Matrix modulation of the toxicity of alkenylbenzenes, studied by an integrated approach using in vitro, in vivo, and physiologically based biokinetic models

Wasma Alhusainy

THESIS COMMITTEE

Promotors
Prof. Dr I.M.C.M. Rietjens
Professor of Toxicology
Wageningen University

Prof. Dr P.J. van Bladeren
Professor of Toxico-kinetics and Biotransformation
Wageningen University

Co-promotor
Dr A. Punt
Assistant professor, Sub-department of Toxicology
Wageningen University

Other members
Prof. Dr R. Witkamp, Wageningen University
Prof. Dr F. Van Schooten, Maastricht University
Dr Van Benthem, The National Institute for Public Health and the environment (RIVM)
Prof. Dr H. Verhagen, Ulster University

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

Matrix modulation of the toxicity of alkenylbenzenes, studied by an integrated approach using in vitro, in vivo, and physiologically based biokinetic models

Wasma Alhusainy

Thesis
Submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 10 December 2013
At 11 a.m. in the Aula.

Wasma Alhusainy

Matrix modulation of the toxicity of alkenylbenzenes, studied by an integrated approach using in vitro, in vivo, and physiologically based biokinetic

models, 200 pages

PhD thesis, Wageningen University, wageningen, NL (2013)

With references, with summaries in Dutch and English

ISBN 978-94-6173-806-6

For my family, Ahmad and Josef
Thank you very much for your endless love and support

TABLE OF CONTENTS

1 Introduction

Background

Alkenylbenzenes are common components of spices and herbs such as tarragon, basil, fennel, mace, allspice, star anise and anise and their essential oils. Major examples of alkenylbenzenes are the compounds estragole (1-allyl-4-methoxybenzene) (Figure 1A) and methyleugenol (1-allyl-3,4-dimethoxybenzene) (Smith *et al.*, 2002)(Figure 1B).

At high dose levels estragole and methyleugenol and their 1′-hydroxy metabolites can induce hepatic tumours in mice (Miller *et al.*, 1983). In 2000, the National Toxicology Program (NTP) has further investigated the carcinogenicity of methyleugenol and concluded that methyleugenol is carcinogenic in both rats and mice (NTP, 2000). Based on the rodent studies with estragole, methyleugenol and structurally related alkenylbenzenes the hepatocarcinogenicity of alkenylbenzenes is ascribed to their bioactivation by cytochrome P450 enzymes leading to the formation of the proximate carcinogen 1′-hydroxy metabolite (Figure 2) (Miller *et al.*, 1983; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Smith *et al.*, 2002). Further bioactivation of the 1′-hydroxy metabolite requires the involvement of sulfotransferases (SULTs) which convert the 1′-hydroxy metabolite to the ultimate carcinogenic 1′-sulfooxy metabolite (Figure 2). The 1′-sulfooxy metabolite is unstable and covalently bind via presumed reactive carbocation intermediate to different endogenous nucleophiles including DNA (Phillips *et al.*, 1981, 1984; Boberg *et al.*, 1983; Miller *et al.*, 1983; Randerath *et al.*, 1984; Fennell *et al.*, 1985; Wiseman *et al.*, 1987; Smith *et al.*, 2002). Because of their genotoxicity the addition of estragole and methyleugenol as pure substances in foodstuffs has been prohibited within the European Union since September 2008 (European Commission, 2008).

A significant difficulty in evaluating the metabolic and toxicological data for alkenylbenzenes is that human exposure to these substances results from exposure to a complex mixture of food, spice, and spice oil constituents which may influence the biochemical fate and toxicological risk of the alkenylbenzenes. For example, a methanolic extract of basil has been shown to inhibit the formation of estragole DNA adducts in human HepG2 cells exposed to the proximate carcinogen 1′-hydroxyestragole (Jeurissen *et al.*, 2008). This inhibition occurred at the level of SULT-mediated bioactivation of 1′-hydroxyestragole into 1′-sulfooxyestragole (Jeurissen *et al.*, 2008).

The objective of this PhD research was to identify the SULT inhibitor(s) present in the basil extract able to inhibit the estragole DNA adduct formation and to identify the presence of other SULT inhibitors in a series of alkenylbenzene-containing herbs and spices. An additional objective was to predict the potential reduction in estragole

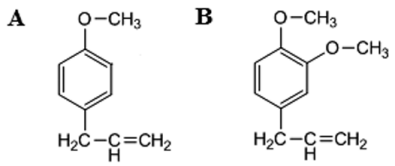


Figure 1. Chemical structures of the alkenylbenzenes A) estragole and B) methyleugenol.

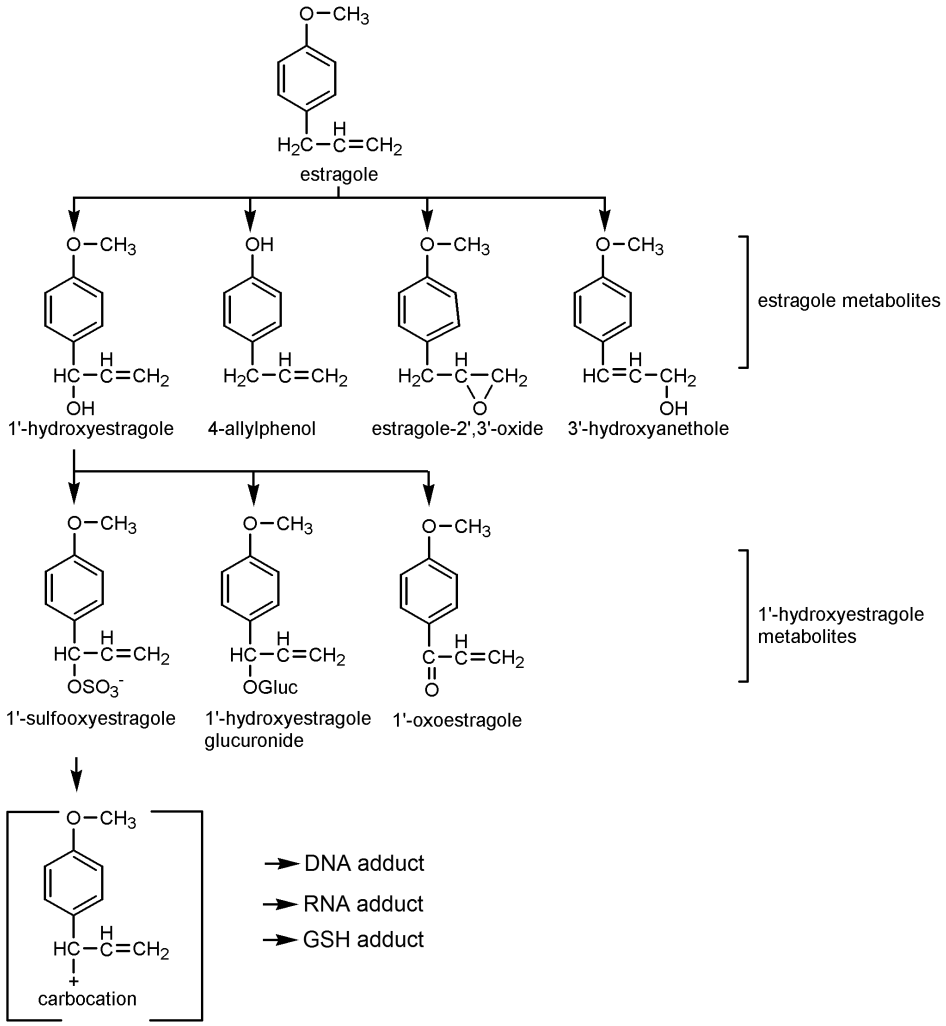


Figure 2. Bioactivation and detoxification pathways of estragole which are also representative for methyleugenol.

and methyleugenol bioactivation when these compounds are consumed with either a single SULT inhibitor or a mixture of SULT inhibitors at relevant dose levels of the alkenylbenzene and the SULT inhibitor(s). An integrated approach of in vitro, in vivo and physiologically based biokinetic (PBBK) models was applied to investigate how the SULT inhibition influences the bioactivation and thus potentially also the toxicity and risk assessment of alkenylbenzenes.

Exposure to alkenylbenzenes

Even though alkenylbenzenes are present in many foods at low levels, including for example oranges, bananas and grapefruit juice, intake of alkenylbenzenes occurs primarily from herbs, spices and their essential oils (Smith *et al.*, 2002). The intake of estragole occurs for example primarily from consumption of foods containing sweet basil, fennel and anise or their essential oils whereas intake of methyleugenol occurs primarily from nutmeg, allspice, sweet basil, and fennel (Smith *et al.*, 2002).

