lung, respectively, and S9 protein yield of 143 mg/g liver

[c] Catalytic efficiency (scaled V_{max}(app)/K_m)

ND not detected

Evaluation of PBK model performance

The performance of the newly developed PBK models for elemicin could not be evaluated against in vivo data because quantitative data on the excretion of the different metabolites formed after elemicin metabolism in rat or humans exposed to elemicin are not available. However, Beyer *et al.* (2006) identified different metabolites of elemicin excreted in rat urine in a qualitative manner. It was demonstrated that the formation of 2′,3′-dihydroxyelemicin, formed after hydrolysis of elemicin-2′,3′-oxide, was the most abundant metabolite found in rat urine samples collected over a 24 h period after administration of a single oral dose of 100 mg/kg bw elemicin (Beyer *et al.*, 2006). In line with these results, the developed PBK model predicted elemicin-2′,3′-oxide to be the major metabolite formed at a dose of 100 mg/kg bw elemicin in rat after 24 h.

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

PBK model predictions

Following an exposure to 0.05 mg/kg bw elemicin, both elemicin and its proximate carcinogenic metabolite 1′-hydroxyelemicin were predicted to be almost completely metabolized within a 10 h period in rat and human (data not shown). At a higher oral dose level of 300 mg/kg bw elemicin, both elemicin

and 1′-hydroxyelemicin are predicted to be metabolized within 24 h (data not shown). Dose levels of 0.05 and 300 mg/kg bw were chosen to allow comparison with the PBK model outcomes previously reported for estragole (Punt *et al.*, 2008 and 2009), and methyleugenol (Al-Subeihi *et al.*, 2011 and 2012). Figure 6A shows the PBK model based predictions for the dose-dependent formation of the different microsomal metabolites of elemicin in rat 24 h after exposure. The percentage of the dose converted to 1′-hydroxyelemicin is predicted to decrease in a dose-dependent manner. Concurrent with the decreased percentage of the dose that undergoes 1′-hydroxylation of the alkene side chain, a 2.4-fold dose-dependent increase in the percentage of the dose that underwent epoxidation was observed. A less than 2-fold decrease in the formation of 3′-hydroxyisoelemicin was observed. The relative formation of M4 changed from 1.4% to 4.1% when the dose increased. The formation of 3-hydroxy-4,5-dimethoxyphenol did not change with increasing dose-levels and was equal to 4.3% of the administered dose. Figure 6B shows the dose-dependent decrease in the formation of the metabolites of 1′-hydroxyelemicin. This reveals a relative decrease in the percentage of the dose ultimately converted into 1′-hydroxyelemicin glucuronide, 1′-sulfoxyelemicin and 1′-oxoelemicin which can be explained by the decrease of the formation of 1′-hydroxyelemicin with increasing dose levels.

Figure 7A reveals that in human the percentage of the dose converted to 1′-hydroxyelemicin equaled 7.9% at a dose of 0.05 mg/kg bw/day and increased to 15.6% at a dose of 300 mg/kg bw/day. Concomitant with the increase in the formation of 1′-hydroxyelemicin, a relative decrease in the formation of 3′-hydroxyisoelemicin and elemicin-2′,3′-oxide is predicted. 3-Hydroxy-4,5-dimethoxyphenol was predicted to change only to a small extent with increasing dose. Figure 7B shows the dose-dependent increase in the formation of 1′-hydroxyelemicin glucuronide and 1′-oxoelemicin and the dose-dependent decrease of 1′-sulfoxyelemicin in human liver 24 h after elemicin exposure.

Comparison of the relative extent of bioactivation of elemicin by rat and human liver revealed that species differences in the formation of 1′-hydroxyelemicin and 1′-sulfoxyelemicin (expressed as nmol/g liver) are limited (<3.8-fold) from a relatively low dose of 10^{-6} mg/kg bw up to dose levels of 100 mg/kg bw (Figure 8).

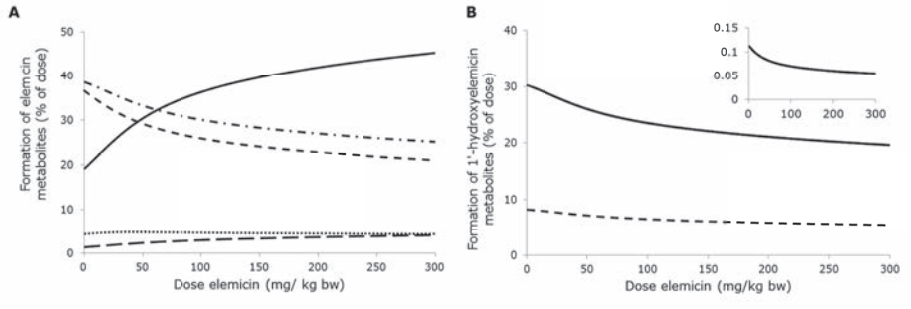

Figure 6. PBK predicted dose-dependent changes in overall formation of A) microsomal metabolites of elemicin in male rat lung and liver and B) metabolites of 1′-hydroxyelemicin in male rat liver. The lines correspond to A) 1′-hydroxyelemicin (···), 3′-hydroxyisoelemicin (- - -), elemicin-2′,3′-oxide (—), M4 (— —) and 3-hydroxy-4,5-dimethoxyallylbenzene (···) and B) 1′-hydroxyelemicin glucuronide (—), 1′-oxoelemicin (- - -) and 1′-sulfoxyelemicin (insert), 24 h after elemicin exposure.

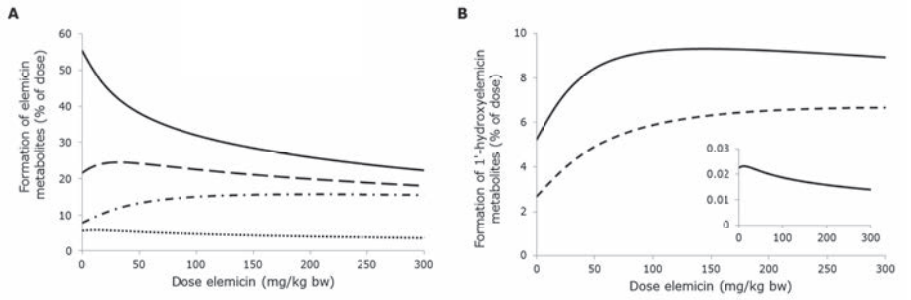

Figure 7. PBK predicted dose-dependent changes in overall formation of A) microsomal metabolites of elemicin and B) metabolites of 1′-hydroxyelemicin in human liver. The lines correspond to A) 1′-hydroxyelemicin (···), 3′-hydroxyisoelemicin (- - -), elemicin-2′,3′-oxide (—) and 3-hydroxy-4,5-dimethoxyallylbenzene (···) and B) 1′-hydroxyelemicin glucuronide (—), 1′-oxoelemicin (- - -) and 1′-sulfoxyelemicin (insert), 24 h after elemicin exposure.

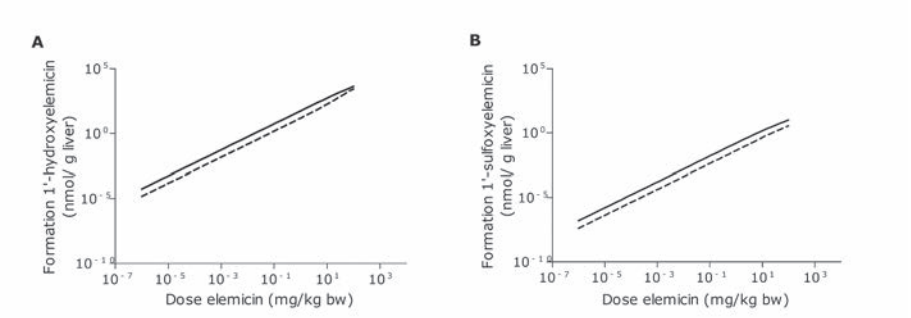

Figure 8. PBK predicted dose-dependent formation of A) 1′-hydroxyelemicin and B) 1′-sulfoxyelemicin in rat (—) and human (- - -) liver 24 h after elemicin exposure.

Sensitivity analysis

A sensitivity analysis was performed to define model parameters that are capable of influencing the formation of 1′-hydroxyelemicin and 1′-sulfoxyelemicin in rat and human liver. For this purpose, normalized sensitivity coefficients were calculated for all parameters at a dose of 0.05 mg/kg bw elemicin. Figure 9 presents the parameters affecting the formation of 1′-hydroxyelemicin (A) and 1′-sulfoxyelemicin (B) that have a normalized sensitivity coefficient higher than |0.1|.

In rat and human liver, formation of 1′-hydroxyelemicin is primarily influenced by the kinetic constants for its formation from elemicin (V_{max,L_HE}, K_{m,L_HE}) (Figure 9A). Figure 9B reveals that the formation of the ultimate carcinogenic metabolite 1′-sulfoxyelemicin is mainly influenced by the kinetic constants for its formation from 1′-hydroxyelemicin (V_{max,L_HES}, K_{m,L_HES}). The kinetic constants for the formation of 1′-hydroxyelemicin glucuronide (V_{max,L_HEG}, K_{m,L_HEG}) were also found to highly influence the formation of 1′-sulfoxyelemicin in rat and human liver. The kinetic constants for formation of 1′-oxoelemicin were predicted to affect the formation of 1′-sulfoxyelemicin in rat and human liver to a smaller extent. These results suggest that glucuronidation of 1′-hydroxyelemicin can be considered as the most important competitive metabolic pathway to sulfonation in rat and human due to its high catalytic efficiency.

Comparison of the PBK model based prediction of bioactivation of elemicin by rat and human to the PBK model predictions for bioactivation of the structurally related compounds estragole and methyleugenol

By defining mode of action based PBK models for elemicin metabolism in rat and human, a read across to data from estragole and methyleugenol was facilitated. On the basis of the PBK models for these structurally related alkenylbenzenes (Al-Subeihi *et al.*, 2011 and 2012; Punt *et al.*, 2008 and 2009), comparisons were made for the dose-dependent formation of the 1′-hydroxymetabolites of these compounds and the formation of their 1′-sulfoxymetabolites. Figure 10 shows the dose-dependent formation of these metabolites in rat as predicted by the respective PBK models. The PBK model based predicted formation of the proximate carcinogenic 1′-hydroxymetabolites was found to be very similar for all three alkenylbenzenes. Up to dose levels of 100 mg/kg bw, the predicted formation of 1′-hydroxyelemicin increased linearly with the dose and was found to be approximately 2-fold higher compared to the formation

of 1′-hydroxyestragole and 1′-hydroxymethyleugenol (Figure 10A). Figure 10B shows the predicted model outcomes for the formation of the ultimate carcinogenic metabolites of estragole, methyleugenol and elemicin. In rat liver, the formation of the 1′-sulfoxymetabolites is expected to be highest for elemicin followed by methyleugenol and estragole in decreasing order.

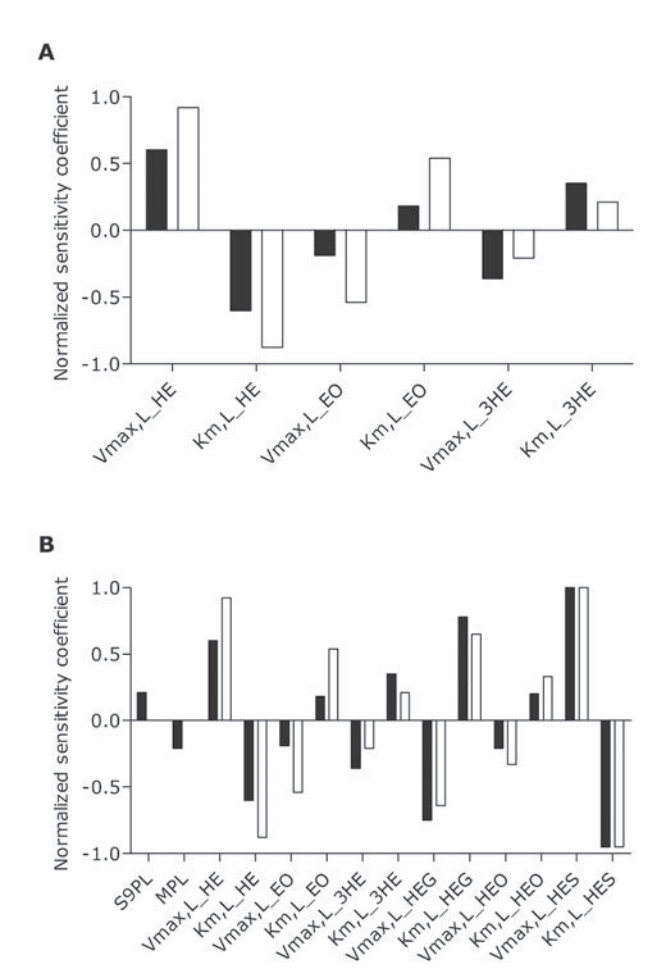

Figure 9. Sensitivity analysis of the predicted formation of A) 1′-hydroxyelemicin and B) 1′-sulfoxyelemicin in rat (black bars) and human (white bars). S9PL = S9 protein yield of the liver, MPL = microsomal protein yield of the liver, Vmax and Km are respectively the maximum rate of formation and the Michaelis-Menten constant for formation of different metabolites in the liver (L) including 1′-hydroxyelemicin (HE), elemicin-2′,3′-oxide (EO), 3′-hydroxyisoelemicin (3HE), 1′-hydroxyelemicin glucuronide (HEG), 1′-oxoelemicin (HEO), and 1′-sulfoxyelemicin (HES).

However, the differences between the formation of these metabolites varies within one order of magnitude for all alkenylbenzenes as it was seen that the formation of 1′-sulfoxyelemicin is 2-fold higher than that of 1′-sulfoxymethyleugenol whereas it is approximately 8-fold higher as compared to the predicted formation of 1′-sulfoxyestragole. For all alkenylbenzenes, the formation of their 1′-sulfoxymetabolite increases linearly up to a dose of 100 mg/kg bw.

Figure 11 shows the predicted dose-dependent formation of the 1′-hydroxymetabolites and 1′-sulfoxymetabolites of elemicin, estragole, and methyleugenol in human liver. The predicted formation of 1′-hydroxyelemicin is similar to the formation of 1′-hydroxymethyleugenol, 11-fold lower than the formation of 1′-hydroxyestragole. Thus, compound differences in the formation of 1′-hydroxymetabolites are limited. Moreover, data reveal that in human at dose levels around the $BMDL_{10}$ (*i.e.* the lower confidence limit of the BenchMark Dose resulting in a 10% extra cancer incidence) down to realistic exposure levels, the formation of the DNA reactive 1′-sulfoxymetabolite is relatively low after elemicin exposure, being 11- and 2-fold lower as compared to the formation of the 1′-sulfoxymetabolites of estragole and methyleugenol, respectively. The formation of 1′-sulfoxymetabolites was found to increase in a linear manner up to dose levels of 10 mg/kg bw.

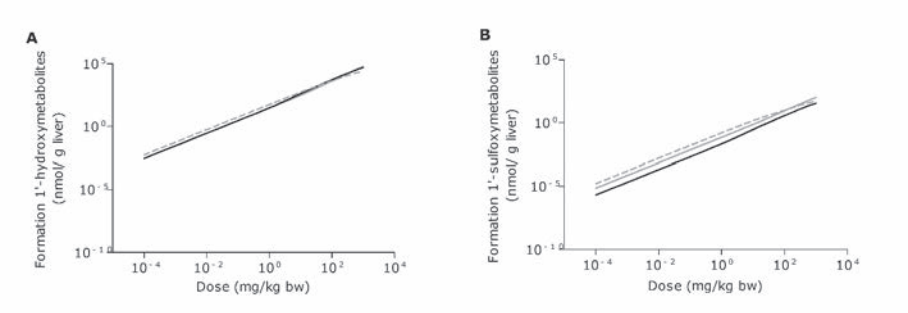

Figure 10. PBK predicted dose-dependent formation of A) 1′-hydroxymetabolites and B) 1′-sulfoxymetabolites of estragole (—), methyleugenol (—) and elemicin (- - -) in male rat liver after 24 h.

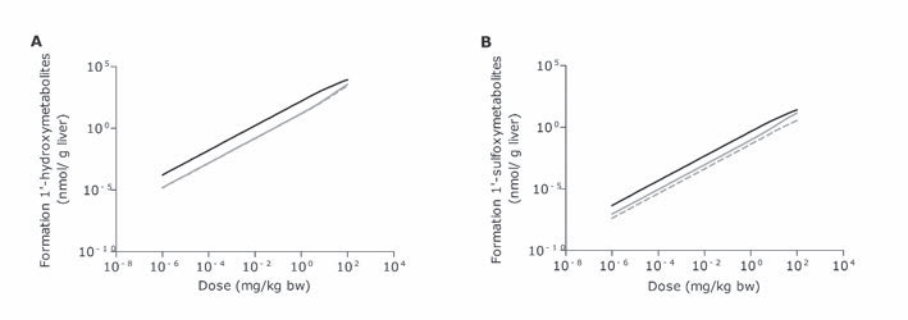

Figure 11. PBK predicted dose-dependent formation of A) 1'-hydroxymetabolites and B) 1'-sulfoxymetabolites of estragole (—), methyleugenol (—) and elemicin (- - -) in human liver after 24 h.

Implications for risk assessment

The Margin of Exposure (MOE) concept was applied to assess the possible risks for human health resulting from the daily exposure to elemicin (EFSA, 2005; JECFA, 2005; Barlow *et al.*, 2006; O'Brien *et al.*, 2006). The MOE is a dimensionless ratio based on a reference point representing a dose causing a low but measurable cancer incidence in experimental animals (*e.g.* a $BMDL_{10}$) which is divided by the estimated daily intake in humans (EFSA, 2005). When the MOE is lower than 10,000, the compound of interest is considered to be of priority for risk management actions and a concern for human health (EFSA, 2005). This value of 10,000 is based on several factors that may cause uncertainty in the MOE including species differences and human variability in kinetics and dynamics among others (EFSA, 2005).

To date, tumor data for elemicin, from which a $BMDL_{10}$ can be derived, are absent in the available literature, hampering the application of the MOE approach in the risk assessment of elemicin. Nevertheless, PBK model predictions indicate that at dose levels in the range of the $BMDL_{10}$ values, the formation of 1'-sulfoxymetabolites of elemicin in human liver is lower as compared to that of the structurally related methoxy allylbenzene derivatives estragole and methyleugenol, facilitating read across from carcinogenicity data on the two alkenylbenzenes to elemicin assuming, as a worst case approximation, that formation of the ultimate carcinogenic metabolite would be linearly related to the tumor incidence. On the basis of these considerations, the specific $BMDL_{10}$ values of estragole and methyleugenol (Table 5) were used to estimate possible $BMDL_{10}$ values for elemicin using the results of the present study showing the formation of the 1'-sulfoxymetabolite of elemicin to be 11- and 2-fold lower

in human liver and, therefore, multiplying the $BMDL_{10}$ values of estragole and methyleugenol by these factors. In a next step, MOE values for elemicin were calculated based on the estimated daily intake (Table 6). For comparison Table 5 presents the MOE values for the other two related methoxy allylbenzenes. The intake estimates used to calculate the MOE values were based on exposure from the use of herbs and spices and products made thereof (SCF 2001a, 2001b). MOE values for estragole and methyleugenol (Table 5) were found to be generally lower than the MOE default of 10,000 and even lower than 1,000 indicating a priority for risk management actions. Table 6 illustrates the results from the read across from $BMDL_{10}$ values of estragole and methyleugenol to elemicin. The results obtained indicate that the daily exposure to elemicin resulting from the use of spices, food, and essential oils is generally of lower priority for risk management than the exposure to the two related alkenylbenzenes resulting from these sources. The intake of elemicin resulting from the use of essential oils is only limited, and specific data are lacking. Assuming that the total intake from nutmeg and mace as spices exceeds the intake from essential oils by a factor 10 at least (WHO, 2009), MOE values equal to 40,000 – 100,000 were found for intake from elemicin from essential oils (Table 6). In addition, MOE values were calculated based on the intake estimates of elemicin resulting from the use of nutmeg-containing PFS based on chemical analysis of these products as well as their proposed uses as described previously (van den Berg *et al.*, 2011). On the basis of the daily intake estimates thus derived, MOE values of 80 – 40,000 were found (Table 6).

Table 5. $BMDL_{10}$ values for estragole and methyleugenol derived from long-term carcinogenicity studies (Miller *et al.*, 1983; NTP, 2000) as calculated previously (van den Berg *et al.*, 2011), corresponding daily exposures from all food sources (SCF 2001a and 2001b) and MOE values.

Compound	Species and sex	Tumors	$BMDL_{10}$ (mg/kg bw/day)	Estimated daily intake (mg/kg bw/day)	MOE[a]
Estragole	Female mice	hepatocellular carcinomas	3.3 - 6.5 (Miller *et al.*, 1983; van den Berg *et al.*, 2011)	0.07 (SCF, 2001b)	50-90
Methyl-eugenol	Male rats	hepatocellular carcinomas	15.3 - 34.0 (NTP, 2000; van den Berg *et al.*, 2011)	0.19 (SCF, 2001a)	80-200
	Female rats	hepatocellular carcinomas	48.8 - 73.6 (NTP, 2000; van den Berg *et al.*, 2011)	0.19 (SCF, 2001a)	300-400

[a] MOE values are rounded to a single significant value

Table 6. Read across of $BMDL_{10}$ values for estragole and methyleugenol to estimated $BMDL_{10}$ values for elemicin based on the lower relative extent of formation of 1'-sulfoxymetabolites compared to its structurally related analogues as demonstrated in the present study, corresponding daily exposures from spices, foods and essential oils (SFE) (WHO, 2009), essential oils only (EO) or plant food supplements (PFS) and resulting MOE values [a].

Compound for read across	$BMDL_{10}$ (mg/kg bw/day)	Fold difference	Corrected $BMDL_{10}$ (mg/kg bw/day)	Estimated daily intake[b] (mg/kg bw/day)	MOE[d]
Estragole	3.3 - 6.5 (Miller *et al.*, 1983; van den Berg *et al.*, 2011)	11	36.5 – 71.5	SFE: 0.017 (WHO, 2009)	SFE: 2,000 – 4,000
				EO: 0.00071 (WHO, 2009)	EO: 50,000 – 100,000
				PFS: 0.002 – 0.40[c]	PFS: 90 – 40,000
Methyl-eugenol	15.3 - 34.0 (NTP, 2000; van den Berg *et al.*, 2011)	2	30.6-68.0	SFE: 0.017 (WHO, 2009)	SFE: 2,000 – 4,000
				EO: 0.00071 (WHO, 2009)	EO: 40,000 – 100,000
				PFS: 0.002 – 0.40[c]	PFS: 80 – 30,000

[a] To make a worst-case estimate only the lowest $BMDL_{10}$ values for methyleugenol were used and data from female rats were discarded from further analysis.
[b] Estimated daily intake from spices, food and essential oils (SFE) and from nutmeg-containing plant food supplements (PFS). Intake data from essential oils only (EO) are based on the mean per capita daily intake of nutmeg, mace and their essential oils within the EU assuming that the intake from spice sources exceeds the intakes from essential oils by a factor 10 at least (WHO, 2009).
[c] Intake estimates were made using the levels of elemicin in commercial available PFS based on chemical analysis and the use of PFS at the dose recommended by the respective manufacturer as described previously (van den Berg *et al.*, 2011).
[d] MOE values are rounded to a single significant value.

Discussion

In the presented chapter, the recently developed mode of action based PBK models for detoxification and bioactivation of the structurally related alkenylbenzenes estragole (Punt *et al.*, 2008, 2009), methyleugenol (Al-Subeihi *et al.*, 2011, 2012), and safrole (Martati *et al.*, 2011, 2012) in male rat and human were extended to elemicin. The newly developed PBK models combine biochemical and physicochemical information of elemicin and physiology of the organism of interest (*i.e.* rat and human), enabling the quantification of detoxification and bioactivation in rat and human at realistic low exposure levels. The development of these models facilitates a read across from data on estragole and methyleugenol, for which in vivo toxicity studies are available, to elemicin a compound for which toxicity data are limited.

Although the PBK models for elemicin defined in the present chapter were able to predict the overall formation of the reactive 1′-sulfoxyelemicin metabolite in the liver of rat and human and compare this overall formation to the overall formation of the 1′-sulfoxymetabolites of the corresponding alkenylbenzenes facilitating a relative comparison, the models did not include further metabolic reactions of the 1′-sulfoxymetabolite such as conjugation with GSH, RNA, DNA, and other cellular macromolecules. Such subsequent steps (as well as DNA repair and reversibility of DNA-adduct formation) should be included in the model when the ultimate aim would be to predict DNA-adduct formation (see, for an example, Paini *et al.*, 2010) but this was beyond the scope of the present study. Comparison of the rat and human PBK model predictions indicated a species-dependent difference in the bioactivation of elemicin. However, the influence of species-differences on the overall metabolic activation of elemicin was limited and comparable to the species-differences in metabolic activation of estragole (Punt *et al.*, 2008; 2009) and methyleugenol (Al-Subeihi *et al.*, 2011, 2012) to the respective 1′-sulfoxymetabolites and also in line with the default factor of 4 which is generally used to describe kinetic differences between species (IPCS, 2010).