Among different alkenylbenzenes, only estragole and methyleugenol are used as flavouring agents intentionally added as pure compounds to compounded flavours. This use is limited to the USA, but annual production volumes are low (491 kg per year for estragole and 77 kg per year for methyleugenol) (Gavin *et al.*, 2008). Realistic daily intake levels of estragole have been estimated to range between 0.01 mg/kg bw/day (Smith *et al.*, 2002) and 0.07 mg/kg bw/day (SCF, 2001a). Realistic daily intake levels of methyleugenol have been estimated to range from 0.014 mg/kg bw/day (Smith *et al.*, 2002) to 0.217 mg/kg bw/day (SCF, 2001b). The intake estimates of the SCF for estragole and methyleugenol were based on theoretical maximum use levels of the two compounds as flavouring substances in various food categories and consumption data for these food categories (SCF, 2001a, b). The intake estimation of the Flavour Extract Manufacturers Association (FEMA) of the United States was based on production volume data for herbs and their essential oils and for the pure compounds to be used as flavouring substances (Smith *et al.*, 2002). Given that at present in the European Union addition of estragole and methyleugenol as pure compounds to individual food categories is no longer allowed, the intake estimates from Smith *et al.* (2002) resulting mainly from herbs and spices may represent more accurate estimates for the current levels of dietary human intake.

In addition to the average daily intake, some specialized eating groups may experience higher exposure levels. For example, people frequently consuming pesto ingest some of the highest levels of methyleugenol. Thus although normal exposure to methyleugenol may be in the order of 0.001-0.01 mg/kg bw/day, exposures of at least 10 times higher may be experienced by this group (Smith *et al.*, 2002).

Absorption, distribution, metabolism and excretion

In vivo studies with rodents and humans indicate that upon oral intake estragole and methyleugenol are rapidly and completely absorbed and that they are predominantly metabolised in the liver (Sutton *et al.*, 1985; Anthony *et al.*, 1987; Sangster *et al.*, 1987; Johnson *et al.*, 2000; Smith *et al.*, 2002; Schecter *et al.*, 2004). In rats, both estragole and methyleugenol are rapidly cleared from the system within 24 h (Solheim and Scheline, 1973; Anthony *et al.*, 1987). In two human subjects given a 100 µg dose of ^{14}C-methoxy labelled estragole by gelatin capsule, approximately 70% of the dose was recovered within 48 h (Sangster *et al.*, 1987). Also, in an experiment with human volunteers who consumed estragole by drinking fennel tea, containing 3.5 mg of estragole, no estragole was detectable in plasma or urine samples pointing at a very fast distribution and complete metabolism in the body. After 0.75 h, less than 0.1% of the estragole dose

could be detected as conjugated 1′-hydroxyestragole in plasma whereas in the urine 0.27% of the estragole dose was detected as conjugated 1′-hydroxyestragole. Excretion happened very quickly, and after 10 h, no metabolite was detectable in the urine (Zeller *et al.*, 2009). Methyleugenol has been detected in the plasma of humans not specifically exposed (Schecter *et al.*, 2004), indicating that methyleugenol and probably other alkenylbenzene derivatives are absorbed from the diet.

The principal metabolic pathways for alkenylbenzene derivatives have been established based on studies in rats, mice and humans and Figure 2 presents an overview of estragole metabolism. This overview is also representative for methyleugenol as well as for other alkenylbenzene derivatives. *O*-demethylation of estragole or methyleugenol results in phenolic derivatives, which may be excreted as sulfonate or glucuronic acid conjugate (Solheim and Scheline, 1973; Anthony *et al.*, 1987). Epoxidation of the side chain yields a 2′,3′-epoxide. This epoxide has been shown to be able to form DNA adducts in vitro, but these adducts are not found in vivo. This has been ascribed to the rapid detoxification of the 2′,3′-epoxide by epoxide hydrolases and/or glutathione-*S*-transferases in vivo (Solheim and Scheline, 1976; Luo *et al.*, 1992; Luo and Guenthner, 1995; NTP, 2000; Guenthner and Luo, 2001). Bioactivation of estragole proceeds by cytochrome P450 enzymes leading to the formation of the proximate carcinogen 1′-hydroxyestragole (Drinkwater *et al.*, 1976; Zangouras *et al.*, 1981; Miller *et al.*, 1983; Anthony *et al.*, 1987; Sangster *et al.*, 1987). Further bioactivation of 1′-hydroxyestragole requires the involvement of sulfotransferases (SULTs) converting 1′-hydroxyestragole to 1′-sulfooxyestragole. The 1′-sulfooxy metabolite is unstable and, via a proposed reactive carbocation intermediate, binds covalently to different endogenous nucleophiles including DNA leading to DNA adduct formation (Phillips *et al.*, 1981; Phillips *et al.*, 1984; Randerath *et al.*, 1984). Conjugation of 1′-hydroxy metabolites with glucuronic acid leads to the formation of a stable metabolite that is excreted in urine and therefore represents an important detoxification route (Solheim and Scheline, 1973; Anthony *et al.*, 1987). In vitro experiments using liver S9 fractions from male rat and human showed that oxidation of 1′-hydroxyestragole is the major detoxification pathway of 1′-hydroxyestragole in human whereas in rat glucurondation of 1′-hydroxyestragole seems to be the major pathway (Punt *et al.*, 2009).

Genotoxicity

Estragole was negative in genotoxicity tests with common strains of *Salmonella typhimurium* with and without metabolic activation (Dorange *et al.*, 1977; Sekizawa and Shibamoto, 1982; To *et al.*, 1982; Zeiger *et al.*, 1987; Zani *et al.*, 1991). Methyleugenol was negative in multiple tests in various strains of *S. typhimurium* and *Saccharomyces cerevisae* (Dorange *et al.*, 1977; Sekizawa and Shibamoto, 1982; Mortelmans *et al.*, 1986; Schiestl *et al.*, 1989; Brennan *et al.*, 1996). The reason for these negative effects is that the sulfonation pathway, which is essential for the genotoxicity, is absent in these in vitro tests. However, based on a recent study where the mutagenicity of different isomeric hydroxylated metabolites

of methyleugenol was studied in an Ames test using the *Salmonella typhimirum* TA100 strain expressing different SULTs it was shown that the different hydroxylated metabolites of methyleugenol were mutagenic in the Ames test and among the human enzymes; SULT1A1 showed the highest activity (Herrmann *et al.*, 2012).

Estragole concentrations of 10^{-3}-10^{-5} M did not induce the formation of chromosomal aberrations in V79 cells with and without metabolic activation and in primary rat hepatocytes (Muller *et al.*, 1994). Methyleugenol produced sister chromatid exchange (SCE) in Chinese hamster ovary cells only in the presence of metabolic activation and at near cytotoxic levels. Therefore, the positive findings likely occurred secondary to cytotoxicity in which release of lysosomal nucleases may have resulted in a false positive response (NTP, 2000). Both substances and their 1′-hydroxy metabolites induce unscheduled DNA synthesis (UDS) in hepatocytes. The UDS observed in hepatocytes following treatment of rodents with parent compounds is most likely the result of CYP450-mediated metabolism of the compounds to 1′-hydroxy metabolites. However, in these studies dose levels at which UDS occurs coincide with hepatocellular cytotoxicity (Chan and Caldwell, 1992; Muller *et al.*, 1994). The majority of in vivo micronucleus induction studies for both compounds produced negative results (NTP, 2000, 2008).

The 1′-hydroxy metabolites of estragole and methyleugenol, and the corresponding sulfonate conjugates of the 1′-hydroxy metabolites form DNA adducts in vivo and in vitro (Smith *et al.*, 2002). In an experiment where adult female CD-1 mice were given 58 mg/kg bw of [^{3}H]-labeled 1′-hydroxyestragole by ip injection and in 9-day-old male or female B6C3F1 mice given 14 mg/kg of labeled estragole by ip injections and sacrificed after 23 h, three adducts were formed resulting from the reaction of the 1′ or 3′ positions (*cis* or *trans* isomers) of estragole at the exocyclic amino group (N^{2}) of deoxyguanosine (Figure 3). An additional adduct was formed by a reaction at the 3′ position of estragole and the (N^{6}) position of deoxyadenosine. The three adducts of estragole deoxyribonucleoside were removed rapidly from mouse liver DNA (Phillips *et al.*, 1981) (Figure 3). A rapid drop in total adduct formation also occurred in a ^{32}P-post-labelling experiment with adult female CD-1 mice within 7 days after dosing with a 2 or 10 mg ip dose of estragole or methyleugenol and this drop was followed by a relatively constant level over the next 140 days (Randerath *et al.*, 1984). The authors noted that the significant decrease in DNA adduct levels was probably related to DNA repair processes (Randerath *et al.*, 1984).