In addition to species differences in the relative extent of bioactivation and detoxification, the newly developed PBK models for elemicin were used to compare the levels of metabolic activation of elemicin, to those for estragole (Punt *et al.*, 2008; 2009) and methyleugenol (Al-Subeihi *et al.*, 2011, 2012) in male rat and human liver. Results reveal that the formation of the proximate and ultimate carcinogenic metabolites of the three alkenylbenzenes of interest in rat is predicted to be comparable within an order of magnitude. In line with these findings, previous studies demonstrated the ability of elemicin, estragole, and methyleugenol to all induce unscheduled DNA synthesis (UDS) in rat hepatocytes (Chan and Caldwell, 1992; Hasheminejad and Caldwell, 1994; Howes *et al.*, 1990). In fact, the UDS responses of all alkenylbenzenes of interest were found to be in close agreement, revealing a dose-dependent increase in UDS from 2.0- up to 2.7-fold as compared to the control at comparable experimental conditions (Chan and Caldwell, 1992; Hasheminejad and Caldwell, 1994; Howes *et al.*, 1990).

In humans the formation of especially the 1′-sulfoxymetabolites of the three alkenylbenzenes are predicted to be lower for elemicin as compared to what was predicted for the 1′-sulfoxy formation for the structurally related

analogues. This difference can be ascribed to the fact that sulfonation of the 1′-hydroxymetabolites of estragole and methyleugenol is up to 3 times more efficient by human liver than that of 1′-hydroxyelemicin. Moreover, metabolic conversion of 1′-hydroxyelemicin toward 1′-hydroxyelemicin glucuronide was found to be 5-10 times more efficient as compared to the glucuronidation of 1′-hydroxymetabolites of estragole (Punt *et al.*, 2009) and methyleugenol (Al-Subeihi *et al.*, 2012) affecting the amount of 1′-hydroxyelemicin in human liver available for sulfonation. Interestingly, glucuronidation of 1′-hydroxymetabolites is more important than the oxidation pathway for elemicin metabolism but not for the structurally related analogues for which the oxidation of the proximate carcinogenic metabolites represents the major detoxification pathway. It is important to note that the oxidation of 1′-hydroxyelemicin to 1′-oxoelemicin might be reversible. However, given the rapid scavenging of 1′-oxoelemicin by GSH as observed in the present study, the ultimate effect may still represent an important detoxification pathway also considering that 1′-oxometabolites of alkenylbenzenes have not been observed to be carcinogenic in vivo in mice (Wiseman *et al.*, 1987; Wislocki *et al.*, 1977). Thus, in the model, it was implicitly assumed that scavenging of the 1′-oxometabolite by GSH would dominate over reduction back to 1′-hydroxyelemicin, allowing as a first approximation to not include these steps explicitly in the model.

Making use of the human PBK model outcomes obtained for the formation of reactive 1′-sulfoxymetabolites of elemicin, estragole, and methyleugenol, a comparison can be made to the relative potency of these compounds to bind to DNA. However, the ability of elemicin to form DNA adducts in cultured human cells has not been studied yet. Punt *et al.* (2007) concluded that sulfonation of 1′-hydroxyestragole is equally efficient in male mice S9 as in human S9 indicating that data derived from mice studies may accurately represent the level of bioactivation by sulfotransferases in human. Thus, in vivo data of DNA adducts formed in mice liver following elemicin, estragole, or methyleugenol exposure can provide an insight in the relative extent of metabolic activation of the three alkenylbenzenes in human. Previous studies demonstrated that the covalent binding of elemicin to female CD-1 mouse liver DNA and B6C3F$_1$ male mice liver DNA was up to 27-fold lower as compared to that of structurally related compounds including estragole and methyleugenol (Phillips *et al.*, 1984; Randerath *et al.*, 1984). Moreover, tumor induction in male B6C3F$_1$ mice given *i.p.* injections of 1′-hydroxyelemicin prior

to weaning was found to be 30-fold lower, on a molar basis, as compared to the results found for its structurally related analogue 1'-hydroxyestragole (Wiseman *et al.*, 1987). Consistent with these results, the PBK model outcomes revealed that in human liver the lowest levels of the ultimate carcinogenic metabolite would be formed after elemicin exposure as compared to its structurally related analogues. In line with these results, it was seen that despite the fact that estragole and methyleugenol were capable of inducing hepatic tumors in male mice, no detectable hepatocarcinogenic activities were observed in mice after administration of elemicin at comparable experimental conditions (Miller *et al.*, 1983). These differences in DNA binding activities and subsequent tumor formation between the different alkenylbenzenes might be explained by the fact that increasing the number of methoxy substituents will decrease the electrophilicity of their 1'-sulfoxymetabolites and/or the corresponding carbocations, which is in line with the fact that it was previously shown that mono- and di-substituted alkenylbenzenes were bound more rapidly to mouse liver DNA and showed higher levels of hepatocarcinogenic activity compared to structurally related analogues exerting multiple methylenedioxy and/or methoxy groups (Miller *et al.*, 1983; Randerath *et al.*, 1984).

On the basis of the results now available for the different alkenylbenzenes, the present study also presents a risk assessment performed for elemicin using the MOE approach. To be able to apply the MOE in the risk assessment of elemicin, for which data on tumor formation are currently not available, $BMDL_{10}$ values of related alkenylbenzenes were corrected, reflecting the lower relative extent of elemicin bioactivation in human liver predicted by the newly developed PBK models. Extrapolation of the $BMDL_{10}$ values for estragole and methyleugenol to a possible $BMDL_{10}$ for elemicin, based on the differences in formation of the related ultimate carcinogenic 1'-sulfoxymetabolites, results in a $BMDL_{10}$ value for elemicin that is at least 30.6 mg/kg bw/day (Table 6) and thus significantly higher than the $BMDL_{10}$ values for the other two alkenylbenzenes (Table 5). This is in line with the fact that Miller *et al.* (1983) and Wiseman *et al.* (1987) showed that elemicin and 1'-hydroxyelemicin had no hepatocarcinogenic activities in mice after *i.p.* injections of up to approximately 60 mg/kg bw while a significant increased incidence in the formation of treatment-related liver tumors was observed upon administration of 1'-hydroxyelemicin at the highest dose tested (*i.e.* 4 injections with a total dose of 106.5 mg/kg bw). The daily intake of estragole and methyleugenol resulted in relatively lower MOE values

as compared to the daily intake of elemicin, indicating the intake of these related methoxy allylbenzenes to be of higher priority for risk management actions. Data also indicated that the use of elemicin-containing PFS is generally considered to be of lower risk for human health as compared to the use of some PFS containing estragole or methyleugenol that were previously shown to present a possible priority for risk management (van den Berg *et al.*, 2011).

Finally, it is of importance to note that the developed PBK models, as other models, are based on assumptions needed to considerably reduce the number of parameters. As a result, some factors are not taken into account, including for example further metabolic reactions of 1′-sulfoxyelemicin, the possible reversibility of oxidation of 1′-oxoelemicin, and extrahepatic metabolic activation of 1′-hydroxyelemicin. Rationales for these assumptions were provided and as a result the outcomes of the developed PBK models for elemicin could be compared to those of the previous developed PBK models for estragole (Punt *et al.*, 2008, 2009), methyleugenol (Al-Subeihi *et al.*, 2011, 2012) which were found to adequately describe the in vivo situation. In view of model simplification and associated uncertainties, conclusions obtained from the developed models should preferably be based on a relative comparison of the relative extent of bioactivation and detoxification as was done in the present study.

Altogether, the results obtained indicate that PBK modeling provides an important insight in the occurrence of species differences in the metabolic activation of elemicin. Moreover, they provide an example of how PBK modeling can facilitate a read across in risk assessment from compounds for which in vivo toxicity studies are available to a compound for which only limited toxicity data have been described, thus contributing to development of alternatives for animal testing.

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Supplementary materials Chapter 4

Table S1. Characteristic NMR chemical shifts.

Compound	Position	Chemical shift (ppm)
elemicin[a]		
	H4/H6	6.49
	H1′	3.33
	H2′	5.97
	H3′a	5.10
	H3′b	5.05
	1-OCH_3, 3-OCH_3	3.82
	2-OCH_3	3.75
1′-oxoelemicin[a]		
	H4/H6	7.34
	H2′	7.38
	H3′a	6.43
	H3′b	5.97
	1-OCH_3, 3-OCH_3	3.93
	2-OCH_3	3.87
elemicin-2′,3′-oxide[a, b]		
	H4/H6	6.58
	H1′a	2.84
	H1′b	2.73
	H2′	3.14
	H3′a	2.79
	H3′b	2.59
	1-OCH_3, 3-OCH_3	3.84
	2-OCH_3	3.75
1′-hydroxyelemicin[c]		
	H4/H6	6.59
	H1′	5.12
	H2′	6.02
	H3′a	5.36
	H3′b	5.20
	1-OCH_3, 3-OCH_3	3.85
	2-OCH_3	3.82
3′-hydroxyisoelemicin[d]	H2/H6	6.64
	H1′	6.55
	H2′	6.31
	H3′	4.35
	OCH_3	3.89

[a] Measurements were performed at 300K using a 1D NOESY pulse sequence at a Bruker Avance III 600 MHz NMR spectrometer equipped with a 5 mm cryoprobe using CD_3OD. The methanol peak was set at 3.33 ppm.
[b] Elemicin-2′,3′-epoxide was, because of the additional presence of elemicin, checked by means of 2D NMR spectroscopy (^1H-^1H COSY, ^1H-^1H TOCSY, ^1H-^{13}C HSQC, ^1H-^{13}C HMBC).
[c] NMR analysis was performed on a Bruker AV-500 NMR spectrometer using $CDCl_3$.
[d] NMR analysis was performed on a Bruker AV-400 MHz spectrometer using $CDCl_3$.

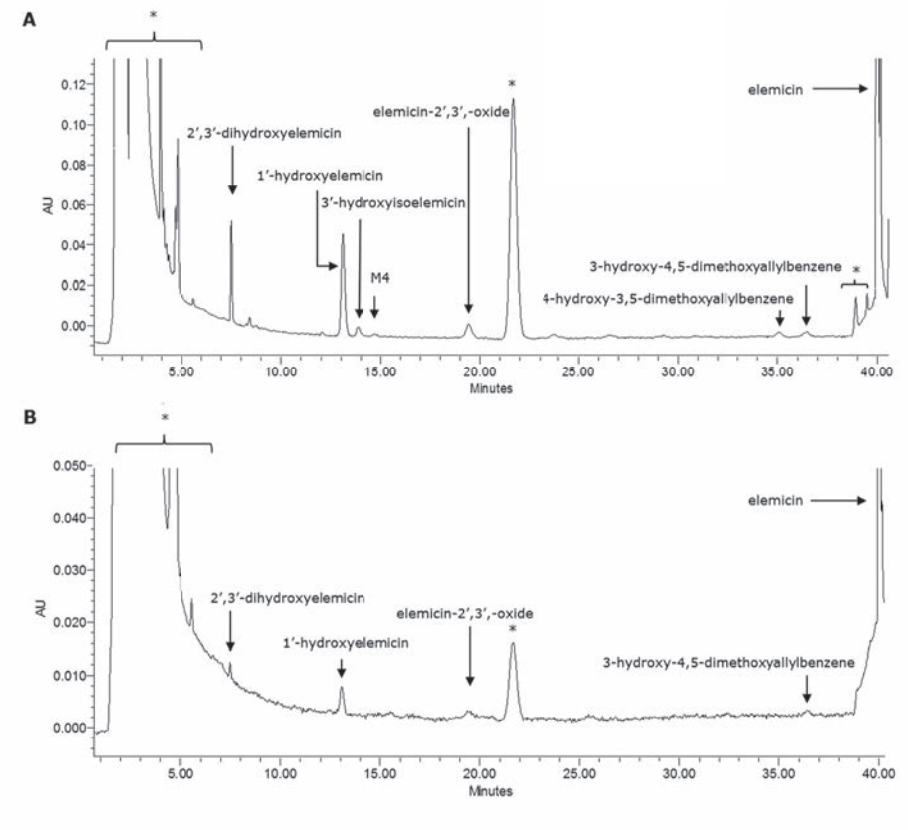

Figure S1. Chromatograms showing the different metabolites of elemicin formed in incubations with A) pooled human mixed gender liver microsomes and B) male rat lung. Incubations were performed using a final concentration of 100 µM elemicin and NADPH as a cofactor for 30 min at 37 ˚C. The peaks marked with an asterisk (*) were also present in blank incubations performed in the absence of the cofactor NADPH.

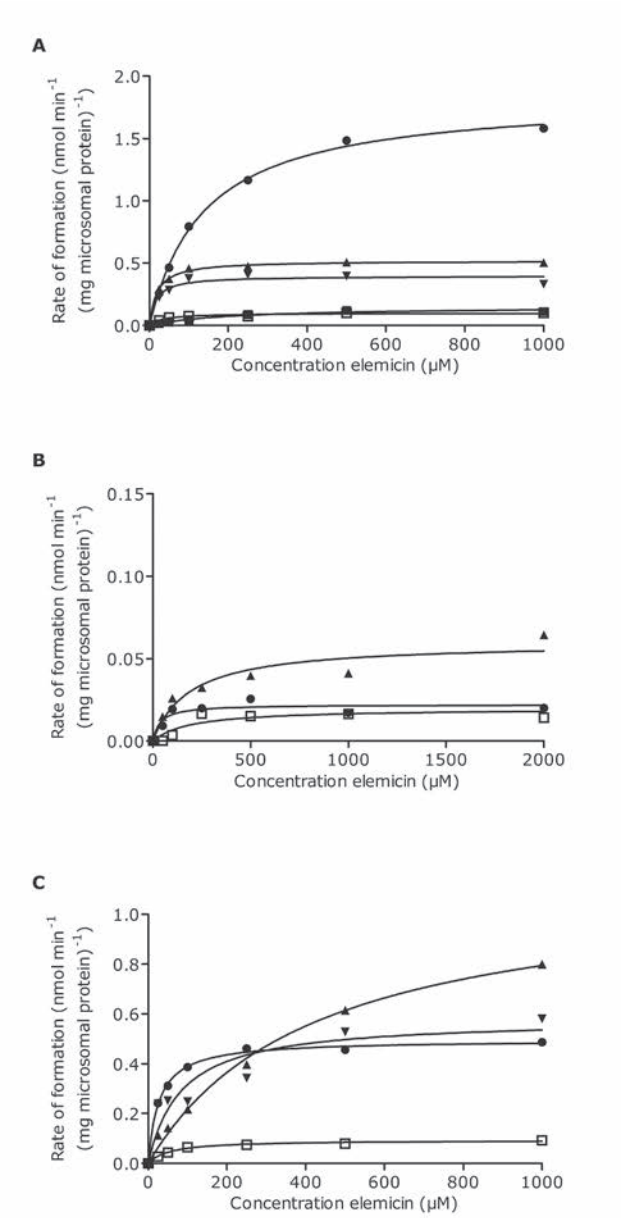

Figure S2. Elemicin concentration dependent rate of formation of different elemicin metabolites in incubations with A) male rat liver and B) lung microsomes and C) mixed gender pooled human liver microsomes and NADPH as a cofactor. Each point represents the mean formation 1′-hydroxyelemicin (▲), 3′-hydroxyisoelemicin (▼), M4 (■) elemicin-2′,3′-oxide (•), 3-hydroxy-4,5-dimethoxyallylbenzene (•) obtained from two individual experiments.

Chapter 5

Matrix-derived combination effect and risk assessment for estragole from basil-containing plant food supplements (PFS)

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Abstract

Basil-containing plant food supplements (PFS) can contain estragole which can be metabolized into a genotoxic and carcinogenic 1'-sulfoxymetabolite. This chapter describes the inhibition of sulfotransferase (SULT)-mediated bioactivation of estragole by compounds present in basil-containing PFS. Results reveal that PFS consisting of powdered basil material contain other compounds with considerable in vitro SULT-inhibiting activity, whereas the presence of such compounds in PFS consisting of basil essential oil was limited. The inhibitor in powdered basil PFS was identified as nevadensin. Physiologically based kinetic (PBK) modeling was performed to elucidate if the observed inhibitory effects can occur in vivo. Subsequently, risk assessment was performed using the Margin of Exposure (MOE) approach. Results suggest that the consequences of the in vivo matrix-derived combination effect are significant when estragole would be tested in rodent bioassays with nevadensin at ratios detected in PFS, thereby increasing MOE values. However, matrix-derived combination effects may be limited at lower dose levels, indicating that the importance of matrix-derived combination effects for risk assessment of individual compounds should be done on a case-by-case basis considering dose-dependent effects. Furthermore, this study illustrates how PBK modeling can be used in risk assessment of PFS, contributing to further reduction in the use of experimental animals.

Introduction

Plant food supplements (PFS) are widely marketed throughout Europe where they are sold in pharmacies, drug stores, health-food shops, supermarkets and via Internet. Despite the fact that the use of botanical products is suggested to be beneficial for overall health, the use of some PFS that are available on the market might raise concerns as several botanicals are known to contain toxic compounds (EFSA, 2012). Examples of naturally present botanical compounds of concern include the group of alkenylbenzenes (EFSA, 2012; van den Berg *et al.*, 2011a, 2011b). In fact, it was recently demonstrated that some -though not all- PFS derived from basil, fennel, nutmeg, sassafras or calamus contain relatively high levels of the alkenylbenzenes estragole, methyleugenol, safrole and/or β-asarone (van den Berg *et al.*, 2011b). Based on the Margin of Exposure (MOE) approach it was even concluded that the use of such products, at dose levels recommended by the respective manufacturers, may lead to a daily intake of these genotoxic carcinogens comparable to dose levels causing malignant tumors in rodents, indicating a priority for risk management and a potential risk for human health (van den Berg *et al.*, 2011b). It is important to note that the available tumor data for these food-borne alkenylbenzenes, which were used to estimate the MOE values, were obtained from long-term rodent studies performed with pure compounds dosed in the absence of a botanical matrix (Miller *et al.*, 1983). It can be argued whether such data represent an adequate basis for the risk assessment for these botanical products (Rietjens *et al.*, 2008).

The genotoxic and carcinogenic activity of alkenylbenzenes has been ascribed to their bioactivation by cytochromes P450, leading to the formation of 1′-hydroxymetabolites, and the subsequent sulfonation of these 1′-hydroxymetabolites by sulfotransferases (SULTs), generating unstable DNA reactive 1′-sulfoxymetabolites (Figure 1) (Anthony *et al.*, 1987; Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Sangster *et al.*, 1987; Solheim and Scheline, 1973; Wiseman *et al.*, 1987). Interestingly, a previous study (Jeurissen *et al.*, 2008) showed that a methanolic basil extract caused a reduction in the level of DNA-adduct formation in incubations with DNA and rat or human liver S9 as well as in human hepatoma HepG2 cells exposed to 1′-hydroxyestragole, demonstrating the importance of the botanical matrix. The major compound in the methanolic basil extract responsible for this in vitro inhibition of the SULT-mediated bioactivation of estragole was found to be the flavonoid nevadensin

(Alhusainy *et al.*, 2010). Recently, it was demonstrated that nevadensin is also able to significantly inhibit the SULT-mediated bioactivation of estragole in vivo, resulting in reduced levels of estragole DNA-adducts in the livers of rats exposed simultaneously to estragole and nevadensin as compared to the estragole DNA-adduct levels in the livers of rats exposed to estragole alone (Alhusainy *et al.*, 2013).

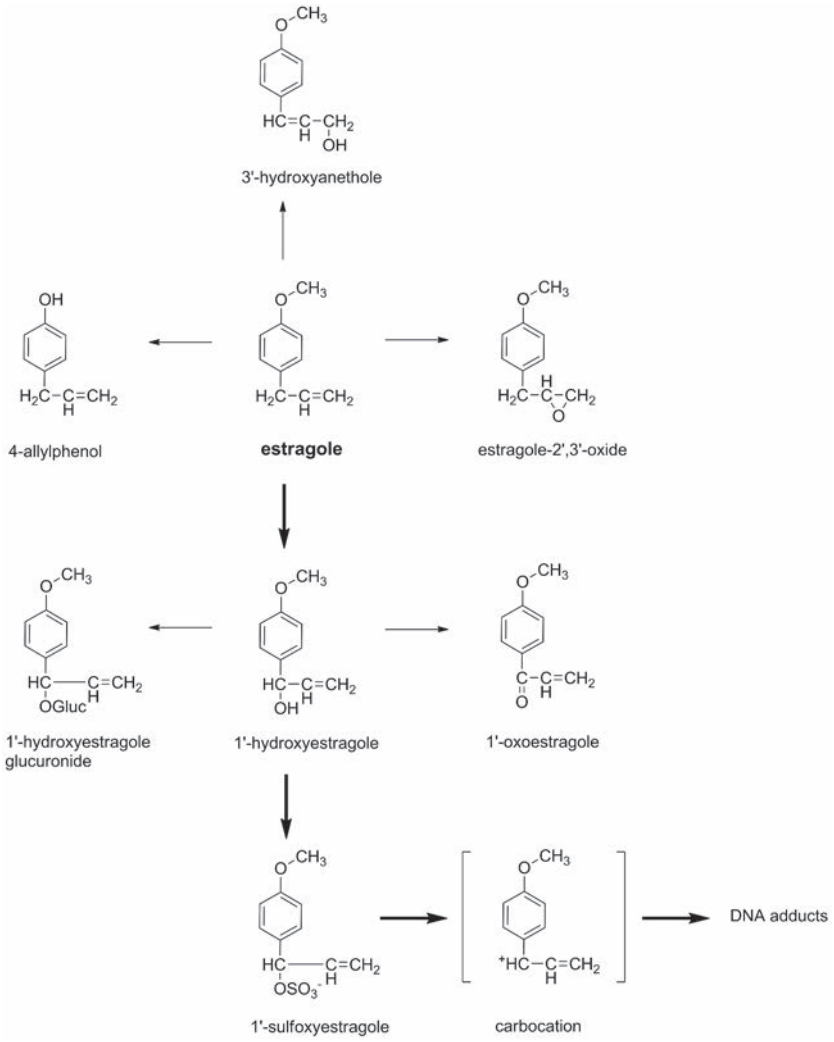

Figure 1. Metabolic pathways of estragole. Arrows in bold indicate the bioactivation to 1′-sulfoxyestragole and the formation of DNA-adducts.

Although a methanolic basil extract could thus markedly inhibit the metabolic bioactivation of estragole, compositional changes might occur when processing and manufacturing PFS. As a result, the level of SULT-inhibition can be different for botanicals used as herbs and spices compared to processed PFS prepared from the same botanical. Therefore, the aims of the present study were (1) to examine the effect on the bioactivation of estragole by compounds present in PFS consisting of powdered basil material or its essential oils for which we previously demonstrated a potential risk for human health (van den Berg *et al.*, 2011b), (2) to identify the most potent SULT inhibitor(s) in these PFS, (3) to perform physiologically based kinetic (PBK) modeling to elucidate if the observed inhibitory effects can occur in vivo and (4) to perform an updated risk assessment for the basil-containing PFS based on the MOE approach taking this matrix-derived combination effect into account.

Materials and methods

PFS and chemicals

Five basil-containing PFS were selected for which a risk assessment was previously made based on the MOE approach, indicating that use of all these basil-containing PFS results in MOE values in the range of 1-1,000 pointing at a high priority for risk management actions and a potential risk for human health (van den Berg *et al.*, 2011b). Two of the selected PFS consisted of powdered basil material and three of the selected PFS were prepared from the essential oil of basil. Characteristics of the selected PFS are presented in Table 1. Basil-containing PFS were purchased via Internet.

Apigenin, 7-hydroxycoumarin (7HC), 7-hydroxycoumarin sulfate (7HCS), tris-(hydroxymethyl)aminomethane, 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and pentachlorophenol (PCP) were purchased from Sigma-Aldrich (Steinheim, Germany). Nevadensin was from Sinova Inc. (Bethesda, USA). Pooled liver S9 from male Sprague-Dawley rats and mixed gender pooled human liver S9 were supplied by BD Gentest (Woburn, United States). Acetic acid was obtained from VWR International (Darmstadt, Germany) and dimethyl sulfoxide (DMSO) was from Acros Organics (Geel, Belgium). Methanol (ULC/MS grade) and acetonitrile (ULC/MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Ultrapure water was obtained from a Barnstead Nanopure Type I ultrapure water system.

Table 1. Levels of estragole and nevadensin in basil-containing PFS included in the present study.