Carcinogenicity

Short term studies in male rats given the alkenylbenzenes estragole or methyleugenol by gavage showed that the incidence of hepatocellular neoplasms for male rats administered 600 mg/kg bw/day of estragole by gavage, 5 days per week, for 93 days is 4/10 for cholangiocarcinomas and 1/10 for adenomas. At 1000 mg/kg bw/day of methyleugenol given by gavage, 5 days per week, for 13 weeks the incidence was 1/10 for adenomas (NTP, 2000; NTP, 2008). In a 2-year NTP study in mice and rats given methyleugenol by gavage daily at dose levels of 37, 75, or 150 mg/kg bw/day, the incidence of hepatocellular adenomas and hepatocellular carcinomas in male

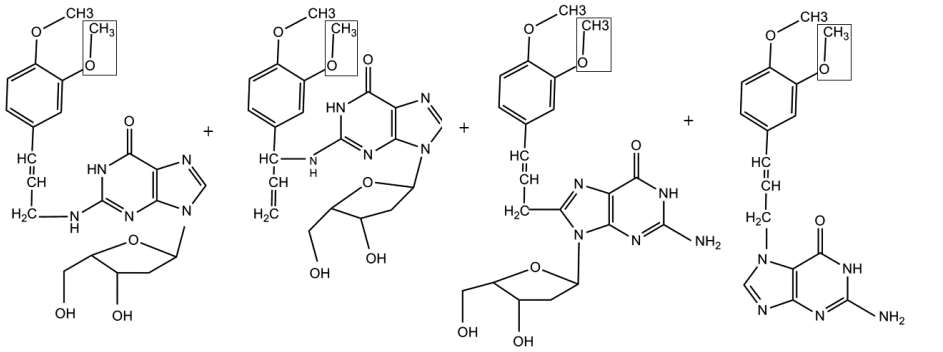


Figure 3. Structure of estragole DNA adducts with 2′-deoxyguanosine as identified by Phillips *et al.* (1981) and Wiseman *et al.* (1985) and DNA adducts of methyleugenol with 2′-deoxyguanosine identified by Al-Subeihi *et al.* (2013) based on the analogy between the two compounds: Estragole and methyleugenol; E-3′-N^2-dGuo, N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine; E-3′-C^8-dGuo, C^8-(*trans*-isoestragol-3′yl)-2′-deoxyguanosine; E-1′-N^2-dGuo, N^2-(estragol-1′-yl)-2′-deoxyguanosine and E-3'-N^7-dGuo, 7-(*trans*-estragol-3′-yl)-2′-guanine. The methoxy group in the boxes are only present in methyleugenol derived adduct.

and female mice and rats increased in a dose dependent way (NTP, 2000). However, because of the evidence of toxicity of methyleugenol in all groups of rats and mice, the study cannot be recognized as conclusive for carcinogenicity at lower, non-toxic doses (Smith *et al.*, 2002). In particular, the hepatic damage undoubtedly altered the metabolism of the compound, and the gastric damage possibly altered its absorption (Smith *et al.*, 2002). Estragole has not been tested in a 2-year carcinogenicity bioassay. However, when administered to male CD-1 mice in the diet during a preweaning period for 12 months, estragole was positive for carcinogenicity and produced hepatic DNA adducts (Miller *et al.*, 1983; Randerath *et al.*, 1984).

Based on the carcinogenic activity of estragole and methyleugenol shown in the study of Miller *at al.* (1983) and the DNA adduct formation by these substances shown in the study of Phillips *et al.* (1984) using the same species and strain, it was concluded that adduct levels of at least 15 pmol/mg of DNA were required for statistically significant tumour formation (Phillips *et al.*, 1984). The authors also noted that, compared with adults, new born mice showed greater sensitivity to alkenylbenzene carcinogenicity (Phillips *et al.*, 1984).

The role of the 1′-sulfooxy metabolite in hepatotoxicity and hepatocarcinogenicity of alkenylbenzenes has also been proven in rodents. This was revealed in mice experiments by co-administration of the specific SULT inhibitor pentachlorophenol (PCP) which resulted in a potent inhibition of hepatic tumour induction upon long-term dietary administration of the closely related alkenylbenzene safrole or its metabolite, 1′-hydroxysafrole (Boberg *et al.*, 1983; Wiseman *et al.*, 1987).

Regulatory status

The safety of human exposure to estragole and methyleugenol at low dietary intake levels has been assessed several times. In 1965, the Expert Panel of the Flavour and Extract Manufacturers Association (FEMA) concluded that estragole and methyleugenol are generally recognized as safe (GRAS) under conditions of intended use as flavouring substances in food (Smith *et al.*, 2002). In 1979, the Panel again evaluated the available data and affirmed the GRAS status of estragole and methyleugenol for use as flavouring substances (Smith *et al.*, 2002). In 2000, the National Toxicology Program (NTP) investigated the carcinogenicity of methyleugenol and concluded that methyleugenol is carcinogenic in both rats and mice (NTP, 2000). In 2001, the FEMA Expert Panel reassessed the available data for methyleugenol and estragole and confirmed that there is no considerable cancer risk resulting from consumption of estragole and methyleugenol as flavouring substances and affirmed the GRAS status at the use levels as flavouring substances (Smith *et al.*, 2002). In the FEMA evaluation it was taken into account that there are experimental data suggesting a non-linear relationship between dose and profiles of metabolism and metabolic activation and that exposure due to use as flavouring substances is relatively low (Smith *et al.*, 2002).

In 2001, the Scientific Committee on Food (SCF) of the European Union published scientific opinions on estragole and methyleugenol in which it was concluded that both compounds are genotoxic and carcinogenic and that reductions in exposure and restrictions in use levels are indicated (SCF, 2001a, b). Based on these findings, the use of estragole and methyleugenol as pure substances in foodstuffs has been prohibited since September 2008 within the European Union (European Commission, 2008). An evaluation performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2008, has indicated that although evidence of carcinogenicity to rodents given high doses of alkenylbenzenes exists, further research is needed to assess the potential risk to human health at relevant dietary exposure levels (JECFA, 2008).

The occurrence of these different expert opinions in risk assessment of alkenylbenzenes originates in part from the absence of scientific agreement to support the unequivocal translation of carcinogenicity data of rodent animal experiments obtained at high levels of exposure to the relevant human situation. Predicting the cancer risk in humans at relevant dietary intake levels requires extrapolation of the animal carcinogenicity data taking into consideration species-, dose- and interindividual variation. Furthermore, it implies extrapolation from rat or mouse studies with high dose levels of the pure compound to the human situation in which exposure at low dose levels occurs within the context of a complex food matrix.

Risk assessment approaches

Alkenylbenzenes such as estragole and methyleugenol occur naturally in botanicals and botanical preparations present in food and therefore they cannot readily be eliminated or avoided. Estimation of the cancer risk from alkenylbenzenes at low dose levels relevant for human intake is essential from a public health point of view. However,

there is no international agreement on how to evaluate the potential risk of genotoxic carcinogens that naturally occur in food. One of the commonly used approaches in risk assessment practices especially in the United States is the linear extrapolation of animal carcinogenicity data obtained at high dose levels to low dose levels which are relevant to human intake. This approach requires definition of a point of departure to be derived from the animal data at high dose levels from which the extrapolation can be performed to zero risk at zero dose level. The point of departure may be the T_{25} which is the dose that increases the tumour incidence by 25% (Barlow *et al.*, 2006; Dybing *et al.*, 2008).

It may also be the so-called Benchmark Dose 10 (BMD_{10}) (the Benchmark Dose that gives a 10% extra cancer incidence) or the lower confidence bound of the $BMDL_{10}$ determined using the Benchmark Dose (BMD) approach (EFSA, 2005; US EPA, 2005). The BMD approach provides a useful tool for deriving a point of departure within the observable dose range by fitting a curve to the dose-response data from an animal bioassay and using this curve fit to estimate the intake level that corresponds to a fixed tumour incidence. The simplest way to estimate the risk associated with the average dietary human intake is to apply linear extrapolation from the BMD_{10} or T_{25} as the point of departure to zero risk at zero dose. Based on this extrapolation a so-called virtual safe dose (VSD) can be derived, corresponding to the dose that results in an additional cancer risk of one in a million upon lifetime exposure, which is generally considered an acceptable risk by risk managers.

There are major problems with linear extrapolation of cancer incidences based on data obtained in animal bioassays at high dose levels that are orders of magnitude higher than the realistic intake levels for the human situation. Such problems are the presence of uncertainties in the shape of the dose-response curve at dose levels relevant for dietary human intake, interspecies variations in metabolism and metabolic activation and the larger interindividual variability within the human population, as compared to the variability in the inbred strains used for the rodent bioassays.