Sample No.	Characteristics of PFS included in the present study (van den Berg *et al.*, 2011b)	Average level of estragole ± StDEV (mg/g supplement) (van den Berg *et al.*, 2011b)	Average level of nevadensin ± StDEV (mg/g supplement)	Ratio (nevadensin: estragole)
1	Supplement consisting of powdered plant material, preparation method unknown. Recommended daily dose equals 1-3 capsules, consistent with 230-690 mg total PFS per day.	1.21 ± 0.11	0.17 ± 0.04	0.14
2	Supplement consisting of powdered plant material, preparation method unknown. Recommended daily dose equals 4-6 capsules, consistent with 1580-2370 mg total PFS per day.	0.20 ± 0.01	0.24 ± 0.06	1.19
3	Supplement consisting of 100% essential oil including 12.5 mg O*cimum basilicum* essential oil per 100 mg supplement (*i.e.* 32.5 mg per capsule). Recommended daily dose equals 6 capsules, consistent with 1560 mg total PFS per day.	183.85 ± 2.24	ND	NC
4	Supplement consisting of 100% essential oil including 3.7 mg *Ocimum basilicum* essential oil per 100 mg supplement (*i.e.* 10 mg per capsule). Recommended daily dose equals 1-4 capsules, consistent with 270-1080 mg total PFS per day.	32.71 ± 1.46	ND	NC
5	Supplement consisting of 100% essential oil including 10.6 mg *Ocimum basilicum* essential oil per 100 mg supplement (*i.e.* 30 mg per capsule). Recommended daily dose equals 2-3 capsules, consistent with 565-848 mg total PFS per day.	241.56 ± 62.02	ND	NC

ND: not detected, NC: not calculated

Preparation of methanolic PFS extracts

The selected PFS consisting of powdered basil material were extracted as described previously (Alhusainy *et al.*, 2010; Jeurissen *et al.*, 2008). In short, 5 g of powdered PFS material was extracted with a mixture of methanol, water and acetic acid (ratio 80:19:1) by stirring the powdered PFS material twice for 2 h at room temperature, each time using 100 mL of the extraction mixture. After filtration of the extract solution through a folded filter (Schleicher & Schuell), the filtrates of each PFS were pooled and evaporated to dryness using a rotary evaporator (Heidolph LABOROTA 4000 efficient, Metrohm USA). The extraction yields were found to range between 21.6% and 23.8%. The dried extracts were subsequently dissolved in methanol to obtain concentrations of 2, 5, and 20 mg/mL. PFS consisting of basil essential oil were directly dissolved in methanol to obtain a concentration of 20 mg/mL followed by sonication (Bandelin Sonorex RK 100) for 10 min at room temperature. Thereafter, dilution series were prepared in methanol to make aliquots of 2 and 5 mg/mL. All stocks were filtered using a 0.2 µM cellulose acetate filter membrane (VWR international) and stored at -20˚C until use in the SULT assay.

SULT assay

The inhibition of SULT activity by the different methanolic basil-containing PFS extracts was measured as described by Alhusainy *et al.* (2010). Since the sulfonated 1'-hydroxymetabolite of estragole is unstable in an aqueous environment, hampering its direct detection by means of HPLC analysis, 7-hydroxycoumarin (7HC) was used as a substrate as previously described (Alhusainy *et al.*, 2010, 2012). Incubations were performed in a total volume of 100 µL with 0.1 M Tris-HCl (pH 7.4) containing (final concentrations) 0.4 mg/mL commercially obtained pooled liver S9 from male Sprague-Dawley rats or mixed gender pooled human liver S9 to 0.1 mM 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and 10, 25 or 100 µg/mL of a methanolic PFS extract (added from a 200 times concentrated stock in methanol). Prior to the addition of 25 µM 7HC (added from a 200 times concentrated stock solution in DMSO), samples were pre-incubated for 1 minute at 37 ºC. Reaction mixtures were incubated for 10 min at 37 ºC and terminated by adding 25 µl ice-cold acetonitrile. Incubations performed in the absence of PFS extract or in the presence of 25 µM of the known SULT inhibitor pentachlorophenol (PCP) (added from a 200 times concentrated stock solution in DMSO) were used as controls. In addition, blank incubations were performed

in the absence of the cofactor PAPS or S9 liver homogenates. In all incubations, the total amount of methanol and/or DMSO was kept below 1% (v/v). All samples were centrifuged at 5 ºC for 5 min at 16,000*g* and the supernatant was stored at -20˚C until HPLC analysis. Experiments were performed in triplicate.

HPLC analysis and quantification of 7HCS

For quantification of 7HCS, 50 µL of each sample obtained in the SULT assay was subjected to HPLC analysis as described previously (Alhusainy *et al.*, 2010, 2012). Chromatographic analysis was performed using a Waters 2695 separation module liquid chromatography system, connected to a 2996 photodiode array detector and an Alltima C18 5 µm column, 150 mm 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 0% acetonitrile to 20% acetonitrile in 2 min, increased to 21% acetonitrile over 10 min and further increased to 100% acetonitrile in 2 min at which it was kept for 1 minute. Starting conditions were reached in the next 2 min and retained for another 10 min. Under the conditions applied, 7HCS eluted at 6.2 min.

Fractionation of methanolic basil-containing PFS extracts

All methanolic basil-containing PFS extracts were subjected to fractionation based on the method described previously (Alhusainy *et al.*, 2010). In short, 50 µL of a concentrated PFS extract (*i.e.* 150 mg/mL) dissolved in methanol were injected into a Waters 600 controller liquid chromatography system connected to a Waters 996 photodiode array detector. Chromatographic separation was performed using an Alltima C18 5 µm column 150 mm x 4.6 mm (Grace Alltech, Breda, The Netherlands). The gradient was made with ultrapure water containing 0.1% (v/v) acetic acid and methanol. The flow rate was 1 mL/min. The mobile phase started with 0% methanol and was increased to 100% methanol within 60 min at which it was kept for 10 min. Starting conditions were reached in 10 min and retained in the next 10 min. In total, 60 fractions with a one minute interval were collected into Eppendorf tubes. Subsequently, the methanolic solvent present in the collected fractions was evaporated under a stream of gaseous nitrogen and the residual water was removed by freeze drying using a Christ Alpha RVC freeze dryer at 0.8-1.2 mbar. For each basil-containing PFS, fractionation was repeated five times and the dried fractions obtained of each

replicate were pooled by reconstituting them in 50 µL methanol. All fractions were tested in the SULT activity assay for their SULT-inhibitory potential.

Identification and quantification of the compounds present in the most potent fractions were accomplished by comparison of their retention times and UV spectra to the retention times and UV spectra of commercially available reference compounds.

UPLC analysis and quantitative detection of nevadensin in PFS consisting of basil

For quantification of nevadensin, the major SULT-inhibiting constituent in the methanolic basil-containing PFS extracts prepared in this study (see results section), 3.5 µL of each methanolic extract was subjected to UPLC analysis (n=3). UPLC analysis was performed on a Waters ACQUITY UPLC H-Class system connected to an ACQUITY UPLC photodiode array detector and a quaternary solvent manager. Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 1.7 µm column, 2.1 × 50 mm. The column was kept at 30°C and the sample manager was set at 10°C. The gradient was made with nanopure water containing 0.1% (v/v) TFA and acetonitrile. The flow rate was 0.6 mL/min and a gradient was applied from 0% acetonitrile to 20% acetonitrile in 0.2 min, increased to 79% acetonitrile over 1.8 min and further increased to 100% acetonitrile in 0.2 min at which it was kept for 0.6 min. Starting conditions were reached in the next 0.2 min and retained for another 1.2 min. The levels of nevadensin were quantified by comparing the peak areas to those in the calibration curve of nevadensin derived using a commercially available reference compound.

PBK modeling, $BMDL_{10}$ analysis and updated risk assessment using the MOE approach

An updated risk assessment of basil-containing PFS was made including the matrix-derived combination effect of nevadensin on the bioactivation of estragole. The reduction in the formation of estragole DNA-adducts (N^2-(*trans*-isoestragol-3'-y1)-2'-deoxyguanosine (E-3'-N^2-dGuo)) in the liver of rat following the combined exposure to estragole and nevadensin was predicted based on the PBK models previously developed for the bioactivation and detoxification of estragole (Punt *et al.*, 2009) including a sub-model describing the absorption, distribution, metabolism and excretion of nevadensin (Alhusainy *et al.*, 2013). The binary estragole-nevadensin PBK model used in this study was previously

validated based on an in vivo study in which rats were exposed to estragole and nevadensin showing that the PBK model based predictions adequately match the measured DNA-adduct levels in the liver of rats (Alhusainy *et al.*, 2013). In the present study, the estragole-nevadensin model (Alhusainy *et al.*, 2013) was run for a period of 24 h using the estragole dose levels administered to mice (Miller *et al.*, 1983) and a relative ratio of estragole and the SULT inhibitor nevadensin reflecting their occurrence in the specific PFS.

Based on the outcomes of the PBK model, the incidence of hepatomas as reported by Miller *et al.* (1983) was refined assuming a linear relationship between the reduced formation of E-3'-N^2-dGuo DNA-adducts and the reduction in the hepatoma incidence. In a next step, refined $BMDL_{10}$ (*i.e.* the lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence) values were calculated by performing a BMD (BenchMark Dose) analysis of the newly refined hepatoma incidences using EPA BMD software (version 2.1.2) as previously described (van den Berg *et al.*, 2011b). MOE values were calculated by dividing the refined $BMDL_{10}$ values by the estimated daily intake of estragole resulting from the use of each specific basil-containing PFS of interest (Table 1) (van den Berg *et al.*, 2011b).

Results

Inhibition of SULT activity in rat and human liver S9 homogenates by methanolic extracts of basil-containing PFS

The inhibition of SULT activity by several methanolic basil-containing PFS extracts in incubations with rat and human liver S9 is shown in Figure 2. All tested methanolic extracts were found to inhibit the metabolic conversion of 7HC to 7HCS in a dose-dependent manner. The extracts prepared from PFS consisting of powdered basil material (*i.e.* PFS1 and PFS2) were found to exert the most potent SULT-inhibiting effects. In fact, in human liver S9 homogenates, PFS2 completely blocked the formation of 7HCS at all concentrations tested (*i.e.* final concentrations of 10, 25 and 100 µg/mL) comparable to the effect observed when incubations were performed using 25 µM of the known SULT inhibitor PCP. In contrast, extracts that were prepared from PFS consisting of the essential oil of basil (*i.e.* PFS3, PFS4 and PFS5) resulted in only a moderate level of SULT-inhibition (Figure 2). Chromatographic analysis of incubations performed in the absence of the cofactor PAPS or S9 liver homogenates demonstrated that under these conditions 7HCS was not formed.

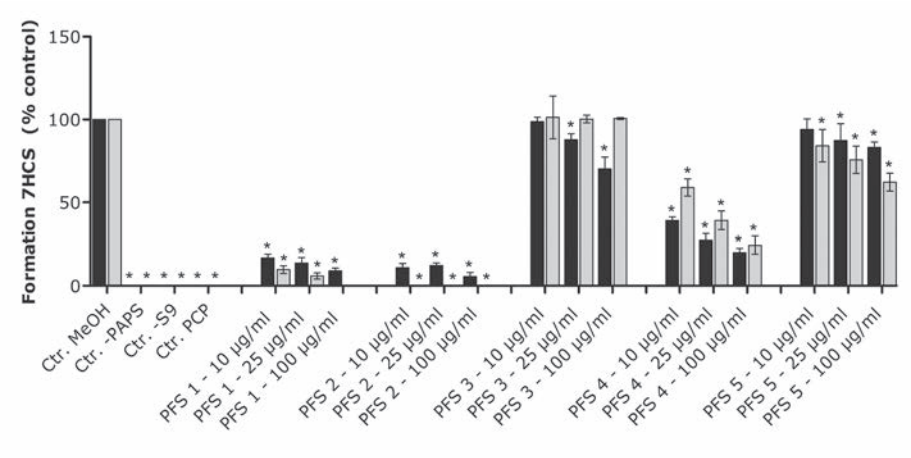

Figure 2. Inhibition of the SULT-mediated conversion of 7HC to 7HCS by methanolic extracts of several basil-containing PFS. Incubations were performed using pooled rat (black bars) or human liver (grey bars) S9 homogenates, 7HC as a substrate and PAPS as a cofactor in the presence or absence of final concentrations of 10, 25 or 100 µg/mL of methanolic basil-containing PFS extracts or 25 µM PCP (positive control). Data are presented as a percentage of the amount of 7HCS formed in incubations without a methanolic PFS extract (*i.e.* control MeOH). An asterix (*) indicates a significant decrease in the formation of 7HCS compared to incubations without SULT inhibitor ($p<0.05$).

Fractionation of methanolic extracts of basil-containing PFS

The methanolic extracts of basil-containing PFS were subjected to fractionation using HPLC. Figure 3 displays the inhibition of the SULT-catalyzed conversion of 7HC to 7HCS by all fractions obtained upon fractionation of methanolic extracts of the powdered basil-containing PFS (*i.e.* PFS1 and PFS2) (Figure 3A and 3B) or from PFS containing basil-derived essential oil (*i.e.* PFS3, PFS4 and PFS5) (Figure 3C-E). A potent SULT-inhibiting effect was shown for several fractions of the methanolic extracts prepared from PFS consisting of powdered basil material (Figure 3A and 3B). Fraction 51 was found to exert the highest level of SULT-inhibiting activity for PFS1 and PFS2 (Figure 3A and 3B). In fact, the formation of 7HCS was completely blocked in the presence of fraction 51. Fraction 50 of PFS1 also resulted in complete inhibition of SULT activity, whereas for PFS2 this fraction resulted in an almost complete SULT-inhibition of 92.9%. In addition, for both methanolic PFS extracts from powdered basil material, fraction 45 and 46 were found to contain potent SULT inhibitors resulting in a level of SULT-inhibition of 92.9-96.1%. Chromatographic analysis of fraction 50 and 51 demonstrated the presence of only one major compound in both fractions which was identified as nevadensin based on comparison of the retention time and UV spectra of

these fractions to those of a commercially available standard of nevadensin. Apigenin was identified in fraction 46 of both PFS consisting of powdered basil material (*i.e.* PFS1 and PFS2), although chromatographic analysis of this fraction also revealed the presence of other minor constituents.

In comparison to the methanolic extracts prepared from PFS consisting of powdered basil material, the level of SULT-inhibition by fractions of methanolic extracts from PFS consisting of basil essential oil was found to be limited amounting to 39.4% or less (Figure 3C-E). Moreover, there was no analogy between the three fractionation patterns with regard to the fractions showing the most potent SULT-inhibiting effect.

PBK prediction of the effect of nevadensin on in vivo estragole DNA-adduct formation

PBK modeling was performed to predict the in vivo effect of the combined exposure to estragole and nevadensin on E-3′-N^2-dGuo formation in rat liver. The binary estragole-nevadensin PBK model used for these analyses was previously validated based on an in vivo study in which DNA-adducts were quantified in the liver of rats simultaneously exposed to estragole and nevadensin (Alhusainy *et al.*, 2013). DNA-adduct formation was modeled using the binary estragole-nevadensin PBK model (Alhusainy *et al.*, 2013) at estragole dose levels used in the estragole rodent bioassay (Miller *et al.*, 1983) on which our previous risk assessment of the basil-containing PFS was based (van den Berg *et al.*, 2011b). Table 2 shows that the level of estragole DNA-adduct formation in rat liver at dose levels of 54 and 107 mg/kg bw/day estragole are predicted by the PBK model to equal 1983 adducts in 10^8 nt and 4114 adducts in 10^8 nt in the absence of nevadensin. In the presence of nevadensin, at a ratio between estragole and nevadensin reflecting their levels in PFS1, a reduction in the formation of estragole DNA-adducts by 65% (*i.e.* 688 adducts in 10^8 nt) and 66% (*i.e.* 1385 adducts in 10^8 nt) was predicted for estragole dose levels of 54 and 107 mg/kg bw/day respectively. Table 2 also shows the predicted PBK model outcomes for the formation of estragole DNA-adducts following an exposure to 54 and 107 mg/kg bw/day estragole in the presence of nevadensin at levels reflecting the ratio between estragole and nevadensin in PSF2.

Results reveal that, at 54 and 107 mg/kg bw/day estragole, the reduction of E-3′-N^2-dGuo DNA-adduct formation is predicted by the PBK model to equal 89% and 88% following the co-administration of estragole and nevadensin at a ratio relevant for their occurrence in PFS2.

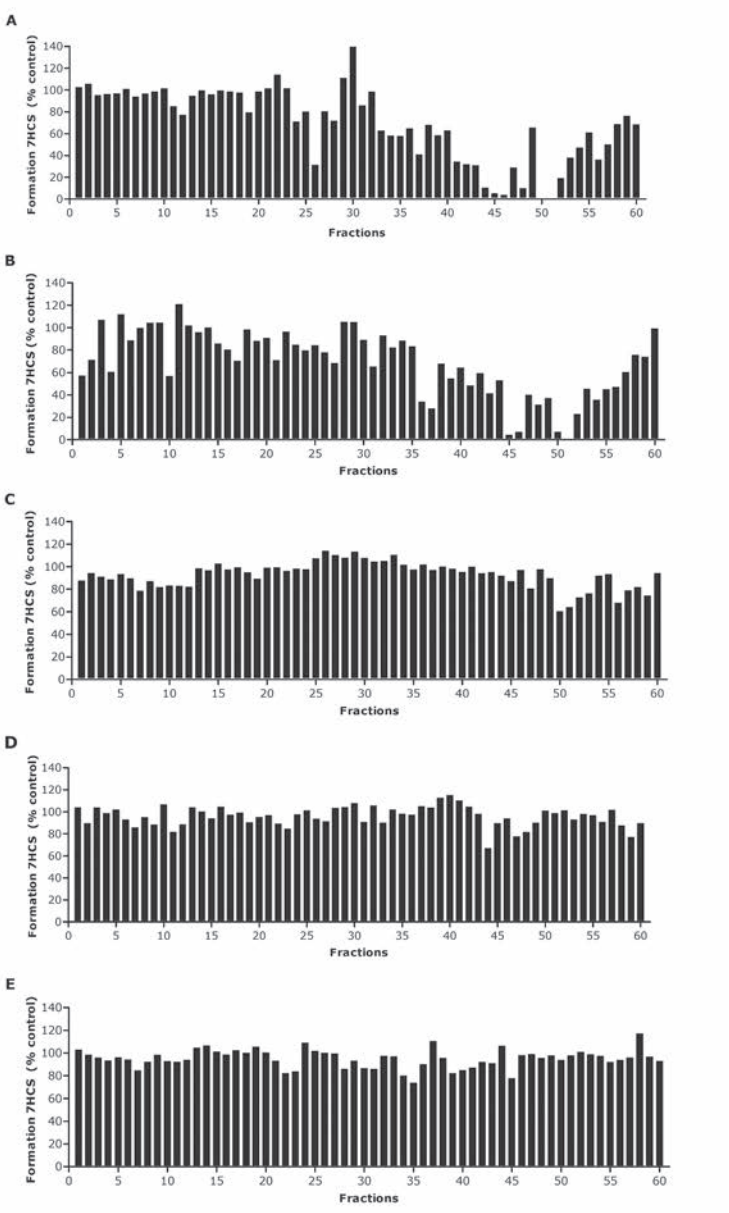

Figure 3. Inhibition of the SULT-mediated conversion of 7HC to 7HCS by several basil-containing PFS fractions collected by HPLC. Incubations were performed using pooled rat liver S9 homogenates, 7HC as a substrate and PAPS as a cofactor in the presence of fractions of A) basil-containing PFS1, B) basil-containing PFS2, C) basil-containing PFS3, D) basil-containing PFS4 or E) basil-containing PFS5. Data are presented as a percentage of the amount of 7HCS formed in incubations without SULT inhibitor (*i.e.* control MeOH).

Extracts of PFS consisting of basil essential oils were able to inhibit SULT activity only to a moderate extent and the effect of this SULT-inhibition was not further investigated. It should be noted that the rodent bioassay by Miller *et al.* (1983) was performed using dose levels of estragole that considerably exceed the levels of exposure to estragole resulting from the use of basil-containing PFS. The levels of DNA-adduct formation at levels of 4.6 and 13.9 µg/kg bw/day estragole, the estimated daily intake of estragole resulting from the use of PFS1 at the recommended daily intake of 1 or 3 capsules, respectively, were predicted by the PBK model to equal 0.1 adducts per 10^8 nt or 0.3 adducts per 10^8 nt. Co-administration of estragole and nevadensin, at a dose reflecting their actual levels in PFS1, was predicted to result in a decrease of estragole DNA-adduct formation by 0.02% and 0.07% following exposure to 4.6 or 13.9 µg/kg bw/day estragole respectively. For PFS2, DNA-adduct formation was predicted by the PBK model to amount to 0.1 adducts in 10^8 nt or 0.2 adducts in 10^8 nt following exposure to 5.4 or 8.1 µg/kg bw/day estragole, respectively, the estimated daily intake of estragole resulting from the use of PFS2 at the recommended daily intake of 4 and 6 capsules, respectively. The formation of estragole DNA-adducts was predicted to decrease by 0.22% and 0.33% at estragole intake levels of 5.4 and 8.1 µg/kg bw/day in the presence of nevadensin at a dose reflecting their actual levels in PFS2 amounting to 6.4 or 9.6 µg/kg bw/day nevadensin respectively. These results demonstrate that the consequences of the in vivo matrix-derived combination effect are especially observed in rodent bioassays at estragole dose levels that cause significant cancer incidences but are predicted to be limited at exposure levels of estragole and nevadensin relevant for the daily use of PFS consisting of powdered basil material. For the extracts consisting of basil essential oil only the PBK predicted levels of DNA-adducts formed upon intake of estragole at dose levels resulting from intake of PSF3, PSF4 and PSF5 at the recommended daily dose levels were calculated and were found to amount to 128, 3-13 and 55-87 in 10^8 nt respectively.

$BMDL_{10}$ analysis and updated risk assessment using the MOE approach

Table 3 presents a summary of the incidence of hepatocellular carcinomas as observed in a long term carcinogenicity study in which rodents were exposed to estragole at dose levels of 54 or 107 mg/kg bw/day without the natural (botanical) matrix being present (Miller *et al.*, 1983). Based on the PBK model predictions for the reduction in estragole DNA-adduct formation in rat liver following the combined exposure to estragole and nevadensin (Table 2),

a refinement of the incidences of hepatocellular carcinomas as reported by Miller *et al.* (1983) was made assuming a proportional reduction in DNA-adduct formation and hepatomas. Table 3 provides a summary of the refined hepatoma incidences thus obtained.

Table 2. Overview of the PBK-predicted formation of estragole DNA-adducts in rat liver in the presence or absence of nevadensin at a ratio of estragole and nevadensin reflecting their occurrence in basil-containing PFS.

Dose estragole (mg/kg bw/day)	Dose nevadensin (mg/kg bw/day)	Adducts in 10^8 nt in the absence of nevadensin as predicted by PBK modeling	Adducts in 10^8 nt in the presence of nevadensin as predicted by PBK modeling	% reduction DNA-adduct formation
Basil-containing PFS1				
0	0	0	0	0
54	8	1983	688	65
107	15	4114	1385	66
Basil-containing PFS2				
0	0	0	0	0
54	64	1983	214	89
107	127	4114	496	88

Based on the refined hepatoma incidences obtained, a BMD analysis was performed resulting in $BMDL_{10}$ values ranging between 21-25 mg/kg bw/day and 63-87 mg/kg bw/day when using the estragole to nevadensin ratios of PFS1 and PFS2 respectively (Table 4) (for details on BMD analysis see Table S1 in the Supplementary Materials of Chapter 5). Subsequently, MOE values were calculated by comparing the newly defined $BMDL_{10}$ values with the estimated daily exposure levels to estragole resulting from the use of the specific basil-containing PFS (*i.e.* 0.005-0.014 mg estragole/kg bw/day and 0.005-0.008 mg estragole/kg bw/day for PFS1 and PFS2, respectively). Table 4 shows the refined MOE values that were obtained in this way. For PFS1 the refined MOE values vary between 2,000 and 5,000 and for PFS2 MOE values were found to amount to 8,000-20,000 (Table 4). These refined MOE values are considerably higher than the MOE values of 200-1,000 and 400-1,000 that were previously derived using the non-refined $BMDL_{10}$ values from tumor data obtained in a rodent study using purified estragole without the natural (botanical) matrix being present (Miller *et al.*, 1983; van den Berg *et al.*, 2011b).

Table 3. Overview of the incidence of hepatomas in rodents following long-term exposure to pure estragole (Miller *et al.*, 1983) and the calculated incidence of hepatomas taking into account the matrix-derived combination effect of basil-containing PFS as derived by PBK modeling.

Dose estragole (mg/kg bw/day)	Dose nevadensin (mg/kg bw/day)	Incidence hepatomas in the presence of estragole only (Miller *et al.*, 1983)	% reduction DNA-adduct formation[a]	Calculated incidence hepatomas after co-administration of estragole and nevadensin
Basil-containing PFS1				
0	0	0/50	0	0/50
54	8	27/48	65	9/48
107	15	35/49	66	12/49
Basil-containing PFS2				
0	0	0/50	0	0/50
54	64	27/48	89	3/48
107	127	35/49	88	4/49

[a] The data used to predict the reduction in DNA-adduct formation in rat liver following the combined exposure to estragole and nevadensin are presented in Table 2.

Table 4. Updated risk assessment based on the MOE approach taking into account the presence of the SULT inhibitor nevadensin in basil-containing PFS (matrix-derived combination effect).

PFS	Daily Intake Estragole (mg/kg bw/day)[a] (van den Berg *et al.*, 2011b)	MOE using data from Miller *et al.*1983 (van den Berg *et al.*, 2011b)	Refined $BMDL_{10}$ taking into account the matrix-derived combination effect (mg/kg bw/day)	Refined MOE taking into account the matrix-derived combination effect[b]
1	0.005 – 0.014	200 – 1,000	21 – 25	2,000 – 5,000
2	0.005 – 0.008	400 – 1,000	63 – 87	8,000 – 20,000
3	4.78	1	ND[c]	ND[c]
4	0.147 – 0.589	6 – 40	ND[c]	ND[c]
5	2.275 – 3.414	1 – 3	ND[c]	ND[c]

[a] Using the dose of PFS intake as recommended by the manufacturer of the basil-containing PFS and the chemically determined levels of estragole in the PFS, daily intake estimates of estragole were obtained assuming a body weight of 60 kg.
[b] Refined MOE = refined $BMDL_{10}$ (mg/kg bw/day) / daily intake estragole (mg/kg bw/day)
[c] ND: Not Determined
Details on BMD analysis can be found in Table S1 in the Supplementary Materials of Chapter 5.

Discussion

In the presented chapter, the consequences of combined exposure to estragole and other compounds present in basil-containing PFS on the SULT-mediated bioactivation of estragole were studied. The results demonstrated that methanolic extracts of PFS consisting of powdered basil material contain potent SULT inhibitors. In line with these findings, a significant inhibition of SULT activity was previously also shown for a methanolic extract prepared from basil as such (Alhusainy *et al.*, 2010, 2012; Jeurissen *et al.*, 2008). It is important to note that results of the present study also revealed that the level of SULT-inhibition by PFS prepared from the essential oil of basil was markedly lower than that of PFS consisting of powdered basil material. In line with these findings, Müller *et al.* (1994) reported that basil essential oil, containing 88% estragole, was found to induce unscheduled DNA Synthesis (UDS) in the same concentration range as pure estragole showing that there was no protective effect of the remaining compounds present in the essential oil. The differences between the observed protective effects of PFS consisting of powdered basil material and PFS consisting of basil essential oils can be explained by the fact that PFS consisting of powdered basil material contain other compounds in addition to estragole that exert a substantial SULT-inhibiting activity whereas the presence of such modulating compounds in PFS consisting of basil essential oil is apparently limited. Thus, assessing the risk for human health resulting from the use of basil-containing PFS, one should take into account the specific processing and manufacturing conditions used since differences in these production processes can induce marked changes in composition, especially when extracting essential oils containing concentrated levels of individual (toxic) ingredients such as estragole.

In the present study, the flavonoids nevadensin and apigenin were identified in the methanolic extracts of PFS consisting of powdered basil material as the compounds responsible for inhibiting SULT activity. In line with these findings, both flavonoids were previously identified as the major SULT-inhibiting constituents in methanolic extracts of intact basil sold in the supermarket for use as a herb (Alhusainy *et al.*, 2010, 2012). Other flavonoids that are reported to be present in some basil varieties have also been demonstrated to exert SULT-inhibiting activities (Alhusainy *et al.*, 2012). These flavonoids include luteolin and catechin. However, it is important to realize that according to literature,

nevadensin is one of the major flavonoids of basil and exists in almost all varieties of the genus *Ocimum basilicum* L. whereas apigenin, luteolin and catechin are present in relatively low quantities or are only present in some basil varieties (Grayer *et al.*, 1996, 2004; Shan *et al.*, 2005). For example, the level of apigenin in different varieties of the genus *Ocimum basilicum* L. was estimated to be up to 71-fold lower compared to the level of nevadensin found in the same basil leaf sample (Grayer *et al.*, 2004). In addition, the K_i value for SULT-inhibition by apigenin was shown to be 175-fold higher than the K_i value for SULT-inhibition by nevadensin (*i.e.* 4 nM) (Alhusainy *et al.*, 2010, 2012) corroborating nevadensin to be a more important SULT inhibitor. Based on these results, the effect of apigenin on SULT-mediated bioactivation of estragole can be expected to be significantly lower than that of nevadensin. Furthermore, nevadensin was not only demonstrated to be a potent inhibitor of SULT activity in vitro, but it was recently also shown to significantly inhibit the SULT-mediated bioactivation and DNA-adduct formation of estragole in vivo (Alhusainy *et al.*, 2013).

Since nevadensin can markedly reduce estragole DNA-adduct formation in vivo (Alhusainy *et al.*, 2013), the adverse effects of estragole are likely to be lower when estragole is consumed in a matrix of basil-containing PFS compared to studies administering pure estragole. Thus, performing a risk assessment for basil-containing PFS as described previously (van den Berg *et al.*, 2011b), by using the $BMDL_{10}$ data obtained from a rodent study with high dose levels of the pure estragole (Miller *et al.*, 1983), may result in an overestimation of the priority for risk management and the potential risk for human health. Therefore, an updated risk assessment of basil-containing PFS using the MOE approach was made in the present study. To this end, studies in rodents should ideally be performed with the botanical or the botanical preparation of interest, allowing the calculation of specific $BMDL_{10}$ values. However, obviously, performing rodent bioassays for all basil-containing PFS is practically unfeasible as the generation of such data is expensive, labor-intensive and above all requires extensive animal testing. Therefore, the present study focused on the chemical identification of the most potent SULT inhibitor in basil-containing PFS and the use of the previously developed and validated estragole-nevadensin PBK model (Alhusainy *et al.*, 2013) to predict the possible in vivo reduction in estragole DNA-adduct formation, taking for each PFS the relative ratio between its levels of estragole and nevadensin into account. To obtain this refined estimate, the possible reduction in the values for the incidence of hepatocellular carcinomas

reported by Miller *et al.* (1983) was predicted using the PBK model, subsequently allowing the calculation of refined $BMDL_{10}$ data. Based on this approach, MOE values could be calculated for the individual PFS. These MOE values were found to be considerably higher than the MOE values that were previously calculated not taking the matrix-derived combination effect into account by using the non-refined $BMDL_{10}$ values (van den Berg *et al.*, 2011b). The results now presented indicate a lower priority for risk management actions for the powdered basil-containing PFS when considering the consequences of the botanical matrix on the bioactivation of estragole. These results indicate that for these PFS the use of $BMDL_{10}$ data derived from long-term carcinogenicity studies using pure estragole results in an overestimation of the potential risk for human health. In contrast, it was concluded that the matrix-derived combination effect of PFS consisting of basil essential oil is only of minor importance suggesting that the use of $BMDL_{10}$ data from studies using pure estragole accurately reflects the potential risk for human health resulting from the use of these PFS.

It should be noted that the estragole doses tested by Miller *et al.* (1983) are not representative for the human situation, since these are dose levels high enough to cause detectable tumor incidences in groups of 50 experimental animals. Risk assessment by the MOE approach is done using rodent tumor data and is thus based on dose levels actually inducing a significant tumor incidence and not necessarily on realistic human intake levels. At these high dose levels used in the Miller *et al.* study the concomitant dose of nevadensin is also high and apparently high enough to reach nevadensin levels in the liver that are in the range of the K_i value of 4 nM for SULT-inhibition (Alhusainy *et al.*, 2010). The presented study however also gives an estimate of the effects at estragole and nevadensin dose levels representing a realistic human intake. The matrix-derived combination effect of nevadensin on the bioactivation of estragole was predicted to be limited at dose levels of estragole and nevadensin relevant for the use of PFS consisting of powdered basil-material. At these dose levels the corresponding dose of nevadensin is not high enough to reach the K_i of nevadensin in vivo. This illustrates that testing compounds in the presence of their natural matrix in rodent bioassays may not necessarily provide better $BMDL_{10}$ values for risk assessment, and that the importance of a matrix-derived combination effect for risk assessment of individual compounds should be done on a case-by-case basis also taking into account analysis of dose-dependent effects on the interactions detected. On the basis of the results

now available and our previous results showing MOE values ranging between 200-1,000 and 1-40 for PFS consisting of powdered basil material or its essential oil, respectively (van den Berg *et al.*, 2011b), it is still concluded that the use of some basil-containing PFS is of priority for risk management actions and a potential risk for human health.

In conclusion, compounds present in especially powdered basil-containing PFS can modulate the bioactivation of estragole, whereas this effect was limited for PFS consisting of basil essential oil suggesting that the matrix-derived combination effect for basil-containing PFS should be judged on a case-by-case basis. Furthermore, the present study provides an example of how PBK modeling can be used in the risk assessment of botanicals and botanical preparations, contributing to further reduction in the use of experimental animals.

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Supplementary Materials Chapter 5

Table S1. Overview of BMD analysis for the induction of hepatomas in female mice using BMDS software version 2.1.2, a BMD of 10% and default settings.

Model	No. of parameters	Log Likeli-hood	p-value	Accepted	BMD_{10} (mg/kg bw/day)	$BMDL_{10}$ (mg/kg bw/day)
Basil-containing PFS1						
Null	1	-60.29				
Full	3	-50.44				
Gamma	1	-50.80	0.69	Yes	34.7	24.7
Logistic	2	-53.73	0.02	No		
LogLogistic	1	-50.68	0.79	Yes	31.7	21.3
LogProbit	2	-50.44	1.00	Yes	13.7	ND
Multistage	1	-50.80	0.69	Yes	34.7	24.7
Multistage-Cancer	1	-50.80	0.69	Yes	34.7	24.7
Probit	2	-53.35	0.03	No		
Weibull	1	-50.80	0.69	Yes	34.7	24.7
Quantal-linear	1	-50.80	0.69	Yes	34.7	24.7
Basil-containing PFS2						
Null	1	-28.14				
Full	3	-25.08				
Gamma	1	-25.21	0.87	Yes	113.4	64.6
Logistic	2	-26.18	0.19	Yes	116.2	87.2
LogLogistic	1	-25.20	0.88	Yes	114.5	63.1
LogProbit	2	-25.08	1.00	Yes	185.5	ND
Multistage	1	-25.21	0.87	Yes	113.4	64.6
Multistage-Cancer	1	-25.21	0.87	Yes	113.4	64.6
Probit	2	-26.07	0.20	Yes	114.7	84.0
Weibull	1	-25.21	0.87	Yes	113.4	64.6
Quantal-linear	1	-25.21	0.87	Yes	113.4	64.6

ND: Not Determined. Benchmark dose computation failed. Lower limit includes zero.

Chapter 6

Chemical analysis of estragole in fennel based teas and associated safety assessment using the Margin of Exposure (MOE) approach

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Abstract

This chapter describes the analysis of estragole in dry fennel preparations and in infusions prepared from them and an associated safety assessment. A wide range of estragole levels of 0.15-13.3 mg/g dry fennel preparation was found. The estragole content in infusions was considerably lower ranging between 0.4 and 133.4 µg/25 mL infusion prepared from 1 g dry material. Infusions prepared from whole fennel fruits contained about 3-fold less estragole compared to infusions prepared from fine cut fennel material. Safety assessment was performed using the Margin of Exposure (MOE) approach comparing available tumor data to the estimated daily estragole intakes from the consumption of 1-3 cups fennel tea. MOE values obtained for adults generally point at a low priority for risk management, especially when one cup of fennel tea is used daily during lifetime. MOE values for use of fennel teas by children were generally <10,000 indicating a priority for risk management. However, limiting such uses to 1-2 weeks, MOE values might be 3 orders of a magnitude higher and there would be no priority for risk management. These results indicate a low priority for risk management actions for use of fennel teas especially for short-term uses proposed for the symptomatic treatment of digestive disorders.

Introduction

For many years, botanicals have formed part of the regular diet as fruits or vegetables, wines, tea, food supplements or as flavors and fragrances added to food. Additionally, numerous botanicals and botanical preparations have a long history of use as traditional herbal medicine. An example of such a botanical used to maintain health or treat symptoms of disease is fennel (*Foeniculum vulgare* Mill.). Fennel based teas are traditionally used in many parts of Europe including France, Germany, Austria, Czech Republic and Poland were they are often used for the symptomatic treatment of digestive disorders alleviating mild spasmodic gastro-intestinal ailments and the relief of symptoms during inflammations of mucous membranes of the upper respiratory tract (EMA, 2008). Resulting from these proposed health-promoting effects, and the natural character of fennel, homemade fennel tea is often used as a remedy for gastrointestinal complaints in infants and young children (Crotteau *et al.*, 2006; Perry *et al.*, 2011). Although fennel based teas have a long history of use and are valued for their beneficial effects, fennel may contain active ingredients of concern, such as the alkenylbenzene estragole (Figure 1) (EFSA, 2012a). Estragole was previously indicated to be genotoxic and carcinogenic (SCF, 2001). In response to these findings, regulatory actions were taken within the EU restricting the use of estragole (Regulation (EC) No. 1334/2008 of the European Parliament and of the Council, 16 December 2008). The potential risks for human health related to the use of estragole-containing fennel based teas to relief gastrointestinal disorders, especially in children, have not (yet) been thoroughly studied and the European Medicines Agency (EMA, former EMEA) concluded that "the use of fennel tea is not recommended in children under 4 years of age due to the lack of adequate data" (EMA, 2008).

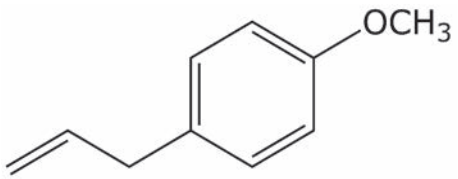

Figure 1. Structural formula of estragole.

A major issue in the safety assessment of estragole and estragole-containing food items is how to provide guidance on the potential risks for human health resulting from the exposure to (low levels of) food-borne compounds that are genotoxic and carcinogenic (EFSA, 2005). The European Food Safety Authority (EFSA) recently indicated that whenever genotoxic and carcinogenic compounds are present in a botanical or botanical preparation of interest, the Margin of Exposure (MOE) approach (EFSA, 2005) can be used to evaluate the potential risk for human health and the priority for risk management actions (EFSA, 2009b). The MOE is a dimensionless ratio that is obtained by comparing exposure levels causing malignant tumors in experimental animals (*e.g.* the $BMDL_{10}$ value, the lower confidence bound of the benchmark dose that gives 10% extra cancer incidence) with the estimated daily intake in humans (EFSA, 2005). In a previous evaluation of the EFSA Scientific Cooperation (ESCO) working group, the MOE was applied to the use of estragole-containing tea prepared from bitter fennel fruits (*F. vulgare* Mill. var. vulgare) (EFSA, 2009a). The exposure to estragole was estimated to equal 1.9-15.8 mg per day corresponding to 33-263 µg estragole/kg bw/day for a person with a body weight of 60 kg (EFSA, 2009a). This theoretical exposure estimate was based on the assumption that 4.5-7.5 g of fennel fruits would be used on a daily basis for the preparation of homemade fennel tea, that fennel fruits contain 5% essential oil, that the essential oil contains 3.5-12% estragole and that the extraction efficiency of the essential oil into the infusion is equal to 25-35% (EFSA, 2009a). The ESCO working group calculated the MOE based on the exposure estimates obtained and $BMDL_{10}$ values derived from the incidence of malignant liver tumors in female CD-1 mice exposed to estragole (EFSA, 2009a; Miller *et al.*, 1983). The MOE values thus obtained were found to be relatively low (*i.e.* MOE values equal to 34-1,000) indicating that the daily use of homemade fennel tea prepared from bitter fennel fruits would be of high priority for risk management actions (EFSA, 2009a). Raffo *et al.* (2011) recently experimentally determined the estragole content in hot water extracts of commercial fennel teas providing a refinement of the exposure estimate made by the ESCO working group. It was demonstrated that the consumption of tea prepared from sweet fennel (*F. vulgare* Mill. Var. dulce), available on the Italian market, that was found to contain the highest estragole level amongst the preparations tested in that study, would result in 3-26 fold lower estragole exposures compared to those estimated by the ESCO working group (Raffo *et al.*, 2011). Based on these lower

exposure levels, Raffo *et al.* (2011) calculated the MOE values to range between 870 and 3210 which is thus higher than the MOE values of 34-1,000 calculated by the ESCO working group (Raffo *et al.*, 2011). However, by being lower than 10,000 these MOE values still point at a priority for risk management.

In general, the use of different exposure estimates (*e.g.* theoretical vs. experimental) may result in different daily intake levels for the same exposure scenario and thus also might provide different advice to risk managers on the potential risk for human health. In fact, limitations in exposure estimates are a major source of uncertainty in safety assessment. Thus, the use of refined intake estimates for estragole is required to facilitate an accurate outcome of the safety assessment of fennel based tea preparations. However, the concentration of estragole may show considerable variations depending on the variety, geographical origin, harvesting techniques and processing methods amongst others (Smith *et al.*, 2002) and high variability of the estragole content in fennel fruits are reported in literature (Dadalioglu and Evrendilek, 2004; EFSA, 2012a; Miraldi, 1999; Ruberto *et al.*, 2000). Although the work of Raffo *et al.* (2011) provided an important refinement of the exposure estimate of estragole resulting from the use of fennel tea, exposure estimates were only based on the use of sweet fennel obtained from the Italian market while in addition to sweet fennel also bitter fennel is widely used to prepare homemade fennel based teas. In this respect, a further refinement of exposure data of estragole resulting from the consumption of homemade fennel tea including different varieties and origins of fennel teas, but also the experimental determination of the extraction efficiency of estragole into the infusion could improve the accuracy of the safety assessment. Hence, the aims of the present study were (1) to make a chemical analysis of estragole in sweet and bitter fennel teas consisting of commercial preparations of fine cut fennel material or whole fruits derived from different geographical origins, and (2) to perform a safety assessment for estragole resulting from drinking fennel tea using the MOE approach, taking into account the previous work by the ESCO working group (EFSA, 2009a) and reported in literature (Raffo *et al.*, 2011).

Materials and methods

Chemicals and materials

A total of 34 fennel tea preparations from different brands were purchased from Internet, obtained from local shops in the Netherlands, Belgium, Germany and Italy or provided by the European Herbal Infusion Association. Fennel tea preparations in the form of teabags, unpackaged fennel consisting of whole fruits or fine cut fennel material, and instant tea granules were included. 7 Fennel tea preparations consisted of sweet fennel material and 8 fennel tea preparations consisted of bitter fennel material. The variety of the remaining samples (n=18) was not specified. Product information as indicated on the label of each product is summarized in Table 1. Estragole (purity 98%) was supplied by Acros organics (Geel, Belgium). Acetonitrile (ULC/MS gradient) and methanol (HPLC supra gradient) were acquired from Sigma Aldrich (Steinheim, Germany). Nanopure water was obtained from a Barnstead nanopure Type I ultra-pure water system.

Methanolic extracts

To allow quantification of the total estragole content in the fennel tea samples expressed per g dry fennel preparation, methanolic extracts were prepared based on the method previously described (Gursale *et al.*, 2010; van den Berg *et al.*, 2011). To correct for the amount of estragole lost in this extraction method, a recovery study was performed as described by van den Berg *et al.* (2011). The average percentage recovery was found to equal 90.6 ± 8.1%. This value was used to correct the total level of estragole in the selected dry fennel preparations for sample recovery.

Hot water extracts

In addition to the methanolic extracts, hot water extracts were made to reflect the preparation of homemade fennel tea. Extracts were prepared based on the method previously described by Raffo *et al.* (2011) with minor modifications. In short, tea extracts were prepared by adding 25 mL freshly boiled water to 1 g of fennel tea material. The mixture of hot water and fennel tea material was incubated for 7 min in a covered beaker and stirred three times with a spoon. After 7 min, all fennel material was removed from the infusion and the extract was cooled down to room temperature.

Table 1. Characteristics of fennel based tea samples used, levels of estragole in dry fennel preparations, in water infusions prepared from the selected fennel samples and extraction efficiencies of estragole into the water infusion.

Sample No.	Variety[a]			Form			Origin	Average ± STDEV (µg estragole/ g dry fennel preparation)[b]	Average ± STDEV (µg estragole/25 mL infusion extracted from 1 g fennel material)	Extraction efficiency of estragole into the infusion (%)
	Sweet	Bitter	Unknown	Fine cut material	Whole fruits	Granules for instant tea				
1	X			X			Turkey	654.3 ± 113.6	3.1 ± 0.1	0.5
2	X				X		Turkey	597.4 ± 19.3	ND	ND
3		X		X			China	929.8 ± 5.9	6.8 ± 0.6	0.7
4		X			X		China	1218.8 ± 212.5	1.1 ± 0.0	0.1
5		X		X			Bulgaria	1769.8 ± 83.2	16.8 ± 2.2	0.9
6		X			X		Bulgaria	1601.6 ± 129.1	2.8 ± 0.4	0.2
7	X			X			China	603.3 ± 65.4	4.9 ± 0.4	0.8
8	X				X		China	604.5 ± 34.5	1.5 ± 0.0	0.2
9			X	X			UK	3833.3 ± 196.6	26.6 ± 4.1	0.7
10			X	X			Netherlands	478.0 ± 37.4	6.8 ± 1.2	1.4
11			X	X			Netherlands	163.8 ± 17.1	3.7 ± 0.5	2.3
12			X	X			Netherlands	958.2 ± 122.3	5.2 ± 3.6	0.5
13			X	X			Germany	913.7 ± 49.5	3.8 ± 1.9	0.4
14			X		X		Austria	1686.9 ± 129.1	3.0 ± 0.7	0.2
15			X		X		Austria	1404.2 ± 87.4	9.3 ± 3.0	0.7
16			X		X		Germany	1453.1 ± 168.7	4.2 ± 1.6	0.3
17			X	X			Germany	627.8 ± 69.0	3.5 ± 1.1	0.6
18			X	X			Belgium	1443.8 ± 65.4	8.8 ± 4.9	0.6
19			X	X			Belgium	158.8 ± 30.4	3.7 ± 1.5	2.3
20			X			X	Belgium	ND	0.4 ± 0.1	ND
21			X	X			Italy	2227.8 ± 245.6	15.6 ± 5.4	0.7
22			X	X			India	150.2 ± 17.0	2.3 ± 0.6	1.5
23			X	X			India	7867.1 ± 1292.1	29.1 ± 8.4	0.4

Table 1 (continued). Characteristics of fennel based tea samples used, levels of estragole in dry fennel preparations, in water infusions prepared from the selected fennel samples and extraction efficiencies of estragole into the water infusion.

Sample No.	Variety[a]			Form			Origin	Average ± STDEV (µg estragole/ g dry fennel preparation)[b]	Average ± STDEV (µg estragole/25 mL infusion extracted from 1 g fennel material)	Extraction efficiency of estragole into the infusion (%)
	Sweet	Bitter	Unknown	Fine cut material	Whole fruits	Granules for instant tea				
24			X	X			Germany	281.9 ± 21.2	5.8 ± 1.2	2.0
25			X	X			Germany	914.2 ± 90.5	1.6 ± 0.4	0.2
26			X			X	Italy	ND	2.5 ± 0.7	ND
27	X			X	X		Unknown	791.0 ± 78.6	5.7 ± 0.6	0.7
28	X			X			Unknown	912.6 ± 31.8	12.8 ± 1.9	1.4
29	X			X			Unknown	638.6 ± 31.8	7.1 ± 0.4	1.1
30		X		X			Unknown	1499.0 ± 106.7	15.8 ± 5.2	1.1
31		X		X			Unknown	2383.5 ± 303.2	33.5 ± 2.2	1.4
32		X		X			Unknown	387.5 ± 18.1	3.3 ± 0.5	0.9
33		X		X			Unknown	871.9 ± 144.5	10.7 ± 1.4	1.2
34			X	X	X		Unknown	13248.7 ± 1390.4	133.4 ± 18.5	1.0

[a] Sweet fennel, *F. vulgare* Mill. Var. dulce; bitter fennel, *F. vulgare* Mill. var. vulgare

[b] Values represent the total level of estragole in the fennel samples and was determined with the methanolic extraction method applied and corrected for sample recovery (see materials and methods section for details).

ND: not determined

Aliquots of the hot water extracts were centrifuged at 16,000*g* for 5 min and the supernatant was stored at −20 °C until ultra performance liquid chromatography (UPLC)-analysis.

UPLC analysis
Before UPLC analysis, aliquots of the methanolic extract solution were diluted in acetonitrile (1:10 v/v). Aliquots of the hot water extracts were analyzed undiluted. For quantification of estragole, 3.5 µl of each sample was subjected to UPLC analysis (*n*=3) as described previously (van den Berg *et al.*, 2011).

Estimation of daily intakes of estragole resulting from the use of fennel based teas
The exposure estimate of estragole resulting from the use of fennel teas was based on the estragole content in the hot water extracts as determined in the present study (see results) together with the assumption that 1.5-2.5 g fennel would be used for the preparation of a cup of fennel tea as described by the ESCO working group (EFSA, 2009a). Intake estimates of estragole were made for the daily consumption of one, two or three cups of fennel based tea assuming a body weight of 70 kg, the default value for adult body weight recently proposed by EFSA (2012b).

Calculation of the Margin of Exposure values
MOE values were calculated by comparing the previously calculated $BMDL_{10}$ values of 3.3-6.5 mg/kg bw/day (van den Berg *et al.*, 2011) for the induction of hepatocellular carcinomas in female mice derived from the data of Miller *et al.* (1983), with the estimated daily intakes of estragole resulting from the use of fennel based teas as determined in the present study. MOE values were rounded to a single significant value.

Results

Chemical analysis of estragole in dry fennel preparations and in fennel based teas
Figure 2 presents, as an example, part of the UPLC chromatogram of a methanolic as well as a hot water fennel extract detecting the presence and level of estragole. Table 1 shows that the total estragole content in the different dry fennel preparations was found to vary considerably ranging between 0.15 and 13.3 mg/g dry fennel preparation. Estragole could not be detected in two

samples because the instant tea granules in those specific samples only partially dissolved in methanol. The analysis revealed that the content of estragole in the hot water extracts was considerably lower than the estragole content found in the methanolic extracts (Figure 2 and Table 1). In fact, results for the hot water extracts from 1 g of fennel material, also presented in Table 1, revealed that the level of estragole was relatively low in these water infusions ranging between 0.4 and 133.4 µg/25 mL infusion (Table 1). In the hot water extract prepared from sample 2, estragole could even not be detected (Table 1). Comparing the concentration of estragole in the hot water extracts to the total concentration of estragole in the dry fennel preparations revealed an extraction efficiency of estragole from fennel into the infusion equal to 0.1-2.3% (Table 1).