An alternative approach that has been suggested by the European Food Safety Authority (EFSA) for the risk assessment of compounds that are both genotoxic and carcinogenic like alkenylbenzenes is the so-called margin of exposure (MOE) approach which can be used to set priorities in risk management (EFSA, 2005). The MOE is defined as the ratio between the lower confidence limit of the benchmark dose that gives 10% extra cancer incidence ($BMDL_{10}$) and the estimated daily intake (EDI) (EFSA, 2005). EFSA considered that, when the MOE is higher than 10,000, the compound is considered to be of low priority for risk management actions (EFSA, 2005). This margin of 10,000 takes into consideration the various uncertainties in the MOE approach, such as interspecies differences and human variability in biokinetics and biodynamics, the shape of the dose-response curve below the experimental data, and human interindividual variability in cell cycle control and DNA repair (EFSA, 2005; Barlow *et al.*, 2006; Dybing *et al.*, 2008). The magnitude of the MOE does not indicate a safe dose of a chemical but indicates whether the estimated daily intake is (or is not) close to dose levels actually causing increased tumour incidences in animal bioassays so that risk management actions might (not) be required (Dybing *et al.*, 2008).

Also, it is worth mentioning that some allometric approaches can provide a useful tool to extrapolate across different species as recommended by the US FDA and US EPA. In this regard two methods can be used to calculate human equivalent doses, one is based on body surface area scaling (body mass raised to the 2/3 power of the test animal relative to humans, $BW^{2/3}$), the other is based on metabolic rate scaling ($BW^{3/4}$). Scaling based on body surface area is considered to be more conservative, and is recommended by the FDA to estimate the maximum safe starting doses in initial clinical trials for therapeutics in adult healthy volunteers (US FDA, 2005). EPA recommends metabolic rate scaling for interspecies extrapolation (US EPA, 2005). For oral exposures, the EPA suggests to scale daily applied doses experienced for a lifetime in proportion to body weight raised to the 3/4 power (metabolic rate scaling) (US EPA, 2005). Using allometric scaling when extrapolating between species is mainly used in the US but is not generally applied by regulatory bodies in Europe.

Generally, extrapolation of animal tumour data at high doses using mathematical modeling in order to obtain estimates of the risk to humans at low exposures has been much debated, since it is not known whether or not the model chosen actually reflects the underlying biological processes. In addition, numerical estimates may be misused or misinterpreted in further risk management and risk communication, if the uncertainties and inaccuracy connected to the model are not communicated (EFSA, 2005). To address major issues of debate in risk assessment strategies new tools such as physiologically based biokinetic (PBBK) modeling can be used. PBBK modeling provides a method to obtain a better mechanistic basis for extrapolations from high to low dose as well as for interindividual and interspecies extrapolations (Andersen and Krishnan, 1994; Clewell *et al.*, 2001; Clewell Iii *et al.*, 2002) as explained in more detail in the section on PBBK modeling below.

PBBK modeling

The main challenge in the current risk assessment strategies is the need to extrapolate the cancer risk from high dose levels at which most animal studies are performed to low dose levels relevant for human dietary intake. Another issue is to extrapolate between species giving that differences in metabolism and metabolic activation might differ substantially between the different species. In addition, the modulating effects by the food matrix in which the food borne-toxicants occur has to be taken into account. A useful tool to address these issues is the use of physiologically based biokinetic (PBBK) modelling. A PBBK model consists of a set of mathematical equations that together describe the absorption, distribution, metabolism and excretion (ADME) characteristics of a compound within an organism. Input data for any PBBK model consist of three types of parameters, namely, physiological, physiochemical and biochemical parameters (Krishnan *et al.*, 2002). Target tissues are generally represented as individual compartments (e.g. liver, lung, or kidney) while non-target tissues are lumped together in one compartments (e.g. slowly perfused tissues such as muscle and skin or richly perfused tissues such brain and spleen). Each compartment has its

own physiological (e.g. blood flows), physicochemical (e.g. partition coefficients) and biochemical (e.g. metabolic rates) parameters. Kinetic parameters for PBBK models, including metabolic parameters, are, at present, often obtained by optimizing the fit of the model to an informative data set (Krewski *et al.*, 1994; Clewell and Clewell Iii, 2008). Alternatively, kinetic parameters might also be derived from in vitro experiments using relative tissue fractions, primary cell cultures, or tissue slices of organs involved in the metabolism of the compound. Some advantages of using in vitro metabolic parameters to define PBBK models include the ability to separately define and analyze individual metabolic processes, such as phase I metabolism and phase II metabolism, or bioactivation and detoxification, and to compare contributions from individual conversions to the overall metabolism across species and between individuals. This is especially of use when limited in vivo data are available as is often the case for humans (Lipscomb and Poet, 2008). PBBK models quantitatively describe actual physiological processes within the relevant tissues of the body (Krishnan *et al.*, 2002).

Output data expected from such PBBK models are, for example, prediction of internal concentrations at a certain dose, that have not been directly measured, (e.g. the predicted plasma or tissue levels of a parent compound or its reactive metabolites over time) in animal bioassays, as well as in human exposure scenarios (Andersen and Krishnan, 1994). Such an internal dose metric is considered to be more closely related to the toxic response than the applied external doses. Extrapolation of the cancer risk from high to low doses and from animals to humans can be performed based on the internal dose metrics rather than the applied external doses (Andersen and Krishnan, 1994). Furthermore, such PBBK models can be developed for different species, which facilitates interspecies extrapolation. In addition, by incorporating equations and kinetic constants for metabolic conversions by samples from different human individuals and/or specific isoenzymes, modeling of interindividual variations and genetic polymorphisms becomes feasible (Bogaards *et al.*, 2000).

Recently, PBBK models have been developed to study the bioactivation and detoxification of the alkenylbenzenes estragole and methyleugenol in rat and human (Punt *et al.*, 2008, 2009; Al-Subeihi *et al.*, 2011, 2012). These PBBK models provide mechanistic insight in the dose-dependent, interspecies and interindividual difference in the metabolism of the two compounds. For example, the estragole PBBK models revealed that the species difference in the ultimate overall bioactivation and formation of the 1′-sulfooxyestragole between rat and human is about twofold and thus smaller than the default factor of 4 generally assumed to reflect interspecies variation in kinetics (Punt *et al.*, 2009) whereas in the methyleugenol PBBK models species differences in the formation of 1′-sulfooxymethyleugenol is negligible despite the significant difference in especially the metabolic pathways of the proximate carcinogenic metabolite 1′-hydroxymethyleugenol between human and male rat (Al-Subeihi *et al.*, 2012). PBBK models of estragole and methyleugenol also showed that the formation of the ultimate carcinogen, the 1′-sulfooxy metabolite, in rat as well as in human from doses as high as the $BMDL_{10}$ at which actual increased tumour incidences are observed in rodent bioassays, down to as low as the virtual safe dose (VSD) is

linear with the dose (Punt *et al.*, 2009; Al-Subeihi *et al.*, 2012) and thus do not provide an argument against linear extrapolation from high dose animal data to the low dose human situation (Rietjens *et al.*, 2010).

Not only the bioactivation of alkenylbenzenes can be studied by these PBBK models but also the modulation of their bioactivation. This can be achieved by integrating the kinetics for this modulation in the PBBK model. Thus the original PBBK models can be modified and updated with newly generated data to allow representation of dose- and species-dependent effects of this modulation (e.g. matrix effect by herbs or their constituents) on the formation of the ultimate carcinogenic metabolite, the 1′-sulfooxy metabolite, in the target organ, the liver.

Objective and outline of the thesis

This PhD research was based on the outcomes from the study of Jeurissen *et al.* (2008) where it was shown that a methanolic extract of basil was able to inhibit the formation of estragole DNA adducts in human HepG2 hepatoma cells exposed to the proximate carcinogen 1′-hydroxyestragole (Jeurissen *et al.*, 2008) and that inhibition takes place at the level of SULT-mediated bioactivation of 1′-hydroxyestragole into 1′-sulfooxyestragole (Jeurissen *et al.*, 2008).

The objective of this PhD research was to identify the SULT inhibitor(s) present in the basil extract able to inhibit estragole DNA adduct formation and to identify the presence of other SULT inhibitors in a series of alkenylbenzene-containing herbs and spices. An additional objective was to predict the reduction in bioactivation of alkenylbenzenes when they are consumed with either a single SULT inhibitor or with a mixture of SULT inhibitors at relevant dose levels of the alkenylbenzene and the SULT inhibitor(s) using an integrated approach of in vitro, in vivo and physiologically based biokinetic (PBBK) models. The later was achieved by using the PBBK models recently developed to study the bioactivation and detoxification of estragole and methyleugenol in rat (Punt *et al.*, 2008; Al-Subeihi *et al.*, 2011) and human (Punt *et al.*, 2009; Al-Subeihi *et al.*, 2012). The estragole and methyleugenol PBBK models were updated based on data obtained in the present thesis to study the inhibition of estragole and methyleugenol bioactivation by the different SULT inhibitors.