Results obtained also show that the variation in the total estragole content as determined for the dry fennel preparations could not be attributed to the type of fennel (*i.e.* sweet or bitter) or the form (*i.e.* fine cut fennel material or whole fennel fruits). In fact, calculating the average estragole content showed the presence of 1.1 ± 0.4 and 1.3 ± 0.2 mg estragole/g dry weight in preparations of sweet (n=7) and bitter (n=8) fennel material, respectively. In addition, dry fennel preparations consisting of fine cut fennel material (n=23) or whole fennel fruits (n=7) were found to contain on average 1.3 ± 0.3 and 1.2 ± 0.2 mg estragole/g dry fennel preparation, respectively. In line with these findings, in water infusions prepared from 1 g sweet or bitter fennel material, the average estragole content was comparable (*i.e.* 9 ± 4 and 11 ± 4 µg estragole/25 mL infusion, respectively) indicating that the estragole content in infusions cannot be attributed to the type of fennel. However, the average estragole content in infusions prepared from whole fennel fruits (*i.e.* 3 ± 1 µg estragole/25 mL infusion) was found to be about 3-fold lower compared to the average content of estragole in infusions prepared from fine cut fennel material (*i.e.* 10 ± 2 µg estragole/25 mL infusion) demonstrating that the extraction efficiency of whole fennel fruits is lower compared to that of fine cut fennel material.

Interestingly, it was shown that the average estragole content in water infusions prepared from fennel material (*i.e.* fine cut fennel material and whole fennel fruits) for which customary selections of the respective levels of estragole were done (samples 1-8, average estragole content of 5 ± 2 µg estragole/25 mL infusion prepared from 1 g dry material) was found to be about 3-fold lower compared to that of the remaining fennel tea preparations (samples 9-34, average estragole content of 14 ± 5 µg estragole/25 mL infusion). In addition, it

was even shown that estragole could not be detected in a water infusion that was prepared from sample 2. These results demonstrate that the use of fennel material containing low levels of estragole can have a marked effect on the estragole content in fennel teas and thus also on the potential risk for human health resulting from the use of fennel based teas.

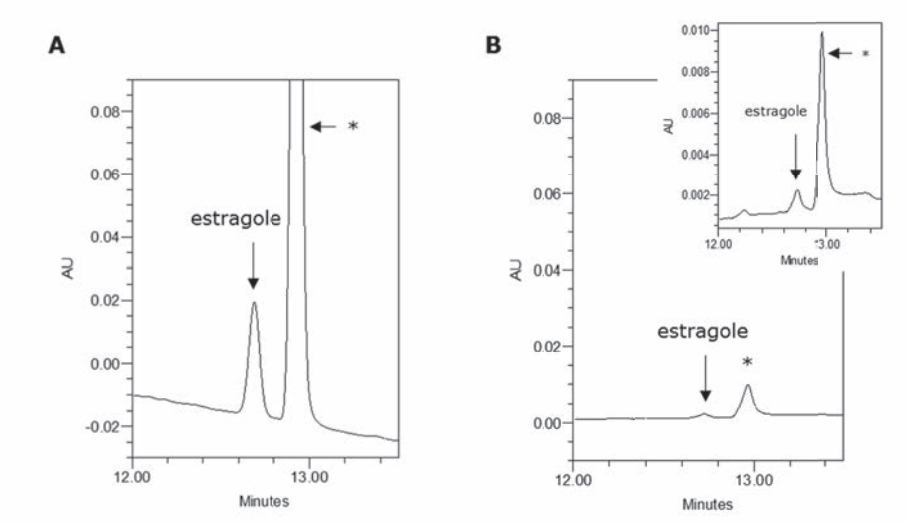

Figure 2. Representative sections of UPLC chromatograms of a methanolic fennel extract (A) and a hot water extract (B). The insert presents the reduced scale of the same chromatogram. To allow chromatographic analysis, methanolic extracts were diluted in acetonitrile (1:10 v/v) whereas hot water extracts were analyzed undiluted. Peaks marked with an asterisk (*) were not identified. Chromatograms were obtained at a wavelength of 225 nm.

Intake estimates and safety assessment of estragole resulting from drinking fennel based tea by adults

Using the estragole content in hot water extracts as determined in the present study, intake estimates up to 4.8 or 14.3 µg estragole/kg bw/day were obtained for a 70 kg person assuming the daily consumption of one or three cups of fennel based tea (Table 2). Table 2 presents MOE values that are equal to 200-70,000 for the use of three cups of fennel based tea on a daily basis (*i.e.* 4.5-7.5 g fennel material) as used in the safety assessment made by the ESCO working group. For 10 of the 34 selected fennel preparations MOE values were calculated to be higher than 10,000 when assuming the use of three cups of fennel based teas as presented in Table 2. Interestingly, the fennel tea preparations for which MOE values lower than 10,000 were found all consisted of fine cut fennel material.

Assuming the daily consumption of one cup of fennel based tea throughout life, MOE values of 10,000 and higher were found for the vast majority (25 out of 34) of the selected fennel tea preparations (Table 2). In general, these fennel preparations consisted of whole fennel fruits or instant tea granules. It should be noted that also when assuming the daily use of one cup of fennel tea, 8 of the 9 samples for which MOE values lower than 10,000 were found consisted of fine cut fennel material and 1 of the 9 samples consisted of a mixture of fine cut fennel material and whole fennel fruits.

Intake estimates and safety assessment of estragole resulting from drinking fennel based tea by infants

Infants are the main consumer group of homemade fennel based tea for symptomatic treatment of mild, spasmodic gastro-intestinal complaints. Therefore, a separate exposure estimate and subsequent safety assessment were made for this consumer group focusing on fennel based preparations that are specifically marketed for infants (*i.e.* sample 17, 18, 20 and 21).

Table 3 shows the exposure estimates for estragole resulting from the use of homemade fennel based teas that are specifically marketed for infants. Exposure estimates were made based on the estragole content in the fennel preparations as determined in the present study in hot water extracts together with the amount of fine cut fennel material present in the tea bag assuming one cup of fennel based tea would be consumed on a daily basis (samples 17, 18 and 21). For sample 20, consisting of instant tea granules, the weight of two whipped off tea spoons (the dose recommended by the respective manufacturer) was used to estimate the daily estragole intake. Exposure estimates reveal a daily intake of 0.5-6.5 µg/kg bw/day, 0.4-4.7 µg/kg bw/day, 0.3-3.6 µg/kg bw/day and 0.2-2.6 µg/kg bw/day for infants of 0-3 months, 3-6 months, 6-12 months and 12-36 months of age, respectively (Table 3). Comparing the $BMDL_{10}$ values for estragole with the exposure estimates obtained, for samples 17, 18 and 21 consisting of fine cut fennel material MOE values were found to generally be below 10,000 indicating a priority for risk management actions (Table 3). However, it should be noted that for sample 20 consisting of instant tea granules MOE values of 10,000 and higher were found when tea prepared from this product would be consumed by infants of 6 months and older.

Table 2. Daily estragole intake estimates resulting from the use of one, two or three cups of fennel tea and corresponding MOE values.

Sample No.	Daily intake of estragole based on the consumption of one cup fennel tea[a] (µg/ kg bw/day)	MOE[b,c]	Daily intake of estragole based on the consumption of two cups of fennel tea[a] (µg/ kg bw/day)	MOE[b,d]	Daily intake of estragole based on the consumption of three cups of fennel tea[a] (µg/ kg bw/day)	MOE[b,e]
1	0.1	30,000 – 70,000	0.1 – 0.2	20,000 – 70,000	0.2 – 0.3	10,000 – 30,000
2	ND	>10,000	ND	>10,000	ND	>10,000
3	0.1 – 0.2	20,000 – 70,000	0.3 – 0.5	7,000 – 20,000	0.4 – 0.7	5,000 – 20,000
4	<0.1	>10,000	≤0.1	>10,000	0.1	30,000 – 70,000
5	0.4 – 0.6	6,000 – 20,000	0.7 – 1.2	3,000 – 9,000	1.1 – 1.8	2,000 – 6,000
6	0.1	30,000 – 70,000	0.1 – 0.2	20,000 – 70,000	0.2 – 0.3	10,000 – 30,000
7	0.1 – 0.2	20,000 – 70,000	0.2 – 0.4	8,000 – 30,000	0.3 – 0.5	7,000 – 20,000
8	≤0.1	>10,000	0.1	30,000 – 70,000	0.1 – 0.2	20,000 – 70,000
9	0.6 – 0.9	4,000 – 10,000	1.1 – 1.9	2,000 – 6,000	1.7 – 2.8	1,000 – 4,000
10	0.1 – 0.2	20,000 – 70,000	0.3 – 0.5	7,000 – 20,000	0.4 – 0.7	5,000 – 20,000
11	0.1	30,000 – 70,000	0.2 – 0.3	10,000 – 30,000	0.2 – 0.4	8,000 – 30,000
12	0.1 – 0.2	20,000 – 70,000	0.2 – 0.4	8,000 – 30,000	0.3 – 0.6	6,000 – 20,000
13	0.1	30,000 – 70,000	0.2 – 0.3	10,000 – 30,000	0.2 -0.4	8,000 – 30,000
14	0.1	30,000 – 70,000	0.1 – 0.2	20,000 – 70,000	0.2 – 0.3	10,000 – 30,000
15	0.2 – 0.3	10,000 – 30,000	0.4 – 0.7	5,000 – 20,000	0.6 – 1.0	3,000 – 10,000
16	0.1 – 0.2	20,000 – 70,000	0.2 – 0.3	10,000 – 30,000	0.3 – 0.5	7,000 – 20,000
17	0.1	30,000 – 70,000	0.2 – 0.3	10,000 – 30,000	0.2 – 0.4	8,000 – 30,000
18	0.2 – 0.3	10,000 – 30,000	0.4 – 0.6	6,000 – 20,000	0.6 – 0.9	4,000 – 10,000
19	0.1	30,000 – 70,000	0.2 – 0.3	10,000 – 30,000	0.2 – 0.4	8,000 – 30,000

Table 2 (continued). Daily estragole intake estimates resulting from the use of one, two or three cups of fennel tea and corresponding MOE values.

Sample No.	Daily intake of estragole based on the consumption of one cup fennel tea[a] (µg/ kg bw/day)	MOE[b,c]	Daily intake of estragole based on the consumption of two cups of fennel tea[a] (µg/ kg bw/day)	MOE[b,d]	Daily intake of estragole based on the consumption of three cups of fennel tea[a] (µg/ kg bw/day)	MOE[b,e]
20	<0.1	>10,000	<0.1	>10,000	<0.1	>10,000
21	0.3 – 0.6	6,000 – 20,000	0.7 – 1.1	3,000 – 9,000	1.0 – 1.7	2,000 – 7,000
22	<0.1	>10,000	0.1 – 0.2	20,000 – 70,000	0.1 – 0.2	20,000 – 70,000
23	0.6 -1.0	3,000 – 10,000	1.2 – 2.1	2,000 – 5,000	1.9 – 3.1	1,000 – 3,000
24	0.1 – 0.2	20,000 – 70,000	0.2 – 0.4	8,000 – 30,000	0.4 – 0.6	6,000 – 20,000
25	<0.1	>10,000	0.1	30,000 – 70,000	0.1 – 0.2	20,000 – 70,000
26	0.1	30,000 – 70,000	0.1 – 0.2	20,000 – 70,000	0.2 – 0.3	10,000 – 30,000
27	0.1 – 0.2	20,000 – 70,000	0.2 – 0.4	8,000 – 30,000	0.4 – 0.6	6,000 – 20,000
28	0.3 – 0.5	7,000 – 20,000	0.5 – 0.9	4,000 – 10,000	0.8 – 1.4	2,000 – 8,000
29	0.2 – 0.3	10,000 – 30,000	0.3 – 0.5	7,000 – 20,000	0.5 – 0.8	4,000 – 10,000
30	0.3 – 0.6	6,000 – 20,000	0.7 – 1.1	3,000 – 9,000	1.0 – 1.7	2,000 – 7,000
31	0.7 – 1.2	3,000 – 9,000	1.4 – 2.4	1,000 – 5,000	2.2 – 3.6	900 – 3,000
32	0.1	30,000 – 70,000	0.1 – 0.2	20,000 – 70,000	0.2 – 0.4	8,000 – 30,000
33	0.2 – 0.4	8,000 – 30,000	0.5 – 0.8	4,000 – 10,000	0.7 – 1.1	3,000 – 9,000
34	2.9 – 4.8	700 – 2,000	5.7 – 9.5	300 – 1,000	8.6 – 14.3	200 – 800

[a] Daily intake estimates of estragole were obtained using the estragole content in water infusions prepared from 1 g fennel material as presented in Table 1, assuming the use of 1.5-2.5 g fennel material for the preparation of a cup of tea (EFSA, 2009a) and a body weight of 70 kg (EFSA, 2012b)
[b] MOE = $BMDL_{10}$ (mg/kg bw/day)/ daily intake estragole (mg/kg bw/day)
[c] MOE assuming a daily intake of one cup of fennel tea
[d] MOE assuming a daily intake of two cups of fennel tea
[e] MOE assuming a daily intake of three cups of fennel tea

ND: not determined

Table 3. Level of estragole in hot water extracts of fennel based tea preparations specifically marketed for infants, corresponding daily intake estimates and MOE values.

Sample No.	Average ± STDEV (µg estragole/ 25 mL infusion)	Daily intake fennel(g)[a]	Daily Intake estragole (µg/kg bw/day)[b]	MOE[c]
Infants 0-3 months of age				
17	3.5 ± 1.1	1.5	1.1	3,000 – 6,000
18	8.8 ± 4.9	1.8	3.3	1,000 – 2,000
20	0.4 ± 0.1	6.0	0.5	6,000 – 10,000
21	15.6 ± 5.4	2.0	6.5	500 – 1,000
Infants 3-6 months of age				
17	3.5 ± 1.1	1.5	0.8	4,000 – 8,000
18	8.8 ± 4.9	1.8	2.3	1,000 -3,000
20	0.4 ± 0.1	6.0	0.4	8,000 – 20,000
21	15.6 ± 5.4	2.0	4.7	700 – 1,000
Infants 6-12 months of age				
17	3.5 ± 1.1	1.5	0.6	6,000 – 10,000
18	8.8 ± 4.9	1.8	1.8	2,000 – 4,000
20	0.4 ± 0.1	6.0	0.3	10,000 – 20,000
21	15.6 ± 5.4	2.0	3.6	900 – 2,000
Toddlers 1-3 years of age				
17	3.5 ± 1.1	1.5	0.4	8,000 – 20,000
18	8.8 ± 4.9	1.8	1.3	3,000 – 5,000
20	0.4 ± 0.1	6.0	0.2	20,000 – 30,000
21	15.6 ± 5.4	2.0	2.6	1,000 – 3,000

[a] Daily intake of fennel is based on the weight of the respective teabags and granules assuming one cup of homemade fennel tea will be used on a daily basis

[b] Daily intake estimates of estragole were obtained assuming a body weight of 4.8 kg, 6.7 kg, 8.8 kg, and 11.9 kg for infants of 0-3 months of age, infants of 3-6 months of age, infants of 6-12 months of age and toddlers of 1-3 years of age respectively (EFSA, 2012b)

[c] MOE = $BMDL_{10}$ (mg/kg bw/day)/ daily intake estragole (mg/kg bw/day)

Discussion

In the present study, a safety assessment for estragole resulting from drinking fennel based tea was made using the MOE approach taking into account the previous work in the field by the ESCO working group (EFSA, 2009a) and reported in literature (Raffo *et al.*, 2011). The results presented show that the consumption of fennel teas generally presents a low priority for risk management actions, especially when one cup of fennel tea is consumed by adults on a daily basis.

Based on the chemical analysis performed in the present study, estragole was detected in the selected 34 fennel samples in quantities equal to 0.15-13.3 mg/g dry fennel preparation. This is comparable to the value resulting

from the assumption made by the ESCO working group of 5% essential oil containing 3.5-12% estragole which would give rise to 1.75-6 mg/g fennel material (EFSA, 2009a). Assuming that 4.5-7.5 g fennel material per day would be used for the preparation of fennel tea as described by the ESCO working group (EFSA, 2009a) and that the extraction efficiency of the essential oil of fennel is 25-35% (EFSA, 2009a), the daily estragole intake would amount to 3-499 µg/kg bw/day for a person with a body weight of 70 kg, which is comparable to the estimate of 33-263 µg/kg bw/day reported by the ESCO working group. However, this indirect worst case approach using levels of estragole in fennel material might represent an overestimation of the actual risk for human health resulting from the use of fennel tea. Raffo *et al.* (2011) indicated that the extraction efficiency of fennel essential oil into the infusion of 25-35% as used by the ESCO working group (EFSA, 2009a) is relatively high leading to an overestimation of the estragole content in the infusion. In fact, Zeller and Rychlik (2006) experimentally determined the extraction efficiency for specific odorants occurring in fennel essential oil into an infusion demonstrating an extraction efficiency of 12% for estragole which is 2-3 fold lower than the extraction efficiency of 25-35% used in the theoretical worst case approach reported by the ESCO working group (EFSA, 2009a). Therefore, in the present study hot water extracts were used representing tea preparation by consumers. Results revealed that the levels of estragole were indeed found to be remarkably lower in the hot water extracts compared to the total estragole content in dry fennel preparations (*i.e.* up to 1,000-fold). The extraction efficiencies obtained were 0.1-2.3% and thus lower compared to the extraction efficiency determined by Zeller and Rychlik (2006). This difference might be explained by the fact that Zeller and Rychlik (2006) prepared tea from freshly comminuted fennel fruits (2.5 g in 150 mL hot water) while in the present study dried fine cut fennel material or intact fennel fruits were used. Raffo *et al.* (2011) previously indicated that estragole levels determined in water infusions prepared from intact fruits were 4-6 times lower compared to the levels in water infusions prepared from freshly comminuted fruits explaining the difference in extraction efficiency. Moreover, it is likely that fresh fennel fruits contain higher levels of volatile constituents including estragole compared to the packaged fennel materials used in the present study as a result of graduate losses of volatile constituents in crushed and/or powdered fennel material upon ageing (EMA, 2008). In fact, in teabags opened for a period of 30 days a 4-10% decrease in the level of essential oil was previously reported (EMA, 2008).

Calculation of the MOE values for the intake of estragole from fennel tea using the levels of estragole in the hot water extracts as determined in the present study resulted in MOE values that are generally above the default of 10,000. In spite of this, for some -though not all- fennel based teas MOE values are still below 10,000 indicating a potential risk for human health and a priority for risk management actions. However, it is important to note that while the daily estragole exposure resulting from the consumption of tea prepared from 1.5 to 2.5 g fennel material three times a day (*i.e.* 4.5-7.5 g fennel/day) might pose a potential risk for human health, the consumption of tea prepared from 1.5 to 2.5 g fennel seeds one time a day does not. This is because in the latter situation MOE values above the default of 10,000 are obtained for the vast majority of samples, *i.e.* 25 out of 34 samples. Important to note is that for water infusions prepared from fine cut fennel material generally relatively low MOE values were obtained. In contrast, MOE values for water infusions prepared from whole fennel fruits or instant tea granules were calculated to be generally higher than 10,000 indicating a low priority for risk management actions. In addition, the present study revealed that MOE values below 10,000 were obtained for infants of 0-3 years of age drinking one cup of fennel tea prepared from fine cut material on a daily basis indicating a priority for risk management. However, it should be noted that for infants of 6 months and older also MOE values above 10,000 were obtained for one sample consisting of instant tea granules. In fact, the label of the respective fennel based tea product recommends to use the product only for infants of 6-36 months of age which, based on the calculated MOE values of 10,000-30,000, would be a low risk. However, it must be emphasized that in the present chapter MOE values are calculated assuming lifetime exposure while homemade fennel based teas are generally only used during periods of gastrointestinal complaints. For this reason, calculation of the MOE values using the intake estimates as daily intake estimates might overestimate the potential risk for human health. However, a general framework for taking intermittent and/or short-term instead of lifetime exposures to genotoxic carcinogens into account in the safety assessment is currently not in place. Felter *et al.* (2011) recently proposed a framework for assessing the risk from less-than lifetime exposures to carcinogens. They proposed to use the principle of Haber's Rule provided that chemical-specific carcinogenicity data are available and that data support a linear dose-response relationship (Felter *et al.*, 2011). Haber's Rule assumes that the acceptable cumulative lifetime exposure can be averaged

over the duration of short-term exposure, suggesting that higher daily intakes are acceptable when short-term exposure is considered (Felter *et al.*, 2011). EMA previously indicated that fennel based teas should not be used for more than two weeks by adults and less than one week by children under the age of 12 (EMA, 2007). Applying Haber's Rule to assess the potential risk for short-term estragole exposure during a period of one week (children) and two weeks (adults) on an estimated life expectancy of 75 years might result in MOE values that are 3 orders of a magnitude higher than those obtained when assuming lifetime (75 years) daily use of fennel based tea. These results indicate that there may be no reason for risk management actions, when consuming three cups of fennel based tea on a daily basis for only two weeks.

Obviously, the calculation of MOE values is based on $BMDL_{10}$ data that are obtained from a long-term carcinogenicity study in which pure estragole was administered to rodents (Miller *et al.,* 1983). However, the presence of a natural botanical matrix might modulate the bioactivation of estragole thereby lowering the potential cancer risk. In fact, Alhusainy *et al.* (2012) previously demonstrated that methanolic extracts from different herbs and spices were able to inhibit the sulfotransferase-mediated bioactivation of estragole. The use of such carcinogenicity data in safety assessment of fennel based teas might thus overestimate the actual risk. However, in the study of Alhusainy *et al.* (2012) it was shown that among the tested alkenylbenzene-containing herbs and spices, fennel did not show any effect on the sulfotransferase enzyme activity. Moreover, it was previously shown that matrix-derived combination effects may be limited at lower dose levels which are relevant for the use of botanicals and preparations made thereof (van den Berg *et al.*, 2013). On the basis of these findings it can be concluded that the used $BMDL_{10}$ values represent an adequate basis for the safety assessment of fennel based teas.

As shown in the present study, the level of estragole in water infusions prepared from fennel can vary considerably depending on the estragole content present in the fennel material used. In addition, the estragole content in the water infusion might also depend on the preparation method used. Raffo *et al.* (2011) demonstrated that the extent of dilution has only a limited effect on the level of estragole extracted in the infusion. In contrast, squeezing the teabag to remove any residual water following the preparation of a fennel infusion was found to result in a considerable increase in the estragole content and was previously experimentally determined by Raffo *et al.* (2011) to equal 15%

and Zeller and Rychlik (2009) even demonstrated an increased extraction of estragole into the infusion equal to 45% (Zeller and Rychlik, 2009). In addition, the use of freshly comminuted fennel fruits was shown to increase the estragole content in the infusion by 4-6 fold compared to the use of intact fennel fruits (Raffo *et al.*, 2011). It should be noted that reduction of the estragole content in fennel and fennel based teas is technically possible (Pank, 2003). This can be done by removing estragole from instant teas through fractionated distillation (Pank *et al.*, 2003). Moreover, Zeller and Rychlik (2006) indicated that reduction of the estragole content in fennel tea would not influence the overall flavor of fennel tea. Reducing the estragole content is thus recommended to lower the potential risk for human health, if any, resulting from the use of fennel based teas. In fact, the estragole content of one of the analyzed fennel teas consisting of instant tea granules that was specifically marketed for infants of 6 months and older (*i.e.* sample 20) was relatively low indicating that such a technology might have been applied to reduce the estragole content. However, although fractionated distillation can markedly reduce the estragole content in instant teas, this procedure cannot be applied to the fennel fruits in their original form which is the form most commonly used in the preparation of fennel based teas (Pank *et al.*, 2003). Breeding new cultivars without estragole is not (yet) possible (Pank *et al.*, 2003). Moreover, breeding new fennel cultivars with reduced estragole levels was found to result in reduced total essential oil contents including trans-anethole, which gives the characteristic flavor to fennel tea, meaning that these cultivars are of lower quality. 8 Of the 34 selected samples were prepared from fennel material obtained by customary selections of the levels of estragole. The average estragole content in the water infusions prepared from the respective fennel tea preparations (samples 1-8) was found to be about 3-fold lower compared to that of the remaining fennel teas. Moreover, estragole could even not be detected in fennel based tea prepared from sample 2. This indicates that the use of fennel material with relatively low estragole levels can have a marked effect on the estragole intake resulting from the use of fennel based teas.

In addition to estragole, the EFSA compendium indicates trans-anethole as a substance of possible concern for human health present in *F. vulgare* Mill. (EFSA, 2012a). For trans-anethole the Joint FAO/WHO Expert Committee on Food Additives (JECFA) derived a temporary acceptable daily intake (ADI) of 0-2.0 mg/kg bw (JECFA, 1998), which can be used to define whether exposure

resulting from proposed uses and use levels will be safe. Zeller and Rychlik (2006) previously demonstrated that trans-anethole is the most abundant odorant in fennel fruit and in fennel based tea (*i.e.* 3600 µg trans-anethole in an infusion extracted from 1 g fennel material). Based on the level of trans-anethole detected by Zeller and Rychlik (2006) and assuming the daily consumption of one cup of fennel tea prepared from 1.5 to 2.5 g fennel material the daily intake of trans-anethole would equal 77-129 µg/kg bw/day for a 70 kg person. Since this value is well below the ADI it can be concluded that the exposure to trans-anethole resulting from the use of fennel based tea is not of concern for human health.