Chapter 1, the present chapter of this thesis, presents an introduction to the bioactivation, detoxification, genotoxicity and carcinogenicity of the alkenylbenzenes estragole and methyleugenol as well as a short introduction to PBBK modeling and the state-of-the-art knowledge on risk assessment strategies and regulatory status for alkenylbenzenes. The aim and contents of the present thesis are also presented.

Chapter 2 describes the identification of the natural SULT inhibitor nevadensin in the methanolic extract of basil responsible for the inhibition of estragole DNA adduct formation by the methanolic basil extract described previously (Jeurissen *et al.*, 2008) and investigates the kinetics for this inhibition using in vitro assays. The effect of the newly identified SULT inhibitor, nevadensin, on the SULT-mediated bioactivation and DNA adduct formation of 1′-hydroxyestragole was investigated in primary rat hepatocytes. In

order to investigate possible in vivo implications of nevadensin on the overall formation of the ultimate carcinogenic metabolite, 1′-sulfooxyestragole, the SULT inhibition by nevadensin was also integrated into the male rat and human estragole PBBK models.

Chapter 3 identifies the presence of other SULT inhibitors in a series of alkenylbenzene-containing herbs and spices and the kinetics of inhibition by each individual SULT inhibitor and by a mixture of SULT inhibitors. The effect of a mixture of SULT inhibitors on the SULT-mediated bioactivation and DNA adduct formation of 1′-hydroxyestragole is investigated in human HepG2 cells. To investigate possible in vivo implications of the different SULT inhibitors on the formation levels of 1′-sulfooxyestragole, the SULT inhibition by the mixtures was also integrated into the male rat and human estragole PBBK models.

Chapter 4 investigates the activity of nevadensin on the SULT-mediated bioactivation and DNA adduct formation of estragole in vivo using male Sprague–Dawley rats. It also describes the development of a new PBBK model for nevadensin in male rat and human. In a next step the estragole and nevadensin PBBK models are connected to form a binary estragole-nevadensin PBBK model based on the type of interaction between estragole and nevadensin defined in the present research. To extend the PBBK model from predicting levels of 1′-sulfooxyestragole formed to predicting levels of DNA adducts formed, the equation describing DNA adduct formation as a function of PBBK predicted levels of 1′-sulfooxyestragole described previously (Paini *et al.*, 2012) was also integrated in the PBBK models. The resulting binary estragole-nevadensin PBBK models allow simulation of the dose- and species-dependent effect of nevadensin on the formation of estragole DNA adducts in the liver of male rat and human.

Chapter 5 investigates the potential of nevadensin to inhibit the SULT mediated bioactivation and subsequent DNA adduct formation of methyleugenol using human HepG2 cells as an in vitro model. To obtain some insight in the in vivo relevance of these observations the physiologically based kinetic (PBBK) model for bioactivation and detoxification of methyleugenol in male rat (Al-Subeihi *et al.*, 2011) was combined with the recently developed PBBK model for nevadensin in male rat to investigate consequences of this co-exposure on the formation of DNA adducts by methyleugenol in vivo.

Chapter 6 investigates whether the nevadensin-mediated reduction in alkenylbenzene DNA adduct formation is accompanied by a reduction in early markers for carcinogenesis using methyleugenol as the model alkenylbenzene. The early markers of carcinogenesis investigated in this chapter were hepatocellular proliferation, formation of hepatocellular altered foci in addition to DNA adduct formation.

Finally, Chapter 7 presents a discussion of the results obtained in this thesis and their implications for the risk assessment of the alkenylbenzenes.

REFERENCES

1. Al-Subeihi, A. A. A., Spenkelink, B., Punt, A., Boersma, M. G., van Bladeren, P. J., and Rietjens, I. M. C. M. (2012). Physiologically based kinetic modeling of bioactivation and detoxification of the alkenylbenzene methyleugenol in human as compared with rat. *Toxicology and Applied Pharmacology* **260**, 271-284.
2. Al-Subeihi, A. A. A., Spenkelink, B., Rachmawati, N., Boersma, M. G., Punt, A., Vervoort, J., van Bladeren, P. J., and Rietjens, I. M. C. M. (2011). Physiologically based biokinetic model of bioactivation and detoxification of the alkenylbenzene methyleugenol in rat. *Toxicology in Vitro* **25**, 267-285.
3. Andersen, M. E., and Krishnan, K. (1994). Physiologically based pharmacokinetics and cancer risk assessment. *Environmental Health Perspectives* **102**, 103-108.
4. Anthony, A., Caldwell, J., Gutt, A. J., and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1'-hydroxyestragole. *Food and Chemical Toxicology* **25**, 799-806.
5. Barlow, S., Renwick, A. G., Kleiner, J., Bridges, J. W., Busk, L., Dybing, E., Edler, L., Eisenbrand, G., Fink-Gremmels, J., Knaap, A., Kroes, R., Liem, D., Müller, D. J. G., Page, S., Rolland, V., Schlatter, J., Tritscher, A., Tueting, W., and Würtzen, G. (2006). Risk assessment of substances that are both genotoxic and carcinogenic. Report of an International Conference organized by EFSA and WHO with support of ILSI Europe. *Food and Chemical Toxicology* **44**, 1636-1650.
6. Boberg, E. W., Miller, E. C., and Miller, J. A. (1983). Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1'-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1'-hydroxysafrole in mouse liver. *Cancer Research* **43**, 5163-5173.
7. Bogaards, J. J. P., Hissink, E. M., Briggs, M., Weaver, R., Jochemsen, R., Jackson, P., Bertrand, M., and Van Bladeren, P. J. (2000). Prediction of interindividual variation in drug plasma levels in vivo from individual enzyme kinetic data and physiologically based pharmacokinetic modeling. *European Journal of Pharmaceutical Sciences* **12**, 117-124.
8. Brennan, R. J., Kandikonda, S., Khrimian, A. P., DeMilo, A. B., Liquido, N. J., and Schiestl, R. H. (1996). Saturated and monofluoro analogs of the oriental fruit fly attractant methyl eugenol show reduced genotoxic activities in yeast. *Mutation Research - Genetic Toxicology* **369**, 175-181.
9. Chan, V. S. W., and Caldwell, J. (1992). Comparative induction of unscheduled DNA synthesis in cultured rat hepatocytes by allylbenzenes and their 1'-hydroxy metabolites. *Food and Chemical Toxicology* **30**, 831-836.
10. Clewell, H. J., Gentry, P. R., Gearhart, J. M., Allen, B. C., and Andersen, M. E. (2001). Comparison of cancer risk estimates for vinyl chloride using animal and human data with a PBPK model. *Science of the Total Environment* **274**, 37-66.
11. Clewell Iii, H. J., Andersen, M. E., and Barton, H. A. (2002). A consistent approach for the application of pharmacokinetic modeling in cancer and noncancer risk assessment. *Environmental Health Perspectives* **110**, 85-93.
12. Clewell, R. A., and Clewell Iii, H. J. (2008). Development and specification of physiologically based pharmacokinetic models for use in risk assessment. *Regulatory Toxicology and Pharmacology* **50**, 129-143.
13. Dorange, J. L., Delaforge, M., Janiaud, P., and Padieu, P. (1977). Mutagenicity of the metabolites of the epoxide diol pathway of safrole and analogues. Study on Salmonella typhimurium. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales* **171**, 1041-1048.
14. Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-Allyl-4-methoxybenzene) and 1'-Hydroxyestragole in the mouse and mutagenicity of 1'- acetoxyestragole in bacteria. *Journal of the National Cancer Institute* **57**, 1323-1331.
15. Dybing, E., O'Brien, J., Renwick, A. G., and Sanner, T. (2008). Risk assessment of dietary exposures to compounds that are genotoxic and carcinogenic-An overview. *Toxicology Letters* **180**, 110-117.