Altogether, the present study showed that extraction efficiencies of estragole into the infusion were considerably lower than the 25-35% extraction efficiency used in the worst case exposure assessment performed by the ESCO working group. In general, extraction efficiencies were found to be lower for samples consisting of whole fennel fruits compared to that of samples consisting of fine cut fennel material indicating that exposure assessments should be done on a case-by case basis taking into account the physical characteristics of the product of interest. In fact, estragole levels were 3-fold lower in water infusions prepared from whole fennel fruits compared to that in water infusions prepared from fine cut fennel material. Also the use of fennel material with relatively low estragole levels obtained by customary selection to prepare fennel based teas resulted in reduced estragole levels of about 3-fold. The results obtained generally point at a low priority for risk management actions for the use of fennel teas, especially when consuming one cup of fennel tea on a daily basis prepared from 1.5 to 2.5 g fennel material. Moreover, assessing the risk from less-than lifetime exposures to estragole resulting from the use of fennel based teas during a period of one to two weeks resulted in MOE values that are 3 orders of a magnitude higher indicating low priorities for risk management actions. Taken together, these results indicate a low priority for risk management actions and a low risk for human health for the use of fennel based teas especially for the short-term proposed uses of fennel based teas for the symptomatic treatment of digestive disorders alleviating mild spasmodic gastro-intestinal ailments.

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Chapter 7

General discussion

General discussion

To adequately support the safe use of plant food supplements (PFS), uniform and well accepted procedures for risk and safety assessment are essential. While many consumers equal 'natural' with 'safe', PFS may contain compounds that are of concern for human health. One of the main bottlenecks in the safety assessment of botanicals and botanical ingredients and thus also for PFS is the fact that some botanicals may contain compounds that are both genotoxic and carcinogenic. The risk assessment of such compounds is complicated and an international scientific agreement on the best practices for risk assessment of these type of compounds is currently lacking (EFSA, 2005). Therefore, the present thesis aimed at testing and validating new concepts that could be of use for the risk and safety assessment of PFS focusing on finding adequate ways to judge the risk or safety of PFS that may contain compounds that are both genotoxic and carcinogenic (**Chapter 1**). This work should ultimately give a better idea on when risk management actions would be needed, but also for which PFS there is no reason for concern even though they do contain limited amounts of compounds that may be genotoxic and carcinogenic. The new concepts tested and the results obtained are discussed below in some more detail.

Selection of the PFS and compounds to be included

Since a major unsolved issue in safety assessment of botanicals is how to deal with PFS that contain compounds that may be genotoxic and carcinogenic (**Chapter 2**), the work started by making a thorough selection of botanical compounds that may be present in PFS and that exert genotoxic and/or carcinogenic properties. This work was described in detail in **Chapter 3**. Compounds were selected based on (1) information on genotoxic and carcinogenic botanical ingredients in the European Food Safety Authority (EFSA) compendium (EFSA, 2012a), (2) evidence of genotoxicity and/or carcinogenicity of plant constituents described in studies performed by the National Toxicology Program and (3) the expert judgment from partners collaborating under the PlantLIBRA project (acronym PLANT food supplements: Levels of Intake, Benefit and Risk Assessment) which is a European collaborative project under the Seventh Framework Programme. Based on this inventory, 30 compounds were selected. Two major groups of botanical genotoxic carcinogens were identified including the group of alkenylbenzenes (*e.g.* estragole, methyleugenol, safrole, β-asarone and elemicin) and the group

of unsaturated pyrrolizidine alkaloids (*e.g.* riddelliine and monocrotaline). Based on consultation with PlantLIBRA partners it was concluded that unsaturated pyrrolizidine alkaloids are currently already regulated and not allowed in PFS and that therefore further work should focus on the group of alkenylbenzenes.

Testing the Margin of Exposure (MOE) concept for risk and safety assessment of PFS
For risk and safety assessment of genotoxic carcinogens, expert groups of EFSA, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the International Life Sciences Institute (ILSI) have recommended the use of the Margin of Exposure (MOE) approach (**Chapter 2**). The MOE is a ratio between (1) a reference point from an animal experiment (*e.g.* a $BMDL_{10}$, the lower confidence limit of the benchmark dose resulting in 10% extra cancer incidence) and (2) the estimated daily intake in humans (EFSA, 2005). MOE values lower than 10,000 are considered as a priority for risk management actions and a concern for human health (EFSA, 2005). In **Chapter 3** the use of the MOE approach was tested in the safety assessment of PFS containing the alkenylbenzenes which were selected as the model compounds of interest. For the alkenylbenzenes estragole, methyleugenol, safrole and β-asarone carcinogenicity studies were available in the literature from which $BMDL_{10}$ values could be derived that are required for the MOE approach. MOE values were calculated by dividing these $BMDL_{10}$ values by the estimated intakes of the compounds of interest. These intake estimates were based on experiments in which we analyzed the levels of the alkenylbenzenes in PFS obtained on the Dutch and Italian market and via Internet consisting of basil-, fennel-, nutmeg-, sassafras-, cinnamon-, or calamus-containing PFS. Using the results of these chemical analyses and the dose of the PFS recommended by the respective manufacturer, intake estimates were made and compared with the $BMDL_{10}$ values to calculate MOE values. These calculations showed that many PFS contain alkenylbenzenes in such low amounts that the corresponding daily intakes result in MOE values up to 200,000 and are thus far above an MOE of 10,000. For these PFS one can conclude that although they contain compounds that are both genotoxic and carcinogenic they do not present a safety concern. However, for other basil-, fennel-, sassafras-, nutmeg- and calamus-containing PFS, MOE values lower than 10,000 were derived and for a few PFS even MOE values below 10 were found indicating that the estimated daily intake of the alkenylbenzenes resulting from use of these PFS is in the range of dose levels causing liver tumors

in experimental animals. From this study it became clear that the MOE approach is highly useful to perform the risk assessment of PFS that contain compounds that are both genotoxic and carcinogenic, especially because it defines priorities for risk management showing that some PFS are safe whereas others may raise concerns.

Testing the mode of action based concept for risk and safety assessment of PFS
A major limitation of the MOE approach is the fact that this approach requires carcinogenicity data to define the $BMDL_{10}$, whereas for many botanical ingredients that are genotoxic and carcinogenic such carcinogenicity data are not available. Therefore, in **Chapter 4** it was investigated whether a mode of action based approach could be used to do a read across from one compound to another and perform a risk assessment by the MOE approach for a compound for which tumor data are absent. To this end a mode of action based physiologically based kinetic (PBK) modeling approach was used to quantify the relative importance of bioactivation and detoxification of the model alkenylbenzene elemicin, for which no tumor data are available. The results obtained were compared to those obtained from the structurally related alkenylbenzenes estragole and methyleugenol for which tumor data are available. Based on this approach, a $BMDL_{10}$ could be estimated for elemicin. Comparing this estimated $BMDL_{10}$ with the estimated daily intake of elemicin from nutmeg-containing PFS resulted in high MOE values, suggesting that the exposure to elemicin due to use of PFS is of low priority for risk management actions.

Testing the matrix-derived combination effect concept for risk and safety assessment of PFS
Safety and risk assessment of PFS is generally performed using data obtained from studies with pure compounds administered in the absence of the natural botanical matrix. However, the toxicity of the botanical compounds of interest may be modulated by the botanical matrix and this may affect the outcomes of the assessment. Therefore, in **Chapter 5** a new science-based approach was applied to take matrix-derived combination effects into account. To this end the effects of compounds present in the matrix of basil-containing PFS on the bioactivation of estragole were characterized and quantified. Basil-containing PFS, for which relatively low MOE values ranging between 1 and 1,000 were obtained (Chapter 3), were selected as a showcase. Results revealed

that methanolic extracts of PFS consisting of powdered basil material could considerably inhibit sulfotransferase (SULT)-activity required for estragole bioactivation, whereas this effect was found to be limited for methanolic extracts of PFS consisting of basil essential oil. Nevadensin was identified as the major compound responsible for the SULT-inhibiting activity of methanolic extracts of PFS consisting of powdered basil material. Next, PBK modeling was performed to predict the in vivo effects of nevadensin on estragole DNA-adduct formation in rat and human liver. A considerable matrix-derived combination effect was predicted to occur in vivo when estragole would be tested in an animal experiment in the presence of the SULT inhibitor nevadensin at ratios detected in PFS. Based on the outcomes, a refined risk assessment was performed resulting in MOE values that were significantly higher than the MOE values derived using the tumor data obtained in the rodent study in which pure estragole was used without the natural botanical matrix being present. PBK modeling also revealed that the matrix-derived combination effects were dose dependent. At dose levels relevant for the use of PFS, which are lower than the levels tested in rodent bioassays, matrix-derived combination effects may be limited. The results provide an example of how PBK modeling can be used to study matrix-derived combination effects and how to take such effects into account evaluating the risks and safety of PFS.

Safety assessment of botanical preparations other than PFS

The model compounds of the present thesis, alkenylbenzenes, are not only present in PFS but consumer exposure may also result from the use of herbal teas instead of PFS. Therefore, in additional studies of the present thesis safety assessment was also performed for botanical preparations in the form of herbal teas that may contain alkenylbenzenes. In **Chapter 6**, an analysis of the levels of the genotoxic and carcinogenic alkenylbenzene estragole in dry fennel preparations and in infusions prepared from packaged fine cut fennel material, whole fennel fruits or instant tea granules were quantified and an associated safety assessment was performed. Based on available tumor data from literature and the estimated daily intakes of estragole that would result from use of fennel based teas with the estragole levels determined in the present study, a safety assessment was performed using the MOE approach. For adults, the MOE values obtained were generally higher than the default of 10,000 indicating a low priority for risk management, especially when consuming 1

cup of fennel tea on a daily basis during lifetime. However, when calculating the MOE values for use of fennel teas specifically marketed for children, values were obtained that were generally below 10,000 indicating a priority for risk management. However, one could argue that these MOE values are based on lifetime exposure whereas for use of fennel based teas for the symptomatic treatment of digestive disorders shorter exposure periods (1-2 weeks) may be more realistic. Assessing the potential risk for human health resulting from short-term exposure to estragole-containing fennel based teas (*i.e.* 1-2 weeks) on an estimated life expectancy of 75 years, may result in MOE values that are 3-orders of magnitude higher than those obtained assuming lifetime exposure. The results obtained point towards a low priority for risk management and a low risk for human health, especially when considering the short-term use of fennel based teas proposed for the relief of gastrointestinal complaints. These results illustrate how to perform a safety assessment for botanical preparations other than PFS, also taking into account short-term exposure relevant for the botanical preparation of interest.

Conclusions

The new concepts tested in this thesis focus on some of the major bottlenecks in risk assessment of PFS in order to improve the scientific basis for risk and safety assessment. The use of the MOE was found to be a valuable approach in the risk and safety assessment of PFS that can be used to set priorities for risk management actions. Moreover, the integrated approach of in vitro studies and mode of action based PBK modeling that was applied in this thesis was found to be a very useful addition to the risk and safety assessment of PFS based on the MOE approach since it facilitates read across thereby contributing to the reduction of the number of experimental animals used for the safety testing of PFS and it also provides a way to take matrix-derived combination effects into account.

Future perspectives

This thesis presents the use of some new concepts in risk assessment of PFS. In the next sections some suggestions are given for steps to be taken in the near future for a further improvement of risk and safety assessment of PFS. Such steps include developing better models for extrapolation in risk assessment of PFS, and incorporating the matrix effects on absorption, distribution,

metabolism and excretion (ADME) characteristics and dynamic characteristics of active chemical substances in the risk assessment minimizing uncertainties in the MOE approach. Moreover, suggestions are given for the regulation of alkenylbenzene-containing PFS and a selection of the preferred concept in risk and safety assessment of botanicals and botanical preparations is made.

Importance of better models for extrapolation in risk assessment of PFS

In risk assessment of genotoxic carcinogens a variety of qualitative and quantitative approaches is used (Barlow and Schlatter, 2010). Quantitative approaches often include dose-response data which are generally obtained from rodent bioassays since epidemiological data are often lacking (EFSA, 2005). However, the use of such data requires quantitative extrapolation to realistic human exposure levels occurring in daily life which are usually much lower than the high dose levels used in standard 2-years rodent bioassays. For example, to quantitatively assess the potential risk for human health linear extrapolation can be applied to obtain a virtual safe dose (VSD) at which the additional lifetime cancer risk would be one in a million. However, low-dose cancer risk extrapolation has been widely discussed (EFSA, 2005) and is currently not generally accepted. In fact, it is often unknown if the mathematical model used for low-dose extrapolation actually describes the biological mechanisms underlying the toxic effect (EFSA, 2005) introducing uncertainties in the risk assessment. Better approaches for low-dose extrapolation are thus required. EFSA previously advised to use the MOE approach for compounds that are both genotoxic and carcinogenic (EFSA, 2005). Although in the present thesis the MOE approach was found to be highly useful to perform the risk assessment of PFS containing compounds that are both genotoxic and carcinogenic, the MOE does not quantify the risk for human health (Barlow *et al.*, 2006). Therefore, future developments should still work on also developing accepted models to do quantitative risk assessment and quantify risks at low dose levels. This may require that sensitivity of endpoints may need to be increased since for example one in a million cancer risks cannot be experimentally determined. In other words, models and assays are required which are sensitive enough to detect effects in humans at realistic exposure levels already occurring in daily life. An example of a new method in risk assessment is the development of genomics-derived biomarkers of effect which could provide insight into responses occurring at low exposure levels relevant for humans which are generally not

detected using conventional toxicity studies (Paules *et al.*, 2011). However, it should be noted that it is unknown whether or not such -omics effects actually represent adverse effects. In addition, PBK models may help to reduce the uncertainty in low-dose extrapolation. PBK modeling allows studying effects at low dose levels relevant for the human situation. In fact, using PBK modeling, the occurrence of (non-)linear effects and/or thresholds can be studied at relatively low dose levels, which often remain undetected in animal experiments. Moreover, PBK models might be extended to physiologically based dynamic (PBD) models to predict tumor incidences occurring at low realistic intake levels to quantify the ultimate carcinogenic effect. The development of such a PBD model requires the description of dynamic characteristics in addition to the kinetic characteristics used in a PBK model. Dynamic characteristics that can be included to describe the mode of action of the compound of interest, such as the alkenylbenzenes, are DNA-adduct formation, DNA repair, gene and protein expression, mutation modulation and ultimately tumor formation (Paini, 2012). Another issue is that the relevance of the carcinogenicity findings in rodent bioassays for human cancer risks may be questioned. To adequately perform risk assessment it is of importance to take various uncertainties into account such as interspecies differences in kinetics and dynamics. To address this bottleneck in risk assessment of genotoxic carcinogens, PBK and PBD models can be used in the near future to quantify species-dependent differences in the kinetics and dynamics of the compound of interest allowing a more reliable interspecies extrapolation. These insights may result in definition of compound specific uncertainty factors that can be used to refine the risk assessment.

In addition, in the present thesis the incidences of hepatomas as observed in a long-term carcinogenicity study were refined based on the outcomes of the PBK model assuming a linear relationship between the reduced formation of estragole DNA-adduct formation predicted by the PBK model and the reduction in the hepatoma incidence. However, such a relation has not yet been established and the PBK models used in this study do not present full insight in such a relationship introducing uncertainties in the risk assessment. Moreover, DNA-adduct formation is often considered as a biomarker of exposure and not as a biomarker of effect (Brink *et al.*, 2009; La and Swenberg, 1996; Paini *et al.*, 2011; Swenberg *et al.*, 2008). To address these uncertainties, PBD models can be developed and used in future risk assessment to provide insights in the relationship between DNA-adduct formation and tumor formation facilitating

better risk and safety assessments of compounds that are both genotoxic and carcinogenic. In fact, based on the insights in the relationship between the reduced formation of estragole DNA-adduct formation and the reduction in the hepatoma incidence obtained by applying PBD models, uncertainty factors used to describe inter- and intraspecies differences in dynamics can be adjusted. For example, to take into account interspecies differences, a default uncertainty factor of 10 is normally used which can be divided into a factor of 4 for kinetics and 2.5 for dynamics (IPCS, 2010). The outcomes of PBK modeling can provide scientific evidence to increase or decrease the default of 4 for kinetics, and the outcomes of PBD modeling can provide scientific evidence to increase or decrease the default of 2.5 for dynamics.

Matrix–derived combination effects in risk and safety assessment of botanicals
Whereas risk assessments generally address the potential risks for human health based on toxicity studies that are performed using individual chemicals, botanicals generally are complex mixtures consisting of numerous chemicals including primary metabolites such as carbohydrates, lipids and proteins, as well as secondary metabolites such as, for example, compounds belonging to the groups of alkaloids, terpenoids, steroids, saponins, phenolics and flavonoids which occur in a wide variety of botanicals (Vanisree *et al.*, 2004). Botanicals may also include smaller groups of secondary metabolites such as the alkenylbenzenes. In principle, the numerous chemicals present in botanicals and botanical preparations may result in interactions that could affect the absorption, distribution, metabolism and excretion (ADME) characteristics as well as the toxicity of the individual active substance. Thus, the use of toxicity data of individual active substances might not represent an adequate starting point for risk assessment of botanicals and botanical preparations. In fact, Schilter *et al.* (2003) previously described that the use of toxicity data obtained from experiments in which individual active substances were used could be questioned when used in the risk assessment of complex botanical materials (Schilter *et al.*, 2003). Possible consequences and examples of botanical matrix-derived combination effects on the ADME characteristics of active chemical substances are described below.

Matrix-derived combination effects on absorption

An aspect that needs consideration is that the botanical matrix can modulate the rate and extent of absorption of the active substance altering its toxicity. For example, as a consequence of the slow and/or incomplete release of the active substance from its botanical matrix the level of bioavailability might be reduced compared to the situation in which the pure compound is administered (Schilter *et al.*, 2003). For example, Al-Subeihi *et al.* (2012) demonstrated that PBK model based predicted venous blood levels of methyleugenol assuming 100% bioavailability were an order of magnitude higher compared to the bioavailability of methyleugenol from gingersnaps served as a breakfast together with orange juice observed in a human intervention study (Al-Subeihi *et al.*, 2012). The PBK based prediction would actually fit the observed data when the bioavailability of methyleugenol from the gingersnaps and orange juice breakfast would have been 13.8%. Another example of reduced bioavailability due to matrix interactions was demonstrated for β-carotene (van het Hof *et al.*, 1999). Van het Hof *et al.* (1999) reported that the bioavailability of β-carotene present in a matrix of mixed vegetables equals 14% compared to that of purified β-carotene. In addition, Adam *et al.* (2002) showed that the bioavailability of ferulic acid is considerably lower in the presence of a food matrix (*e.g.* cereals). In fact, in rats only 3% of the ingested dose of ferulic acid was recovered in urine following the exposure to ferulic acid in a complex cereal matrix of *Triticum durum* whereas about 50% of the ingested dose was recovered in urine following the exposure to ferulic acid enriched semipurified diets (Adam *et al.*, 2002). In contrast, Chen *et al.* (1997) demonstrated that the rate of absorption of (-)-epigallocatechin-3-gallate (EGCG) was about 4-fold higher in rats following the combined exposure to EGCG and decaffeinated green tea (equivalent dose of EGCG of 15 mg/kg) in comparison to the situation in which pure EGCG (75 mg/kg) was intragastrically administered to the rats (Chen *et al.*, 1997). Moreover, it is of importance to note that several flavonoids have been reported to inhibit various transporters located in the basolateral or apical membrane in cells of the intestinal epithelial barrier affecting the bioavailability of chemical compounds upon oral uptake (Brand *et al.*, 2006). Inhibition of apical transporters that transport compounds out of the intestinal cells back to the intestinal lumen may facilitate systemic uptake, whereas inhibition of basolateral transporters that transport compounds from the intestinal cell to the systemic circulation may oppose bioavailability. Flavonoids are present in a wide variety of botanicals

and therefore it is likely that these compounds may affect the bioavailability of substances of concern occurring in the botanical or botanical preparation of interest.

Effects on distribution

Matrix-derived combination effects can also occur at the level of distribution. Chen *et al.* (1997) revealed that the distribution of EGCG was different when pure EGCG (10 mg/kg) was administered intravenously to rats than when given intravenously in combination with decaffeinated green tea (equivalent dose of EGCG of 1.8 mg/kg) (Chen *et al.*, 1997). Results showed that the concentrations of EGCG found in the plasma and the area under the plasma concentration versus time curve of total EGCG (*i.e.* free and conjugated EGCG) were lower following the intravenous exposure to pure EGCG compared to the exposure to EGCG in a matrix of decaffeinated green tea. In addition, a larger distribution volume was observed following the treatment with pure EGCG than in the situation in which rats were exposed to decaffeinated green tea consisting of EGCG. The authors concluded that these differences might be due to a faster distribution of EGCG in the body following exposure to pure EGCG compared with the exposure to EGCG in a matrix of decaffeinated green tea (Chen *et al.*, 1997). An underlying reason for this observation could be that the parent EGCG is more rapidly conjugated, which may result in a faster distribution and excretion of metabolites, when exposed to pure EGCG compared to EGCG administered in a matrix of decaffeinated green tea (Chen *et al.*, 1997).

Effects on metabolism

In addition to the possible alterations in the rate and extent of absorption and distribution, the botanical-derived matrix may have an effect on the metabolism of the active chemical substance(s) of interest. Effects can occur at the level of phase I and phase II metabolism. Previous studies demonstrated the inhibitory effects of some flavonoids (*e.g.* naringin, naringenin, kaempferol and quercetin) and furanocoumarin (*e.g.* bergamottin and 6',7'-dihydroxybergamottin) occurring in grapefruit juice on the activity of cytochrome P450 3A4 (CYP3A4) (De Castro *et al.*, 2006; Ho *et al.*, 2001). The grapefruit juice-mediated inhibition of human CYP3A4 activity in the small intestine or the liver, may reduce the level of pre-systemic metabolism of prescription drugs by CYP3A4 thereby increasing their oral bioavailability (Bailey *et al.*, 1998).

Another example of botanical ingredients that can interact with the pharmacokinetics of prescription drugs are compounds present in St. John's wort. When concomitantly consumed, St. John's wort can induce CYP3A4 activity resulting in a decrease of plasma levels of several prescription drugs including alprazolam, irinotecan and indinavir (Gurley *et al.*, 2005; Markowitz *et al.*, 2003; Mathijssen *et al.*, 2002; Piscitelli *et al.*, 2000). In addition, St. John's wort may interfere with the efficacy of oral contraceptives (Hall *et al.*, 2003; Murphy *et al.*, 2005).

Furthermore, a matrix-derived combination effect was previously shown for the bioactivation of the alkenylbenzene estragole at the level of sulfotransferase mediated bioactivation. Estragole and its structurally related analogues are not genotoxic or carcinogenic as such, but have to be metabolized into a 1'-sulfoxymetabolite that can covalently bind to DNA (Boberg *et al.*, 1983; Phillips *et al.*, 1981; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Wiseman *et al.*, 1985; Wiseman *et al.*, 1987). Jeurissen *et al.* (2008) showed that the level of estragole bioactivation and DNA-adduct formation was significantly reduced in incubations with rat and human liver S9 and in the human HepG2 liver cell line following co-exposure to 1'-hydroxyestragole, the proximate carcinogen of estragole, and a methanolic basil extract (Jeurissen *et al.*, 2008). The flavonoid nevadensin was identified as the major compound responsible for this observed in vitro inhibition of estragole bioactivation and subsequent DNA-adduct formation by the methanolic basil extract (Alhusainy *et al.*, 2010). In a subsequent study in which rats were simultaneously dosed with estragole and nevadensin it was shown that nevadensin is also able to significantly inhibit DNA-adduct formation in vivo (Alhusainy *et al.*, 2013). The results obtained in this thesis also indicate that data derived from carcinogenicity studies administering pure estragole to rodents in the absence of other botanical compounds may result in an overestimation of the adverse effects (Chapter 5). However, it should be noted that the matrix-derived combination effect may be dose-dependent. In fact, in Chapter 5 it was shown that while the matrix-derived combination effect was observed at dose levels used in rodent bioassays, the effect may be only limited at realistic human exposure levels resulting from the use of PFS. The effects observed when botanicals and botanical preparations including PFS would be tested in rodent bioassays would thus not be representative for the human situation and does not provide a good starting point for risk assessment. Based on the results of the present thesis it may even be concluded that the

experiments with the pure compound may provide a better starting point for the risk assessment than testing of the botanical itself. This indicates that the incorporation of the matrix-derived combination effect in the risk assessment of botanicals and botanical preparations should be done on a case-by-case basis taking into account analysis of the dose dependency of the interactions detected.

Effects on excretion

Combined exposure may not only affect the level of formation of the metabolites responsible for induction of adverse effects but may also alter formation of metabolites that can be excreted. Although limited studies are available in literature describing matrix-derived effects on excretion, an example of such an effect can be found in the study reported by Chen *et al.* (1997) who showed that the presence of a matrix of decaffeinated green tea influenced the elimination rate of EGCG in rats. In fact, in rats EGCG was found to be more rapidly eliminated following the intravenous or intragastric administering of pure EGCG compared to that following the concomitant exposure with decaffeinated green tea (Chen *et al.*, 1997). Competition for the binding sites in metabolizing enzymes by polyphenols present in decaffeinated green tea might result in lower metabolic conversion and consequently a decreased level of elimination compared with pure EGCG (Chen *et al.*, 1997).