16. EFSA (2005). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. *EFSA Journal* **282**, 1-31.
17. European Commission (EC) (2008). Regulation (EC) No 1334/2008 of the Europian Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC.
18. Fennell, T. R., Wiseman, R. W., Miller, J. A., and Miller, E. C. (1985). Major role of hepatic sulfotransferase activity in the metabolic activation, DNA adduct formation, and carcinogenicity of 1'-hydroxy-2',3'-dehydroestragole in infant male C57BL/6J x C3H/HeJ F1 mice. *Cancer Research* **45**, 5310-5320.
19. Gavin, C., Williams, M. C., and Hallagan, J. B. (2008). Poundage and Technical Effects Update Survey, 2005. Washington, D.C.: The Flavor and Extract Manufacturers Association of the United States, 2008.
20. Guenthner, T. M., and Luo, G. (2001). Investigation of the role of the 2',3'-epoxidation pathway in the bioactivation and genotoxicity of dietary allylbenzene analogs. *Toxicology* **160**, 47-58.
21. Herrmann, K., Engst, W., Appel, K. E., Monien, B. H., and Glatt, H. (2012). Identification of human and murine sulfotransferases able to activate hydroxylated metabolites of methyleugenol to mutagens in Salmonella typhimurium and detection of associated DNA adducts using UPLC-MS/MS methods. *Mutagenesis* **27**, 453-462.
22. JECFA (2008). Sixty-ninth Meeting, Rome, Italy, 17–26 June 2008. (JECFA, Ed.), (http://www.who.int/entity/ipcs/food/jecfa/summaries/summary69.pdf).
23. Jeurissen, S. M. F., Punt, A., Delatour, T., and Rietjens, I. M. C. M. (2008). Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1 '-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. *Food and Chemical Toxicology* **46**, 2296-2302.
24. Johnson, J. D., Ryan, M. J., Toft Ii, J. D., Graves, S. W., Hejtmancik, M. R., Cunningham, M. L., Herbert, R., and Abdo, K. M. (2000). Two-year toxicity and carcinogenicity study of methyleugenol in F344/N rats and B6C3F1 mice. *Journal of Agricultural and Food Chemistry* **48**, 3620-3632.
25. Krewski, D., Withey, J. R., Ku -f, L., and Andersen, M. E. (1994). Applications of physiologic pharmacokinetic modeling in carcinogenic risk assessment. *Environmental Health Perspectives* **102**, 37-50.
26. Krishnan, K., Haddad, S., Béliveau, M., and Tardif, R. (2002). Physiological modeling and extrapolation of pharmacokinetic interactions from binary to more complex chemical mixtures. *Environmental Health Perspectives* **110**, 989-994.
27. Lipscomb, J. C., and Poet, T. S. (2008). In vitro measurements of metabolism for application in pharmacokinetic modeling. *Pharmacology and Therapeutics* **118**, 82-103.
28. Luo, G., and Guenthner, T. M. (1995). Metabolism of allylbenzene 2',3'-oxide and estragole 2'3'oxide in the isolated perfused rat liver. *Journal of Pharmacology and Experimental Therapeutics* **272**, 588-596.
29. Luo, G., Qato, M. K., and Guenthner, T. M. (1992). Hydrolysis of the 2',3'-allylic epoxides of allylbenzene, estragole, eugenol, and safrole by both microsomal and cytosolic epoxide hydrolases. *Drug Metabolism and Disposition* **20**, 440-445.
30. Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Research* **43**, 1124-1134.
31. Mortelmans, K., Haworth, S., Lawlor, T., Speck, W., Tainer, B., and Zeiger, E. (1986). Salmonella mutagenicity tests: II. Results from the testing of 270 chemicals. *Environmental Mutagenesis* **8**, 1-119.
32. Muller, L., Kasper, P., Muller-Tegethoff, K., and Petr, T. (1994). The genotoxic potential in vitro and in vivo of the allyl

benzene etheric oils estragole, basil oil and trans-anethole. *Mutation Research - Mutation Research Letters* **325**, 129-136.

33. NTP (2000). National Toxicology Program on toxicology and carcinogenesis studies of Methyleugenol (CAS NO. 93-15-12) in F344/N rats and B6C3F1 mice (Gavage Studies). DRAFT NTP-TR-491; NIH Publication No. 98-3950., 1-412.
34. NTP (2008). National Toxicology Program on toxicology and carcinogenesis studies of estragole in F344/N rats and B6C3F1 mice. U.S. Dept Of health Human services. NIH Publication TOX-82.
35. Paini, A., Scholz, G., Boersma, M. G., Spenkelink, A., Schilter, B., van Bladeren, P. J., Rietjens, I. M. C. M., and Punt, A. (2012). Evaluation of interindividual human variation in bioactivation and DNA binding of estragole in liver predicted by physiologically based biodynamic (PBBD) and Monte Carlo modeling. In *Generation of in vitro data to model dose dependent in vivo DNA binding of genotoxic carcinogens and its consequences: the case of estragole.*, pp. 70-85.
36. Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. *Cancer Research* **41**, 176-186.
37. Phillips, D. H., Reddy, M. V., and Randerath, K. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. *Carcinogenesis* **5**, 1623-1628.
38. Punt, A., Freidig, A. P., Delatour, T., Scholz, G., Boersma, M. G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2008). A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat. *Toxicology and Applied Pharmacology* **231**, 248-259.
39. Punt, A., Paini, A., Boersma, M. G., Freidig, A. P., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2009). Use of physiologically based biokinetic (PBBK) modeling to study estragole bioactivation and detoxification in humans as compared with male rats. *Toxicological Sciences* **110**, 255-269.
40. Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis* **5**, 1613-1622.
41. Rietjens, I. M. C. M., Punt, A., Schilter, B., Scholz, G., Delatour, T., and van Bladeren, P. J. (2010). In silico methods for physiologically based biokinetic models describing bioactivation and detoxification of coumarin and estragole: Implications for risk assessment. *Molecular Nutrition and Food Research* **54**, 195-207.
42. Sangster, S. A., Caldwell, J., and Hutt, A. J. (1987). The metabolic disposition of [methoxy-14C]-labelled trans-anethole, estragole and p-propylanisole in human volunteers. *Xenobiotica* **17**, 1223-1232.
43. SCF (2001a). Opinion of the Scientific Committee on Food on Estragole (1-allyl-4-methoxybenzene). European Commission, Health and Consumer Protection Directorate. General, Report Series 10, Directorate C, Scientific Opinions, Brussels, Belgium. Obtained January 10, 2008, at http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.
44. SCF (2001b). Opinion of the Scientific Committee on Food on methyleugenol (4-allyl-1,2-dimethoxybenzene.
45. Schecter, A., Lucier, G. W., Cunningham, M. L., Abdo, K. M., Blumenthal, G., Silver, A. G., Melnick, R., Portier, C., Barr, D. B., Barr, J. R., Stanfill, S. B., Patterson Jr, D. G., Needham, L. L., Stopford, W., Masten, S., Mignogna, J., and Tung, K. C. (2004). Human consumption of methyleugenol and its elimination from serum. *Environmental Health Perspectives* **112**, 678-680.
46. Schiestl, R. H., Chan, W. S., Gietz, R. D., Mehta, R. D., and Hastings, P. J. (1989). Safrole, eugenol and methyleugenol induce intrachromosomal recombination in yeast. *Mutation Research* **224**, 427-436.
47. Sekizawa, J., and Shibamoto, T. (1982). Genotoxicity of safrole-related chemicals in microbial test systems. *Mutation Research* **101**, 127-140.
48. Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers,

A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - Methyl eugenol and estragole. *Food and Chemical Toxicology* **40**, 851-870.

49. Solheim, E., and Scheline, R. R. (1973). Metabolism of alkenebenzene derivatives in the rat. I. p Methoxyallylbenzene (estragole) and p methoxypropenylbenzene (anethole). *Xenobiotica* **3**, 493-510.
50. Solheim, E., and Scheline, R. R. (1976). Metabolism of alkenebenzene derivatives in the rat. II. Eugenol and isoeugenol methyl ethers. *Xenobiotica* **6**, 137-150.
51. Sutton, J. D., Sangster, S. A., and Caldwell, J. (1985). Dose-dependent variation is the disposition of eugenol in the rat. *Biochemical Pharmacology* **34**, 465-466.
52. Swanson, A. B., Chambliss, D. D., Blomquist, J. C., Miller, E. C., and Miller, J. A. (1979). The mutagenicities of safrole, estragole, eugenol, trans-anethole, and some of their known or possible metabolites for Salmonella typhimurium mutants. *Mutation Research* **60**, 143-153.
53. To, L. P., Hunt, T. P., and Andersen, M. E. (1982). Mutagenicity of trans-anethole, estragole, eugenol, and safrole in the Ames Salmonella typhimurium assay. *Bulletin of Environmental Contamination and Toxicology* **28**, 647-654.
54. US EPA (2005). Guidelines for Carcinogen Risk Assessment and Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens. (Chapter 3.1.3. Cross-species Scaling Procedures).
55. US FDA (2005). Department of Health and Human Services, and Center for Drug Evaluation and Research (CDER) (2005). Guidance for industry. Estimating the maximum safe starting dose in intitial clinical trials for therapeutics in adult healthy volunteers.
56. Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F1 mice. *Cancer Research* **47**, 2275-2283.
57. Zangouras, A., Caldwell, J., Hutt, A. J., and Smith, R. L. (1981). Dose dependent conversion of estragole in the rat and mouse to the carcinogenic metabolite, 1'-hydroxyestragole. *Biochemical Pharmacology* **30**, 1383-1386.
58. Zani, F., Massimo, G., Benvenuti, S., Bianchi, A., Albasini, A., Melegari, M., Vampa, G., Bellotti, A., and Mazza, P. (1991). Studies on the genotoxic properties of essential oils with Bacillus subtilis rec-assay and Salmonella/microsome reversion assay. *Planta Medica* **57**, 237-241.
59. Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., Mortelmans, K., and Speck, W. (1987). Salmonella mutagenicity tests: III. Results from the testing of 255 chemicals. *Environmental Mutagenesis* **9**, 1-110.
60. Zeller, A., Horst, K., and Rychlik, M. (2009). Study of the metabolism of estragole in humans consuming fennel tea. *Chemical Research in Toxicology* **22**, 1929-1937.

2

Identification of nevadensin as an important herb-based constituent inhibiting estragole bioactivation and physiologically based biokinetic modeling of its possible in vivo effect

Based on:
Alhusainy W, Paini A, Punt A, Louisse J, Spenkelink A, Vervoort J, Delatour T, Scholz G, Schilter B, Adams TB, van Bladeren PJ and Rietjens IMCM

Toxicology and Applied Pharmacology 245 (2010), 179-190

ABSTRACT

Estragole is a natural constituent of several herbs and spices including sweet basil. In rodent bioassays, estragole induces hepatomas, an effect ascribed to estragole bioactivation to 1′-sulfooxyestragole resulting in DNA adduct formation. The present paper identifies nevadensin as a basil constituent able to inhibit DNA adduct formation in rat hepatocytes exposed to the proximate carcinogen 1′-hydroxyestragole and nevadensin. This inhibition occurs at the level of sulfotransferase (SULT)-mediated bioactivation of 1′-hydroxyestragole. The *K*i for SULT inhibition by nevadensin was 4 nM in male rat and human liver fractions. Furthermore, nevadensin up to 20 μM did not inhibit 1′-hydroxyestragole detoxification by glucuronidation and oxidation. The inhibition of SULT by nevadensin was incorporated into the recently developed physiologically based biokinetic (PBBK) rat and human models for estragole bioactivation and detoxification. The results predict that co-administration of estragole at a level inducing hepatic tumours in vivo (50 mg/kg bw) with nevadensin at a molar ratio of 0.06, representing the ratio of their occurrence in basil, results in almost 100% inhibition of the ultimate carcinogen 1′-sulfooxyestragole when assuming 100% uptake of nevadensin. Assuming 1% uptake, inhibition would still amount to more than 83%. Altogether these data point at a nevadensin-mediated inhibition of the formation of the ultimate carcinogenic metabolite of estragole, without reducing the capacity to detoxify 1′-hydroxyestragole via glucuronidation or oxidation. These data also point at a potential reduction of the cancer risk when estragole exposure occurs within a food matrix containing SULT inhibitors compared to what is observed upon exposure to pure estragole.

INTRODUCTION

Ocimum basilicum L., or sweet basil is an important botanical in the production of essential oils, medicinal products and for providing the culinary market with pot, dry and fresh herbs (Lawrence, 1992). It contains aromatic compounds that belong to the group of alkenylbenzenes which make up the larger part of its essential oil (De Vincenzi *et al.*, 2000). According to the Scientific Committee on Food (SCF), several of these alkenylbenzenes, including estragole (Figure 1) which makes up 20–88% of basil essential oil, have been shown to be genotoxic and carcinogenic in rodent studies and therefore, restrictions in their use were indicated (SCF, 2001a, b, c). The European Medicines Agency (EMEA) released a public statement on the use of herbal medicinal products containing estragole concluding that estragole is a naturally occurring genotoxic carcinogen, but that at the low levels of exposure resulting from consumption of herbal medicinal products, it does not pose a significant cancer risk (EMEA, 2005). This conclusion is consistent with that of Smith *et al.* (2002) who published a safety assessment on alkenylbenzenes derivatives, including estragole, used as flavouring substances.

The European Food Safety Authority (EFSA) suggested the so-called margin of exposure (MOE) to be used to set priorities in risk management with respect to compounds that are both genotoxic and carcinogenic (EFSA, 2005). This MOE is defined as the ratio between the lower confidence limit of the benchmark dose that gives 10% extra cancer incidence ($BMDL_{10}$) and the estimated daily intake (EDI). Based on the EDI of estragole from different food sources (EDI=0.07 mg/kg bw/day) (SCF, 2001a), and the $BMDL_{10}$ which can be derived from available tumour data and ranges from 9 to 33 mg/kg bw/day, the MOE for estragole amounts to 129–471 (Rietjens *et al.*, 2010). According to EFSA a MOE lower than 10,000 as in the case of estragole can be considered a priority for risk management (EFSA, 2005). This is also illustrated by the fact that the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has put estragole (as well as some other alkenylbenzenes) on the list of compounds to be re-evaluated (JECFA, 2008).

Figure 1 presents an overview of the bioactivation and detoxification pathways of estragole (Punt *et al.*, 2009 as adapted from Smith *et al.* (2002)). Bioactivation of estragole proceeds by initial metabolic hydroxylation by cytochrome P450 enzymes leading to the formation of the proximate carcinogen 1′-hydroxyestragole which has been detected in the β-glucuronidase treated urine of men dosed with 1 µg estragole/kg bw (Sangster *et al.*, 1987).

Further bioactivation of 1′-hydroxyestragole requires the involvement of sulfotransferases (SULTs) which convert 1′-hydroxyestragole to the ultimate carcinogen 1′-sulfooxyestragole. This sulfonated metabolite is unstable and degrades in aqueous environment to a reactive carbocation capable of binding covalently to different endogenous nucleophiles including glutathione, protein, RNA and DNA (Phillips *et al.*, 1981; Phillips *et al.*, 1984; Randerath *et al.*, 1984). The role of the 1′-sulfooxy metabolites of alkenylbenzenes in the formation of hepatic macromolecular adducts and tumour formation has been also demonstrated in vivo (Boberg *et al.*, 1983).

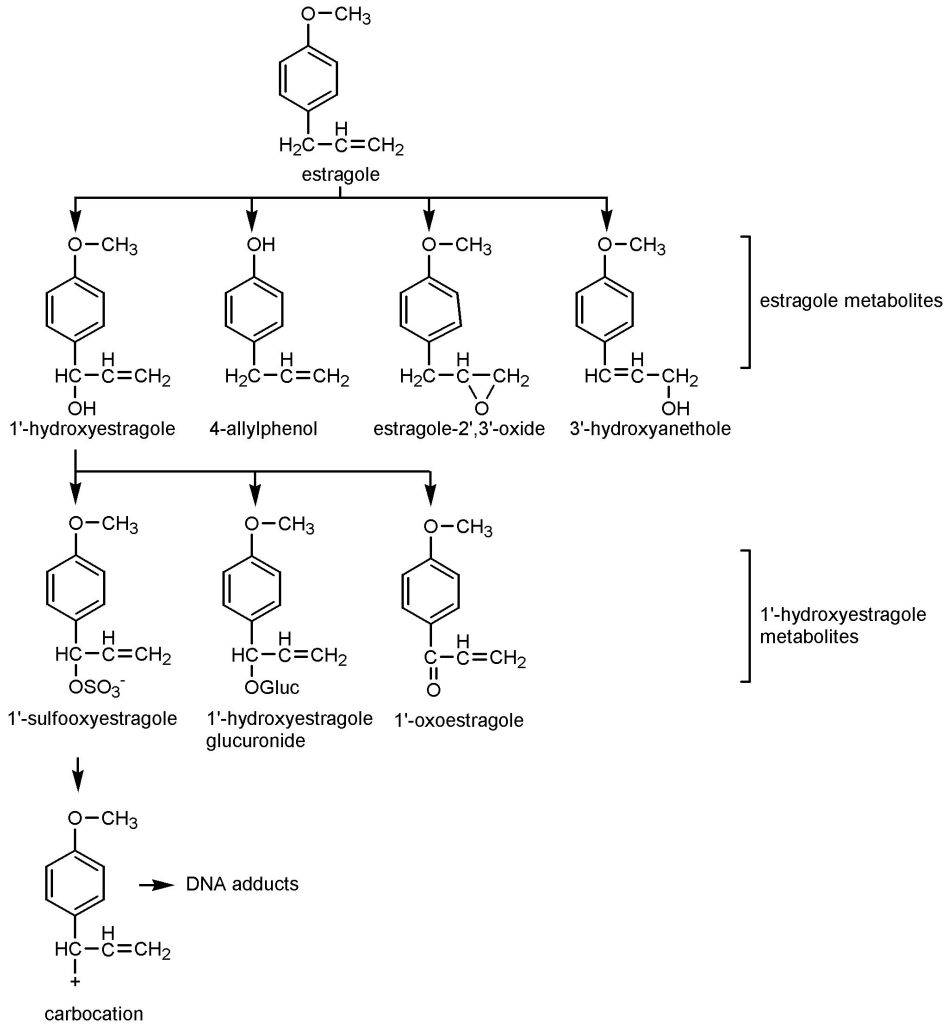


Figure 1. Bioactivation and detoxification pathways of estragole.