In addition, also in the process of excretion of several botanical ingredients transporters might be involved. As a result, compounds that exert an effect on transporters may also affect excretion.

Conclusion

Based on these findings, it can be concluded that matrix-derived combination effects can have a marked effect on ADME characteristics of chemical substances potentially affecting toxicity. Therefore, risk assessment should not only focus on individual substances but should take into account possible consequences of the botanical matrix-derived combination effects on the ADME characteristics and on dynamic processes to accurately determine the risk for human health. An example of how matrix-derived combination effects at the level of metabolism can be included in the risks and safety assessment of PFS is provided in Chapter 5 of the present thesis.

Uncertainties in the MOE approach

The results of the present thesis demonstrate that the MOE approach can be of use in risk assessment and setting priorities for risk management of PFS. When applying this MOE approach an MOE value of 10,000 or above is generally considered as a low priority for risk management actions. This value of 10,000 is based on the following considerations and uncertainties; (1) a factor 100 to take into account species differences and human variability in kinetics and dynamics, (2) a factor of 10 for inter-individual human variability in cell cycle control and DNA-repair and (3) a factor of 10 for the reason that the reference point is not equivalent to a no observed adverse effect level (NOAEL) and effects can occur at lower doses (EFSA, 2005). EFSA previously stated that the safety factors used to describe species differences and human variability in kinetics and dynamics can be adjusted based on the availability of appropriate chemical specific data (EFSA, 2005). However, a clear guidance on how this should be done is not provided. The results obtained in the present thesis indicate that species-dependent effects in the metabolism of the compound of interest can be obtained by developing PBK models for different species allowing a reliable interspecies extrapolation. The PBK model outcomes obtained in Chapter 4 of the present thesis revealed that the relative extent of bioactivation of elemicin in human was 3.8-fold lower than in rat at concentrations relevant for the daily human exposure to elemicin through the daily diet. Resulting from the fact that the bioactivation of elemicin in human was lower compared to that of rat, using the default approach can be considered as too conservative and the default factor of 4 for species differences in kinetics (IPCS, 2010) can thus be removed from the total MOE default factor of 10,000. As a result, a default MOE of 2,500 could be considered assessing the risk for human health resulting from the daily exposure to elemicin through the human diet. In fact, one could argue if the uncertainty factor for interspecies differences can even be 3.8-fold lower since bioactivation of elemicin in humans is not 4-fold higher or equal compared to rat but even 3.8-fold lower. This would result in a relatively low MOE value of about 650. This presents an example how PBK modeling can be integrated in risk assessment to provide a scientific basis on whether or not and how to adjust the default value of 10,000 used in the MOE approach, using so-called compound specific uncertainty factors.

Another issue to consider is that although EFSA previously stated that a lower MOE presents a higher priority for risk management actions, guidance on

banding MOE values is not provided. ILSI previously proposed to use logarithmic intervals (*i.e.* 1–100; 100-1,000; 1,000–10,000; and 10,000-100,000) for banding MOE values (ILSI, 2009). However, an international consensus is still lacking. Banding MOE values might improve communication between risk assessors and risk managers facilitating better priority setting for risk management actions. For example, based on the results obtained in Chapter 3, it can be concluded that in some cases the daily dose of estragole resulting from the use of basil-containing PFS was equivalent to the dose causing malignant tumors in experimental animals (*i.e.* MOE of 1). In addition, also considerably higher MOE values of 3,000-7,000 were found for fennel-containing PFS consisting of estragole. Although being all lower than the default of 10,000, and leading to the conclusion there is a priority for risk management, it may be considered that these MOE values indicate a different priority for risk management actions. To facilitate such a differentiated approach, banding of MOE values would be required to indicate if the priority for risk management actions is for example very high, high, moderate, low or negligible.

Generally, MOE values are calculated using $BMDL_{10}$ data obtained from long-term carcinogenicity studies. EFSA previously indicated that the use of such long-term carcinogenicity studies to derive an MOE might be conservative when short-term exposure is considered (EFSA, 2005). Assessing the potential risk for human health resulting from short-term exposure might be challenging as a general framework for the safety assessment of short-term exposures to genotoxic carcinogens is lacking. Nonetheless, based on a workshop by a committee of the ILSI Health and Environmental Sciences Institute held in 2009, a framework for assessing the risk from less-than lifetime exposures to carcinogens was proposed (Felter *et al.*, 2011). Based on the framework proposed, the potential risk for human health resulting from less-than lifetime exposures to carcinogens can be assessed using the principle of Haber's Rule (Felter *et al.*, 2011). Applying Haber's Rule, the availability of chemical-specific carcinogenicity data and data that support a linear dose-response relationship are a prerequisite (Felter *et al.*, 2011). Haber's Rule assumes that the acceptable cumulative lifetime exposure can be averaged over the duration of short-term exposure, suggesting that higher daily intakes are acceptable when short-term exposure is considered (Haber, 1924). Chapter 6 of this thesis describes the application of Haber's Rule in the MOE approach to assess the potential risk for short-term estragole exposure via fennel based tea for the symptomatic

treatment of digestive disorders alleviating mild spasmodic gastro-intestinal ailments. MOE values were estimated to be three orders of a magnitude higher when fennel based tea would be used for a period of only 1 or 2 weeks relevant for the use of fennel based tea, compared to lifetime (75 years) daily use of fennel based tea. These results indicate that assuming life-time daily exposure in safety assessment of fennel based teas might overestimate the actual risk. The framework proposed can also be applied in the risk assessment of alkenylbenzene-containing PFS. It is important to note that the duration of consumption of (specific) PFS remains unclear adding uncertainty to the exposure assessment. Assuming that a PFS is used for a period of only 6-months on an estimated lifetime expectancy of 75 years, MOE values would be derived that are 150-fold higher than those presented in Chapter 3. Table 1 shows the MOE values obtained assuming lifetime exposure and those assuming short-term exposure (6-months) obtained by applying Haber's Rule. The results obtained indicate that there may be no reason for risk management actions for the vast majority of PFS (19 out of 24) when consumed for only 6-months daily during lifetime (Table 1; bold figures). However, results clearly show that even when assuming short-term exposure especially the use of PFS consisting of essential oils is of priority for risk management actions and is a potential risk for human health. Thus, in cases where the duration of exposure is much shorter than lifetime exposure, the use of the MOE based on lifetime exposure estimates might overestimate the actual risk for human health. These results demonstrate the need for an internationally agreed scientific framework to assess the short-term dietary intakes of genotoxic carcinogens present in botanicals and botanical preparations using the MOE approach.

Based on the considerations described above it is concluded that it should be encouraged to further refine the MOE approach to minimize uncertainties, ultimately improving the quality of risk assessment.

Suggestions for regulation of alkenylbenzene-containing PFS

Within the EU, the addition of pure estragole, methyleugenol (Regulation (EC) No 1334/2008 of the European Parliament and of the Council 16 December 2008), safrole and β-asarone (Council Directive 88/388/EEC of 22 June 1988) to food is currently prohibited. In addition, for several food categories including dairy products, fish products and non-alcoholic beverages maximum levels of these alkenylbenzenes, naturally present in flavorings and food

ingredients with flavoring properties have been defined (Regulation (EC) No 1334/2008 of the European Parliament and of the Council 16 December 2008).

Table 1. MOE values for different alkenylbenzene-containing PFS assuming lifetime exposure or short-term exposure. MOE values lower than 10,000 are printed in bold.

Botanical in supplement		MOE assuming lifetime exposure (75 years) (van den Berg *et al.*, 2011)	MOE assuming short-term exposure (6-months)
Basil			
	1*	E: 1	**E: 150**
		ME: 700-3,000	ME: 100,000-500,000
	2*	E: 6-40	**E: 900-6,000**
		S: 400-5,000	S: 60,000-800,000
		ME: 5,000-7,000	ME: 800,000-10,000,000
	3	E: 200-1000	E: 30,000-200,000
		ME: 20,000-200,000	ME: 3,000,000-30,000,000
	4*	E: 1-3	E: 200-500
		ME: 700-5,000	ME: 100,000-800,000
	5	E: 400-1,000	E: 60,000-200,000
		ME: 1,000-10,000	ME: 200,000-2,000,000
Fennel			
	1	E: 200-300	E: 30,000-50,000
	2	E: 200-700	E: 30,000-100,000
	3	E: 40-80	**E: 6,000-10,000**
	4	E: 90-200	E: 10,000-30,000
	5	E: 2,000-7,000	E: 300,000-1,000,000
	6	E: 3,000-7,000	E: 450,000-1,000,000
	7	E: 700-1,000	E: 100,000-200,000
Sassafras			
	1	S: 2,000-10,000	S: 300,000-200,0000
	2	S: 100-300	S: 20,000-50,000
		ME: 20,000-70,000	ME: 3,000,000-10,000,000
	3	S: 600-2,000	S: 90,000-300,000
	4	S: 600-5,000	S: 90,000-800,000

Table 1 (continued). MOE values for different alkenylbenzene-containing PFS assuming lifetime exposure or short-term exposure. MOE values lower than 10,000 are printed in bold.

Botanical in supplement	MOE assuming lifetime exposure (75 years) (van den Berg *et al.*, 2011)	MOE assuming short-term exposure (6-months)
Nutmeg		
1	S: 50-400	S: 8,000-60,000
	ME: 1,000-10,000	ME: 200,000-2,000,000
2	S: 9-100	S: 1,000-20,000
	ME: 200-6,000	ME: 30,000-900,000
3	S: 200-400	S: 30,000-60,000
	ME: 2,000-8,000	ME: 300,000-1,000,000
4	ME: 2,000-9,000	ME: 300,000-1,000,000
Calamus		
1	BA: 600-4,000	BA: 90,000-600,000
2	BA: 1,000-7,000	BA: 200,000-1,000,000
3	BA: 20-30	**BA: 3,000-5,000**
4	BA: 600-1000	BA: 90,000-200,000

* Botanicals consisting of essentials oils
E, estragole; ME, methyleugenol; S, safrole; BA, β-asarone

In contrast, such regulations are not in place for the use of alkenylbenzene-containing PFS. This is remarkable since some of the PFS selected and analyzed in Chapter 3 such as the PFS consisting of basil essential oil were found to contain highly concentrated levels of estragole (*i.e.* 32.71-241.56 mg estragole/g PFS). For these PFS, MOE values below 10 were derived indicating that the daily exposure to estragole resulting from the use of these PFS is in the range of dose levels causing liver tumors in experimental animals. Moreover, even when considering short-term exposure of only 6-months throughout lifetime, relatively low MOE values would be derived (*i.e.* 150-6,000) for PFS consisting of basil essential oil indicating a priority for risk management actions (Table 1). These results point towards the need for better regulation and quality control of PFS containing alkenylbenzenes. In line with regulation (EC) No 1334/2008 of the European Parliament and of the Council, maximum levels could also be established for the presence of alkenylbenzenes in PFS to protect human health. Such a regulation would restrict the use of PFS containing highly concentrated levels of alkenylbenzenes such as PFS consisting of the essential

oils of alkenylbenzene-containing botanicals. This would facilitate authorities to take regulatory actions to remove alkenylbenzene-containing PFS from the market that are of concern for human health.

Selection of the preferred approach in risk and safety assessment of botanicals and botanical preparations

In the present thesis different concepts that could be of use for the risk and safety assessment of PFS were tested which included the MOE concept, the mode of action based concept and the matrix-derived combination effect concept. Consequently, various advantages of the use of these concepts were defined. Moreover, some limitations came across when testing the MOE concept, the mode of action based concept and the matrix-derived combination effect concept for risk and safety assessment of PFS.

Table 2 summarizes the advantages and limitations that were encountered when using the MOE approach in the risk assessment of PFS. The main limitations of the MOE concept seem to be related to data availability and variability, introducing uncertainties up to one or even more orders of magnitude in the MOE approach or even making use of the approach impossible. However, whenever data are available the MOE concept proved to provide an easy way to set priorities in the risk management of genotoxic and carcinogenic botanical ingredients for (1) different compounds, (2) different sources of exposure including PFS and teas amongst others and (3) different groups within the population *e.g.* children and adults.

Table 3 provides an overview of the advantages and limitations that were met when using the mode of action concept using PBK models to evaluate the risks of low dietary exposure levels. The main limitation of PBK modeling seems to be related to the validation of the developed model in the absence of appropriate in vivo data quantifying the concentrations of the formed metabolites in blood or urine. Despite this limitation, PBK models were found to be a useful method in evaluating the possible risk for human health resulting from the exposure to elemicin since the developed models can quantify the relative importance of bioactivation and detoxification facilitating a read across from rat to human and from one compound to another. The use of such models can considerably contribute to further reduction in the use of experimental animals and provides a way to obtain clear insights in bioactivation and detoxification at relatively low exposure levels which remain undetected in animal studies.

Some advantages and limitations were defined in the incorporation of the matrix-derived combination effects in the risk assessment of PFS by means of PBK modeling which are presented in Table 4. The major limitation to the use of PBK models to take matrix-derived combination effects into account in the risk assessment of PFS seems to be related to the fact that only few PBK models have currently been developed for botanical ingredients. In spite of this limitation, PBK models were found to be a valuable tool in incorporating the matrix-derived combination effect for the bioactivation of estragole from basil-containing PFS in the risk assessment of these products.

Another approach that can be used in the risk assessment of genotoxic carcinogens is the Threshold of Toxicological Concern (TTC) concept that is based on setting a generic human exposure threshold value for chemicals below which there is a low probability of adverse effects on human health (Kroes *et al.*, 2004). According to this approach, a level of exposure below the TTC represents an insignificant risk for human health and the approach can be applied to many chemicals with the relevant TTC being defined on the basis of available toxicity data from structurally related compounds (Kroes *et al.*, 2004). Therefore, the TTC approach can even be applied for chemicals of unknown toxicity on the condition that their chemical structure is known. For substances containing a structural alert for possible genotoxicity a TTC of 0.15 µg/day is suggested which equals 0.0025 µg/kg bw/day for a 60 kg person (Kroes *et al.*, 2004). However, a TTC should not be considered for high potency genotoxic chemicals including aflatoxin-like compounds, N-nitroso-compounds and azoxy-compounds since compound-specific risk assessment is required for these groups (Kroes *et al.*, 2004). Table 5 shows the estimated daily intake of estragole, methyleugenol, safrole and β-asarone resulting from the use of PFS as determined in Chapter 3. Comparing these estimated daily intakes of the alkenylbenzenes with the TTC value of 0.0025 µg/kg bw/day for a 60 kg person, it can be concluded that the use of such alkenylbenzene-containing PFS exceeds the defined TTC for genotoxic compounds thus suggesting that the absence of a potential risk for human health cannot be assumed. EFSA previously stated that "it is possible to make an approximate comparison between the MOE approach and the TTC approach.

Table 2. Overview of advantages and limitations of the MOE concept in risk assessment of PFS.

Advantages	*Limitations*
1. The use of the MOE approach provides a basis for priority setting that can be used by risk managers.	1. The MOE depends strongly on the quality of the exposure data and the method for defining the intake estimates. Different methods used to estimate the daily exposure to a particular compound may result in large variations in the calculated MOE.
2. The use of the MOE concept was found to be a practical approach that is easy to use and the results are relatively easy to interpret in terms of risk for human health.	2. Dietary intake estimates of botanical ingredients through intake of PFS are not available and analytical data on the actual level of the genotoxic and carcinogenic ingredients in the different PFS are often lacking hampering an accurate exposure estimate.
3. The MOE approach takes into account human exposure data as well as carcinogenicity data.	3. The dietary intake estimates of botanical ingredients are often complicated by varying amounts of these compounds found in botanicals belonging to the same species. Especially for botanicals and thus also for PFS, the level of a specific ingredient may differ depending on the plant maturity at harvest and climatic conditions among others (Smith *et al.*, 2002). These differences can result in diverse MOE values.
4. The MOE approach allows priority setting between different genotoxic carcinogenic ingredients in PFS.	4. For several genotoxic carcinogens found in PFS, carcinogenicity data are not available.
5. The MOE approach provides a way to compare different exposure scenarios of a particular substance. Thus, the risks arising from the intake of genotoxic and carcinogenic botanical ingredients from PFS may be evaluated and compared to the risk resulting from exposure to the same genotoxic carcinogens as a result of the use of teas, herbs and spices or from the use of the botanical ingredients as flavoring ingredients.	5. Available carcinogenicity studies may not be suitable for deriving a $BMDL_{10}$ value for several reasons including a limited amount of tested concentrations of the compound of interest, small groups of animals and/or short study durations.
6. The MOE approach can take into account differences between specified groups within the population. Dietary intake estimates, and thus the MOE calculated, may relate to the whole population or specified groups such as children, pregnant women or individuals who are highly exposed.	6. In the present thesis, adjustments were made to the carcinogenicity data allowing a correction of the dose for the length of treatment and observation period. Different methods for performing normalization of tumor data may result in different MOE values based on the same cancer data.
	7. Another factor for variation may be the actual selection of the $BMDL_{10}$ from the range of $BMDL_{10}$ values obtained with different models available within the software.

Table 3. Overview of advantages and limitations in the use of PBK models to evaluate the risks of low dietary exposure levels.

Advantages	*Limitations*
1. Animal experiments are often conducted using high dose levels. PBK modeling allows studying effects at low levels relevant for the human situation. In fact, the occurrence of (non-)linear effects and/or thresholds can be studied at relatively low dose levels, which remain undetected in animal experiments. This is a very important advantage of PBK modeling.	1. Developing a PBK model is relatively time consuming due to the need of a large data set of parameters.
2. Species-dependent effects in the metabolism of the compound of interest can be quantified by developing PBK models for different species allowing a reliable inter-species extrapolation. This is another very important advantage of the PBK modeling.	2. The developed PBK models describe the effects of individual botanical chemicals while botanicals and botanical preparations contain a wide variety of ingredients.
3. Although several botanical substances might share similar structural characteristics, compound-dependent effects in metabolic activation and detoxification may occur. Mode of action based PBK modeling can quantify the relative importance of metabolic activation and detoxification of these compounds facilitating a read across form one compound to another. Using such data, the risks of low (realistic) human dietary exposure to genotoxic carcinogens for which the toxicological database is limited can be evaluated without the need for an in vivo carcinogenicity study.	3. Not for all chemicals of interest in vivo studies are available, hampering the validation of the defined model.
4. PBK modeling facilitates an insight in the involvement of different organs in the metabolism of the compound of interest which remains unidentified when using data derived from animal experiments.	4. Carcinogenesis is a multistep process which does not only depend on toxico-kinetic characteristics but also on the toxico-dynamic processes. Nevertheless, PBK models might be extended to physiologically based dynamic (PBD) models (Blaauboer, 2003; Paini *et al.*, 2010).
5. Estimations of the in vivo levels of a chemical or its active metabolite(s) in relevant tissues can be predicted using only in vitro data and *in sillico* approaches ultimately adding to the replacement, reduction and refinement of animal testing.	
6. Although not performed in the work presented in this thesis, effects of genetic polymorphisms and inter-individual variations can be modelled (Bogaards *et al.*, 2000; Punt *et al.*, 2010; Rietjens *et al.*, 2011; Rostami-Hodjegan and Tucker, 2007).	
7. The effect on the bioactivation of genotoxic carcinogens by other botanical ingredients (matrix-derived combination effect) can be incorporated into the developed PBK models.	

Table 4. Overview of advantages and limitations in the incorporation of matrix-derived combination effects in the risk assessment of PFS by means of PBK modeling.

Advantages	*Limitations*
1. The effect on the bioactivation of an active ingredient of concern by other botanical ingredients can easily be incorporated into (already developed) PBK models.	1. Chemical identification of the botanical ingredient exerting a protective effect against tumor formation can be difficult and relatively time consuming resulting from the fact that botanicals and botanical preparations often comprise a complex mixture containing a wide variety of compounds.
2. PBK modeling can be used to elucidate if the protective effects observed in in vitro experiments also can occur in vivo, thus adding to the replacement of animal studies.	2. Moreover, not for all botanical ingredients pure compounds are (commercially) available hampering quantification.
3. PBK modeling can be performed for individual PFS, taking into account the relative ratio between the active principle (*e.g.* estragole) and the identified botanical ingredient exerting a protective effect against tumor formation (*e.g.* nevadensin) relevant for the specific PFS of interest.	3. PBK models are developed for a limited number of botanical ingredients only. Developing a PBK model can be time consuming due to the need of a large data set of parameters.
4. Based on the outcomes of PBK modeling refined $BMDL_{10}$ values can be defined that take into account the possible in vivo consequences of a botanical matrix on the adverse effects of the active principle. Such refined $BMDL_{10}$ values can subsequently be used to calculate refined MOE values without the need for rodent bioassays performed with the specific botanical or botanical preparation of interest. This will ultimately add to the replacement, refinement and reduction of animal testing.	4. Moreover, not for all botanical ingredients of interest in vivo studies are available, hampering the validation of the defined PBK model.
5. Information on the dose-response relationship is often derived from studies administering high levels of the compound of interest to rodents. However, based on PBK modeling effects at low doses relevant for the human situation can be studied.	5. In the present thesis, the incidence of hepatomas as observed in a long-term carcinogenicity study was refined based on the outcomes of the PBK model assuming a linear relationship between the reduced formation of estragole DNA-adduct formation predicted by the PBK model and the reduction in the hepatoma incidence. However, such a relation has not yet been established and the PBK models used in this study do not present full insight in such a relationship introducing some uncertainties in the risk assessment.

For a substance with an MOE of 10,000 based on a tumor incidence of 10%, and assuming the dose-response is linear, the human exposure would correspond to an upper bound risk of less than 1 in 100,000 (10^{-5} risk). The TTC value of 0.15 µg/day is derived by linear extrapolation down to a 1 in a million risk (10^{-6} risk). Thus, substances with an exposure below the TTC value, if they were to be tested and were shown to be genotoxic carcinogens, they can be expected to have MOE values of 100,000 or more." (EFSA, 2012b). In line with this, Table 5 shows that whenever the TTC for genotoxic compounds was found to be exceeded, MOE values of 100,000 or lower were generally found. Moreover, in line with the results from the MOE concept, results obtained using the TTC concept indicated a priority for risk management actions at the proposed use levels of PFS containing the alkenylbenzenes estragole, methyleugenol, safrole or β-asarone. However, some differences can be indicated for specific PFS especially for methyleugenol-containing PFS. For example, the exposure to methyleugenol resulting from the use of a sassafras containing PFS is suggested to be a low concern for human health based on the MOE approach. On the contrary, making use of the TTC approach, the exposure to this compound was found to exceed the TTC of 0.0025 µg/kg bw/day indicating the need for compound-specific toxicity data. These discrepancies can be explained by the fact that the MOE uses 1 in 10^{-5} risk while the TTC uses 1 in 10^{-6} risk as the cut off. An additional reason for this discrepancy is the inclusion of data from carcinogenicity studies when using the MOE approach, while the TTC approach does not make a distinction between the carcinogenic potencies for specific compounds. Table 6 describes several advantages and disadvantages of the use of the TTC approach in safety assessment of botanicals and/or botanical preparations that contain compounds that are genotoxic and carcinogenic. In general, the TTC concept can be a practical approach for the safety assessment of botanical ingredients. However, the use of the TTC approach, but also the use of the MOE approach, greatly depends on the quality of daily intake estimates for a particular chemical reflecting the major drawback of the use of these approaches in the risk assessment of the consumption of botanicals and botanical preparations including PFS since appropriate human exposure estimates resulting from the use of such products are scarce.

Table 5. Estimated daily intakes of estragole, methyleugenol, safrole or β-asarone present in a variety of PFS and corresponding MOE values (van den Berg *et al.*, 2011).

Botanical in PFS	Estragole		Methyleugenol		Safrole		β-asarone	
	Daily intake (µg/kg bw/day)	MOE	Daily intake (µg/kg bw/day)	MOE	Daily intake (µg/kg bw/day)	MOE	Daily intake (µg/kg bw/day)	MOE
Basil (*n*=5)	5 – 4,780	1 – 1,000	0.3 - 22	700 – 200,000	1 - 5	400 – 5,000	ND	NC
Fennel (*n*=7)	1 – 84	40 – 7,000	ND	NC	ND	NC	ND	NC
Nutmeg (*n*=4)	ND	NC	5 - 65	200 – 10,000	12 - 217	9 - 400	ND	NC
Sassafras (*n*=4)	ND	NC	1	20,000 – 70,000	0.4 - 19	100 – 10,000	ND	NC
Calamus (*n*=4)	ND	NC	ND	NC	ND	NC	3 - 365	20 – 7,000

ND Not detected (detection limit 0.02 mg/g supplement); NC Not Calculated
NOTE: although the default value for adult body weight recently proposed by EFSA (2012b) is equal to 70 kg, daily intakes are calculated for a 60 kg person to allow comparison with values presented in Chapter 3.

However, when sound intake estimates are available, the TTC approach can be used as a "preliminary step in the risk assessment process to aid in the assessment of whether chemical-specific toxicity data are necessary" ultimately resulting in a reduction of time, costs, expertise and animal use when human intakes are found to be below the TTC for the respective chemical of interest (Kroes *et al.*, 2004). Since a major limitation of the MOE approach was found to be the lack of carcinogenicity studies, the TTC approach could be used to conclude on the safety of the intake of PFS containing genotoxic and carcinogenic ingredients in the absence of carcinogenicity data.

Altogether, the concepts tested in the present thesis were found to provide adequate ways to judge the risk or safety of PFS that contain compounds that are both genotoxic and carcinogenic. The MOE concept is a practical approach that can be used to set priorities for risk management actions. Moreover, to perform risk assessment of PFS containing genotoxic carcinogens, various approaches such as mode of action based PBK modeling and read across can even be integrated in the MOE concept. This facilitates risk assessment for compounds for which the toxicological database is only limited and the incorporation of matrix-derived combination effects in risk assessment on a case-by-case basis. Whenever carcinogenicity studies are lacking and read across from one compound to another is not possible, safety assessment of genotoxic and carcinogenic botanical ingredients can be performed using the TTC approach.

Table 6. Overview of advantages and limitations of the TTC concept in risk assessment of PFS.

Advantages	*Limitations*
1. The TTC approach is easy to use.	5. In order to conclude on the safety of the intake of a botanical ingredient of concern, the established TTC values need to be compared to the estimated daily intake of the particular compound, which underlines the need for appropriate exposure data.
2. Based on the TTC risk assessment can be performed for compounds with unknown toxicity data on the condition that the chemical structure of the compound of interest is known. Thus even when no compound specific tumor data are available, risk assessment is possible representing an advantage of the use of the TTC approach.	6. Dietary intake data of botanical ingredients are generally not available in literature. Moreover, estimating the intake of botanical ingredients is often hampered by poor material characterization, a lack of chemical analysis of botanicals and botanical preparations including PFS as well as by the fact that varying amounts of the compounds of interest that can be found in plants belonging to the same species.
3. The TTC approach can be used as a "preliminary step in the risk assessment process to aid in the assessment of whether chemical-specific toxicity data are necessary" ultimately resulting in a reduction of time, costs, expertise and animal use when human intakes are found to be below the TTC for the respective chemical of interest (Kroes *et al.*, 2004). In other words, the TTC can be used to set priorities for the need of toxicity data and avoids the performance of unnecessary toxicity testing.	7. The TTC for the class of carcinogenic chemicals is based on the carcinogenic potencies, derived from the analysis of carcinogenic potencies of 709 substances from 3500 experiments of the Carcinogenic Potency Database (CPDB) (Gold, 1984 ; Gold *et al.*, 1984). In the CPDB the potency of each chemical was expressed in terms of the dose that caused cancer in 50% of the test animals (TD_{50}'s) at the end of their lifespan (corrected for background tumors in controls) in the most sensitive species and sex. Nevertheless, the TTC approach does not take into account compound specific carcinogenic potencies. As a result, the TTC concept does not take into account the fact that carcinogenic properties for different genotoxic and carcinogenic compounds may differ by up to several orders of magnitude. Thus, the TTC concept does not take into account in the risk assessment of these compounds that they do not exert the same risk for human health at a given level of exposure. However, it is noteworthy to underline that simple linear extrapolation from the TD_{50} results in more conservative estimates of 10^{-6} risk level than would be estimating using other models (*e.g.* multistage); all of the compounds were analysed assuming there is no threshold in the dose-response; simple linear low-dose extrapolation is conservative because the possible effects of the DNA repair and cell proliferation rates (which are influenced by dose) may deviate from linearity, the shape of the dose-response relationship, and the approach assumes that all biological processes involved in the generation of tumors at high dosages are linear over a 500,000-fold range of extrapolation.
4. It is possible to apply the TTC concept for special subgroups of the population by adopting a TTC value for that specific subpopulation.	

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Chapter 8

Summary

To date, the consumption of botanicals and preparations made thereof, such as plant food supplements (PFS) is increasing. Although such products generally have a high acceptance by consumers, PFS may contain compounds that are of possible concern for human health (EFSA, 2012). For example, some botanicals are known to contain compounds that are both genotoxic and carcinogenic (EFSA, 2012). However, the risk assessment of PFS containing genotoxic carcinogens is accompanied with difficulties (EFSA, 2005). Moreover, an international scientific agreement on the best testing strategy for risk assessment of such compounds is not (yet) in place (EFSA, 2005). Therefore, the aim of this thesis was to test and validate new concepts for the risk assessment of PFS containing genotoxic carcinogens.

Chapter 1 provides a general introduction to PFS, an introduction to the concepts tested, and an introduction to the model compounds tested in the present thesis. Moreover, Chapter 1 presents the aim and outline of the present thesis.

An overview of the different approaches currently in use for risk and safety assessment of botanicals and botanical preparations is presented in **Chapter 2**. In addition, examples are provided of botanical compounds that are of potential concern for human health because of their genotoxic and carcinogenic properties or other mode of actions, neurotoxic effects or interactions with prescription drugs.

Chapter 3 describes the application of the Margin of Exposure (MOE) concept for the risk assessment of PFS from Internet and the Dutch and Italian market. First, 30 botanical compounds with genotoxic and/or carcinogenic characteristics were selected based on available literature data. Most compounds that were judged to be both carcinogenic and genotoxic could be classified into two categories of botanical ingredients, namely the alkenylbenzenes and unsaturated pyrrolizidine alkaloids. In a next step, $BMDL_{10}$ values (*i.e.* the lower confidence limit of the benchmark dose resulting in a 10% increase in tumor incidence) were calculated for the alkenylbenzenes estragole, methyleugenol, safrole and β-asarone. In addition, intake estimates were made based on results from chemical analyses together with the recommended daily dose levels of the respective PFS indicated on the label. Finally, risk assessment was performed based on the MOE approach dividing the $BMDL_{10}$ values by the estimated daily intakes. For several PFS, MOE values higher than 10,000 were found indicating a low priority for risk management. In addition, for other

PFS MOE values lower than 10,000 were obtained indicating a priority for risk management. For PFS consisting of basil essential oil MOE values were even found to be lower than 10. This means that the daily estragole intake resulting from the use of such basil-containing PFS is comparable to the dose levels at which tumor incidences were detected in rodents. These PFS should be a priority for risk management actions.

In a next step, mode of action based physiologically based kinetic (PBK) models were made for the alkenylbenzene elemicin in rat and human for which limited toxicity data is available (**Chapter 4**). Based on the results obtained, it was concluded that interspecies differences in bioactivation of this compound were limited. Moreover, the differences in the formation of the ultimate carcinogenic metabolites of elemicin and its structurally related analogues estragole and methyleugenol were also found to be limited. Based on these data and read across from available animal data for estragole and methyleugenol, $BMDL_{10}$ values were estimated for elemicin. Next, a risk assessment was performed using the MOE approach indicating that the elemicin intake resulting from use of herbs and spices and products made thereof such as PFS is of lower priority for risk management actions compared to the priority for risk management actions as defined for its structurally related analogues. Based on the results described in Chapter 4, it can be concluded that PBK modeling can provide a scientific basis for read across from one botanical compound to another thereby facilitating risk assessment. As a result, risk assessment can be performed for compounds for which only limited toxicity data are available without the need of performing animal bioassays.

Subsequently, the effects on the bioactivation of estragole by compounds present in basil-containing PFS was studied (**Chapter 5**). Methanolic extracts of several PFS consisting of powdered basil material or basil essential oil were tested for their effect on in vitro sulfotransferase (SULT)-inhibiting activity. Interestingly, all methanolic extracts from basil-containing PFS were found to be able to inhibit SULT activity. However, the level of SULT-inhibiting activity was found to be more pronounced in PFS consisting of powdered basil material compared to that of PFS consisting of basil essential oil. Nevadensin was identified as the botanical compound responsible for SULT-inhibition in powdered basil PFS. Subsequently, the in vivo effect on estragole DNA-adduct formation was predicted by PBK modeling. The PBK model predictions thus derived were used to refine the incidence of liver tumors reported in

literature which we used in our previous risk assessment for basil-containing PFS as described in Chapter 3. Results obtained revealed that the effects of estragole will be reduced in rodent bioassays when high doses of estragole and nevadensin would be administered simultaneously at ratios detected in PFS. However, results also revealed that the matrix-derived combination effect may be limited at lower dose levels of estragole and nevadensin relevant for the use of PFS indicating that the effect of the botanical matrix on the bioactivation of estragole should be tested at realistic intake levels.

In a next step, a chemical analysis and safety assessment was performed for estragole in fennel based teas (**Chapter 6**). In dry fennel preparations estragole levels of 0.15–13.3 mg/g dry fennel preparation were found whereas the estragole content was found to be much lower in water infusions (*i.e.* 0.4-133.4 µg/25 mL infusion prepared from 1 g dry material). Based on these findings, the extraction efficiency of estragole into the infusion was determined to equal 0.1-2.3%. Interestingly, estragole levels were found to be about 3-fold lower in water infusions prepared from whole fennel fruits compared to water infusions prepared from fine cut fennel material. In a next step, an estimate of the daily intake of estragole resulting from the use of fennel teas was made based on chemical analysis and assuming the daily use of 1-3 cups fennel tea. Subsequently, MOE values were calculated by dividing the $BMDL_{10}$ values for estragole, as obtained in Chapter 3 of this thesis, by the estimated estragole intakes resulting from the daily use of fennel teas. Results reveal that the daily use of fennel based teas is generally of low priority for risk management (MOE >10 000), especially when consuming one cup of fennel tea on a daily basis throughout life. However, the use of fennel based teas by children was found to be a priority for risk management. Nevertheless, when fennel based tea is used for a short period of 1-2 weeks, proposed for the symptomatic treatment of digestive disorders, MOE values would be higher than 10,000 indicating a low priority for risk management.

Chapter 7 of this thesis presents a general discussion of the results obtained. In addition, future perspectives are raised to further improve risk and safety assessment of PFS.

Overall, the work presented in this thesis shows that the MOE concept is a useful approach in risk and safety assessment of PFS containing genotoxic carcinogens that can be used to prioritize risk management actions. In addition, integrating various approaches such as mode of action based PBK modeling and read across in the MOE concept was found to facilitate risk and safety assessment of botanical compounds even in the absence of a full set of toxicity data.

Chapter 9

Samenvatting

Momenteel neemt in Europa het gebruik van planten en plantaardige producten zoals plantaardige voedingssupplementen toe. Hoewel zulke producten doorgaans een hoge acceptatiegraad hebben bij de consument, kunnen plantaardige voedingssupplementen toxische ingrediënten bevatten en dat geeft reden tot zorg (EFSA, 2012). Zo kunnen bijvoorbeeld natuurlijke ingrediënten aanwezig zijn die zowel genotoxisch als carcinogeen zijn (EFSA, 2012). De risicobeoordeling van plantaardige voedingssupplementen die genotoxische en carcinogene ingrediënten bevatten is niet gemakkelijk. Zo is er momenteel geen internationale overeenkomst over de beste strategie voor de risicobeoordeling van dergelijke plantaardige ingrediënten en preparaten (EFSA, 2005). Het doel van dit proefschrift was het testen en valideren van nieuwe concepten die gebruikt kunnen worden in de risicobeoordeling van plantaardige voedingssupplementen die genotoxische en carcinogene ingrediënten bevatten.

Hoofdstuk 1 geeft een algemene inleiding over voedingssupplementen, een introductie over de in het proefschrift geteste methoden voor risicobeoordeling van plantaardige voedingssupplementen en een introductie over de in het proefschrift onderzochte genotoxische en carcinogene ingrediënten. Hoofdstuk 1 presenteert tevens het doel van dit proefschrift en geeft een overzicht van de hoofdstukindeling.

Een overzicht van de verschillende benaderingen die momenteel in gebruik zijn voor de risico- en veiligheidsbeoordelingen van planten en plantaardige producten is beschreven in **hoofdstuk 2**. Tevens worden er in hoofdstuk 2 een aantal voorbeelden gegeven van plantaardige ingrediënten die aanleiding geven tot zorg vanwege hun genotoxische en carcinogene eigenschappen of andere werkingsmechanismen, zoals neurotoxische effecten of interacties met geneesmiddelen.

Hoofdstuk 3 beschrijft de toepassing van de zogenaamde Margin of Exposure (MOE)-methode in de risicobeoordeling van plantaardige voedingssupplementen die verkregen zijn via internet en de Nederlandse en Italiaanse markt. Allereerst werden er 30 stoffen met genotoxische en/of carcinogene eigenschappen geselecteerd op basis van de beschikbare literatuur. Het overgrote deel van deze stoffen behoorde tot een van twee belangrijke categorieën van toxische plantaardige ingrediënten, te weten de alkenylbenzenen en de onverzadigde pyrrolizidine alkaloïden. Vervolgens werden $BMDL_{10}$-waarden (dat wil zeggen het laagste betrouwbaarheidsinterval

van de benchmark dosis die resulteert in een stijging van 10% van het aantal tumoren) berekend voor de alkenylbenzenen estragole, methyleugenol, safrole, en bèta-asarone. Ook werden er innameschattingen gemaakt. Deze innameschattingen waren gebaseerd op de resultaten verkregen via een chemische analyse en de aanbevolen dagelijkse dosis van het betreffende plantaardige voedingssupplement zoals aangegeven op het etiket. Gebruik makend van deze gegevens werd er een risicobeoordeling uitgevoerd met behulp van de MOE-methode door de $BMDL_{10}$-waarden te delen door de dagelijkse innames. Voor een aantal plantaardige voedingssupplementen was de MOE-waarde >10.000, wat een lage prioriteit voor risicomanagement impliceert. Echter, voor andere supplementen werden MOE-waarden <10.000 verkregen, wat een prioriteit voor risicomanagement impliceert. Voor plantaardige voedingssupplementen bestaande uit de essentiële oliën van basilicum werden zelfs MOE-waarden <10 gevonden. Dit wil zeggen dat de dagelijkse estragole-inname als gevolg van het gebruik van zulke supplementen vergelijkbaar is met de dagelijkse dosis estragole waarbij levertumoren in proefdieren zijn gevonden. Deze plantaardige voedingssupplementen zijn een prioriteit voor risicomanagement.

Vervolgens werd een zogeheten physiologically based kinetic (PBK) model geconstrueerd voor de alkenylbenzeen elemicine (**hoofdstuk 4**). Voor elemicine zijn in de literatuur slechts weinig toxicologische data beschikbaar. Op basis van de verkregen resultaten kon geconcludeerd worden dat de speciesverschillen in de bioactivering van elemicine slechts beperkt zijn. Ook de verschillen tussen de vorming van de uiteindelijke carcinogene metaboliet van elemicine en de vorming van de vergelijkbare carcinogene metabolieten van de structuurgerelateerde analogen estragole en methyleugenol, bleken beperkt. Gebruik makend van deze gegevens en van read-across naar de aanwezige data uit dierstudies uitgevoerd met estragole of methyleugenol, werd een schatting van de $BMDL_{10}$-waarde voor elemicine gemaakt. Vervolgens werd een risicobeoordeling uitgevoerd met behulp van de MOE-methode. Op basis van deze risicobeoordeling werd geconcludeerd dat de inname van elemicine door het gebruik van kruiden en specerijen, en producten die daarvan gemaakt zijn, zoals plantaardige voedingssupplementen, een lagere prioriteit voor risicomanagement hebben in vergelijking met de structuurgerelateerde analogen estragole en methyleugenol. Gebruik makend van de resultaten zoals verkregen in hoofdstuk 4, kan geconcludeerd worden dat het gebruik van PBK-

modellen een wetenschappelijke basis vormt voor read-across van het ene plantaardige ingrediënt naar het andere, structureel verwante, plantaardige ingrediënt waardoor een risicobeoordeling uitgevoerd kan worden voor een bestanddeel waarvoor slechts weinig toxicologische data aanwezig zijn, zonder dat er gebruik gemaakt hoeft te worden van proefdieren.

Hoofdstuk 5 beschrijft de effecten op de bioactivering van estragole door andere bestanddelen in basilicum-bevattende supplementen. Van elk van de geselecteerde basilicum-bevattende supplementen werd een methanolextract gemaakt. Deze extracten werden vervolgens getest op het effect op de sulfotransferase (SULT)-activiteit. SULT-activiteit werd door alle geteste extracten geremd. Echter, SULT-activiteit werd sterker geremd door supplementen die basilicum in poedervorm bevatten in vergelijking met supplementen die de essentiële oliën van basilicum bevatten. In supplementen, bestaande uit basilicum in poedervorm, werd nevadensin geïdentificeerd als het plantaardige bestanddeel dat verantwoordelijk was voor de remming van SULT-activiteit. Vervolgens werd PBK-modellering toegepast om de *in vivo* effecten op estragole DNA adductvorming te voorspellen. Deze voorspellingen werden gebruikt om de incidentie van levertumoren, zoals beschreven in de literatuur, te verfijnen. De verkregen resultaten lieten zien dat de effecten van estragole lager zullen zijn wanneer hoge concentraties estragole en nevadensin gelijktijdig toegediend worden in ratio's zoals ze ook voorkomen in plantaardige voedingssupplementen. Echter, de resultaten toonden ook aan dat dit effect beperkter is wanneer concentraties estragole en nevadensin gebruikt worden die worden bereikt bij het gebruik van plantaardige voedingssupplementen. Dit benadrukt dat de effecten van de plantaardige matrix op de bioactivering van estragole voor realistische innames getest zou moeten worden.

Hoofdstuk 6 beschrijft de chemische analyse en veiligheids-beoordeling voor estragole in venkelthee. De concentraties estragole in droge venkelpreparaten waren gelijk aan 0.15-13.3 mg/g droog venkelpreparaat. De hoeveelheid estragole was veel lager in waterinfusies, namelijk 0.4-133.4 µg/25 mL infusie gemaakt van 1 g droog venkelmateriaal. De extractie-efficiëntie van estragole in de infusie is dus gelijk aan 0.1-2.3%. De hoeveelheden estragole in waterinfusies, gemaakt van intact venkelmateriaal, was 3 keer lager dan de hoeveelheid estragole in waterinfusies gemaakt van fijngesneden venkelmateriaal. In een vervolgstap werd er een schatting gemaakt van de dagelijkse inname van estragole als gevolg van het drinken van venkelthee.

Dit werd gedaan door gebruik te maken van de resultaten verkregen via chemische analyse en ervan uitgaande dat er dagelijks 1-3 koppen venkelthee geconsumeerd worden. MOE-waarden werden berekend door de $BMDL_{10}$-waarden voor estragole, zoals verkregen in hoofdstuk 3 van dit proefschrift, te delen door de geschatte estragole-inname via het gebruik van venkelthee. Op basis van deze veiligheidsbeoordeling werd geconcludeerd dat het dagelijks gebruik van venkelthee over het algemeen van lage prioriteit voor risicomanagement is (MOE >10.000), voornamelijk wanneer dagelijks 1 kop venkelthee geconsumeerd wordt. Het gebruik van venkelthee door kinderen is wel een prioriteit voor risicomanagement. Echter, in het geval van de behandeling van symptomatische klachten als gevolg van spijsverteringsstoornissen wordt meestal geadviseerd venkelthee te gebruiken voor een periode van 1-2 weken. De MOE-waarden voor een dergelijk kortdurend gebruik van venkelthee zouden 3 orden van grootte hoger zijn en dus >10.000 hetgeen een lage prioriteit voor risicomanagement impliceert.

Hoofdstuk 7 presenteert een discussie van de verkregen resultaten. Daarnaast worden toekomstige perspectieven beschreven om de risico- en veiligheidsbeoordeling van plantaardige voedingssupplementen verder te verbeteren.

Alles bijeen laat het werk, zoals gepresenteerd in dit proefschrift, zien dat de MOE-methode geschikt is voor de risico- en veiligheidsbeoordeling van plantaardige voedingssupplementen die carcinogene en genotoxische bestanddelen bevatten. De MOE-methode kan gebruikt worden om prioriteit voor risicomanagement aan te geven. Bovendien kan met name de combinatie van verschillende methoden, zoals het gebruik van PBK modellering en read-across met de MOE methode, van nut zijn voor de risico- en veiligheidsbeoordeling, en de mogelijkheid bieden een risico- en veiligheidsbeoordeling uit te voeren zelfs voor plantaardige voedingssupplementen waar geen toxicologische informatie voor aanwezig is.

Appendices

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Suzanne van den Berg
Wageningen, June 2014

Curriculum vitae

Suzanne J.P.L. van den Berg was born on November 17, 1984 in Nijmegen and grew up in Wijchen. After graduating from pre-university education, she started a study Biomedical Sciences at the Radboud University Nijmegen. During her Master, she performed an internship at the department of Toxicology of Wageningen University on the matrix effect by herbal ingredients reducing the bioactivation and carcinogenic risk of the genotoxic and carcinogenic alkenylbenzenes present in herbs, spices and essential oils. In addition, she performed a minor internship at the laboratory of Rheumatology Research & Advanced Therapeutics at the Radboud University of Nijmegen Medical Centre on the role of S100A8 and S100A9 proteins in M1-M2 macrophage biology. She received her MSc degree in Biomedical Sciences in September 2010 with a major in Toxicology, a minor in Nutrition and Health and a second minor in Human Pathobiology. From July 2010 to June 2014, she worked as a PhD student at the department of Toxicology of Wageningen University under the supervision of prof. dr. ir. Ivonne M.C.M. Rietjens and dr. ir. Ans Punt. Her research focused on the risk and safety assessment of botanicals and botanical preparations including plant food supplements which resulted in the present thesis. Her PhD research was part of the PlantLIBRA project, a European collaborative project under the Seventh Framework Programme. She was awarded with the PlantLIBRA Young Investigator's Award which aimed to encourage professional development of young scientists involved in the PlantLIBRA project. During her PhD, she attended several PlantLIBRA meetings and (international) conferences. In addition, she followed a number of postgraduate courses in toxicology required for registration as a toxicologist with the Netherlands Society of Toxicology and EUROTOX. Moreover, she was involved in several additional activities during the period of her PhD as she was a member of the Education Committee Toxicology at the department of Toxicology of Wageningen University, she was a co-organizer of the international PhD study excursion to Switzerland and Italy for PhD-students from the department of Toxicology, Wageningen University, and she was a co-organizer of the 35th Anniversary Meeting of the Netherlands Society of Toxicology. Since March 2013, she has been working part-time as a toxicological risk assessor food safety at TNO, Zeist. After completing her PhD, she will continue working at TNO on a full-time basis.

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List of publications in preparation

Malameh, A., Alajlouni, A., Boersma, M.G., Soffers, A.E.M.F., Hao, B., van den Berg, S.J.P.L., Rietjens, I.M.C.M. Mode-of-action based risk assessment of the botanical food-borne alkenylbenzene myristicin.

Alajlouni, A., Malameh, A., Boersma, M.G., Soffers, A.E.M.F., van den Berg, S.J.P.L., Rietjens, I.M.C.M. Mode-of-action based risk assessment of the botanical food-borne alkenylbenzene apiol.

Guzzon, A., Tuomisto, J., van den Berg, S.J.P.L., Rietjens, I.M.C.M., Di Lorenzo, C., Restani, P., Bucchini, L. Integrated approach to risk and benefit assessment of botanicals: application to cinnamon essential oil.

Tyrakowska, B., van den Berg S.J.P.L., Soffers, A.E.M.F., Rietjens, I.M.C.M. Matrix and combination effects in absorption, distribution, metabolism and excretion (ADME) of food born toxic compounds.

Overview of completed training activities

Discipline specific activities
Harmonized literature review workshop (PlantLIBRA project) (2010)
Risk assessment, Postgraduate Education in Toxicology (PET) (2011)
Organ toxicology, PET (2012)
Epidemiology, PET (2012)
Legal and regulatory toxicology, PET (2012)
Medical and forensic toxicology, PET (2012)
Toxicogenomics, PET (2013)
Ecotoxicology, PET (2013)

Meetings
PlantLIBRA meeting, Milan, Italy (2010) (oral presentation)
51st Meeting of the American Society of Toxicology, San Francisco, USA (2012) (poster presentation)
WMFmeetsIUPAC international conference, Rotterdam, The Netherlands (2012) (oral presentation)
RIKILT: Planttoxinen en plantinhoudstoffen, Wageningen, The Netherlands (2012) (oral presentation)
NVWA, Utrecht, The Netherlands (2013) (oral presentation)
RIVM/ NVWA: Workshop Kruiden(producten): goed geregeld of kan het beter? Bilthoven, The Netherlands (2013)
53rd Meeting of the American Society of Toxicology, Phoenix, USA (2014) (poster presentation)
PlantLIBRA conference: integrating botanical science for safer products, Vienna, Switzerland (2014) (oral presentation)

General courses
Competence assessment, Wageningen Graduate Schools (WGS) (2011)
Media training, Ton van Rhoon, Quintrix BV (2011)
Philosophy and ethics of food science and technology, WGS (2011)
VLAG PhD week (2011)
Interpersonal communication for PhD students, WGS (2012)
LC-MS course, department of Toxicology of Wageningen University (2012)
Project and time management, WGS (2012)
Personal coach – effective behavior, Meijer & Meijaard (2013)

Optionals
Preparing PhD research proposal
Attending scientific presentations, department of Toxicology of Wageningen University (2010 - 2014)
PhD study excursion to Switzerland and Italy, department of Toxicology of Wageningen University (2011)
Organizing PhD study excursion, department of Toxicology of Wageningen University (2011)

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