Estragole has been shown to induce hepatomas in rodent bioassays when dosed as a pure compound at high dose levels (Miller *et al.*, 1983). Therefore, an issue that remains to be solved is whether these animal studies using high levels of pure compounds without the normal food matrix being present, represent a good starting point for the risk assessment of these botanical constituents. Phillips *et al.* (1981) reported observing two major and two minor liver DNA adducts in adult female CD-1 mice and preweanling B6C3F1 mice given an intraperitoneal injection of 1′-hydroxyestragole. These four adducts were (I) N^2-(*trans*-isoestragol- 3′-yl)deoxyguanosine, (II) N^6-(*trans*-isoestragol-3′-yl)deoxyadenosine, (III) N^2-(estragole-1′-yl)deoxyguanosine and (IV) N^2-

(*cis*-isoestragol-3′-yl)deoxyguanosine. Later work (Fennell *et al.*, 1985) investigated in vivo adduct formation in 12-day old mice dosed with 1′-hydroxyestragole and confirmed formation of three of these adducts. However, two additional adducts of guanine at positions C-8 (V) and N-7 (VI) were observed and characterized. In our subsequent work on DNA adduct formation, focus was on formation of the major DNA adduct N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine (E-3′-N^2-dGuo).

Recently, we demonstrated that formation of E-3′-N^2-dGuo in incubations with liver S9 fractions in the presence of the proximate carcinogen 1′-hydroxyestragole and the cofactor for SULT-mediated conversion 3′-phosphoadenosine-5′-phosphosulfate (PAPS), and 2′- deoxyguanosine could be inhibited by a methanolic basil extract (Jeurissen *et al.*, 2008). The inhibition of the major estragole DNA adduct (E-3′-N^2-dGuo) formation by the basil extract was also found in intact HepG2 human hepatoma cells exposed to 1′-hydroxyestragole (Jeurissen *et al.*, 2008). Because similar inhibition took place when using the well-known competitive-SULT inhibitor pentachlorophenol (PCP) and because the inhibition was not observed in incubations with the direct electrophile 1′-acetoxyestragole, it was concluded that the inhibition by the basil extract occurs at the level of the SULT-mediated bioactivation of 1′-hydroxyestragole to 1′-sulfooxyestragole (Jeurissen *et al.*, 2008). Although it remains to be established whether the SULT-mediated inhibition by the methanolic basil extract demonstrated in vitro would also occur in vivo, the results suggest that the likelihood of bioactivation and subsequent adverse effects may be lower when estragole is consumed in a matrix of other basil components than what would be expected on the basis of experiments using estragole as a single compound (Jeurissen *et al.*, 2008).

The aims of the present study were i) to identify the active compound(s) present in the basil extract that are responsible for the inhibition of DNA binding of 1′-hydroxyestragole by inhibiting the SULT-mediated bioactivation of 1′-hydroxyestragole to 1′-sulfooxyestragole and ii) to predict the possible effects of combined exposure by incorporating the kinetics for SULT inhibition into our recently developed physiologically based biokinetic (PBBK) models for formation of 1′-sulfooxyestragole in the liver of rat (Punt *et al.*, 2008) and human (Punt *et al.*, 2009). The possible effects of the identified inhibitor(s) on the detoxification of 1′-hydroxyestragole by glucuronidation and oxidation (Figure 1) were quantified in vitro as well. This is important because only when the SULT-mediated bioactivation of 1′-hydroxyestragole is inhibited without affecting the detoxification pathways of 1′-hydroxyestragole, the SULT inhibition can be expected to shift metabolism in favour of detoxification at the cost of bioactivation.

MATERIALS AND METHODS

Materials and chemicals

1′-Hydroxyestragole and 1′-acetoxyestragole are carcinogenic compounds and caution should be taken when handling them.

Dried basil (Silvo; Papendrecht, The Netherlands) was purchased from a local supermarket, 7-hydroxycoumarin (7HC; 99%), 7-hydroxycoumarin sulfate (7HCS; 95%), pentachlorophenol (PCP; 98%), chromatography grade acetonitrile and methanol were obtained from Sigma-Aldrich (Steinheim, Germany). Tris-hydroxymethylaminomethane (tris) was from Gibco BRL Life Technologies (Paisley, Scotland), dimethylsulfoxide (DMSO; spectroscopic grade, 99.9%) and pro analysis grade acetic acid were purchased from VWR Merck (Darmstadt, Germany).

Nevadensin was purchased from Apin Chemicals (Milton, UK). Pooled male rat liver S9 and microsomes (Sprague–Dawley) and pooled human liver S9 and microsomes were purchased from BD Gentest (Woburn, MA). PAPS was purchased from Fluka (Buchs, Switzerland). ENZYLINE™ LDH Optimise 10 kit lactate dehydrogenase (LDH) was purchased from Bio Merieux (Lyon, France). William's E medium, L-glutamine, hepes pH 7.4, ITS+, 15,000 U penicillin/streptomycin, dexamethasone, 5% fetal bovine serum (Hi-clone), and Earle's balanced salt solution (EBSS) were purchased from Sigma (Basel, Switzerland). Matrigel was obtained from Biomedical Products (Bedford, MA). 1′-Hydroxyestragole was synthesized as described previously (Drinkwater *et al.*, 1976) based on a method described by (Borchert *et al.* (1973) for the synthesis of 1′-hydroxysafrole. Structural confirmation by means of GC–MS was carried out as described in our previous work (Punt *et al.*, 2007).

Preparation of methanolic basil extract

A methanolic basil extract was prepared according to the method described previously (Jeurissen *et al.*, 2008) with minor modifications. In short, basil extract was prepared by stirring 5 g of dried basil twice for 2 h at room temperature, each time with 100 mL of a mixture of methanol, ultra-pure water, and acetic acid (ratio 80:19:1). The extracts obtained were filtered using a folded filter (Schleicher & Schuell) and the pooled filtrates were evaporated to dryness under vacuum using a Rotavapor apparatus (Heidolph LABOROTA 4000 efficient, Metrohm USA). The extraction yield was about 10%–20% (*w/w*) (Jeurissen *et al.*, 2008). Thereafter, three concentrated extract stocks in methanol were prepared (2, 5 and 20 mg/mL). For fractionation, a concentrated stock of 150 mg/mL dissolved in a mixture of ultra-pure water and methanol (ratio 1:1) was prepared and all stocks were stored at −20°C until use. All stocks were filtered using a 0.45 µM FP filter (Schleicher & Schuell, Dassel, Germany).

Measurement of SULT activity

To detect inhibition of SULT activity by basil extract or by fractions of basil extract, 7HC was used as a substrate instead of 1′-hydroxyestragole because the metabolite resulting from the sulfonation of 7HC is stable in aqueous solution and can be detected

and quantified by HPLC–UV (Otake *et al.*, 2002; Wang *et al.*, 2005) whereas the highly reactive sulfonated metabolite of 1′-hydroxyestragole degrades in aqueous environment upon formation (Drinkwater *et al.*, 1976; Boberg *et al.*, 1983; Wiseman *et al.*, 1987).

Pooled male rat liver S9 fractions at a final concentration of 0.4 mg protein/mL were incubated with 25 μM 7HC added from a 200 times concentrated stock solution in DMSO and 0.1 mM PAPS in a total volume of 100 μL with 0.1 M Tris–HCl buffer (pH 7.4) for 10 min at 37°C in the absence or presence of increasing concentrations of methanolic basil extracts (10, 25, and 100 μg/mL, added from 200 times concentrated stock solutions in methanol). The reactions were terminated by adding 25 μL of ice-cold acetonitrile, and the samples were centrifuged at 16,000 g for 5 min (Eppendorf centrifuge, type 5415C, Hamburg, Germany). The supernatants were subjected to HPLC–UV analysis to quantify the amount of the sulfonated metabolite, 7HCS, formed. Control incubations without basil extract or in the presence of 25 μM of the known SULT inhibitor, PCP, (added from a 200 times concentrated stock solution in DMSO) were performed.

HPLC–UV analysis and quantification of 7HCS

The supernatants obtained from the SULT activity incubations were analyzed by HPLC–UV on a M600 Waters liquid chromatography system connected to an Alltima C18 5 μm column, 150 mm×4.6 mm (Grace Alltech, Breda, The Netherlands). The gradient was made with ultra-pure water containing 0.1% (*v*/*v*) acetic acid and acetonitrile and the flow rate was set to 1 mL/min. The acetonitrile percentage was increased from 0% to 20% in 2 min, increased to 21% in 10 min, increased to 100% in 2 min, and kept at 100% for 1 min. Initial conditions were retained in 2 min, and then the system was equilibrated at these conditions for 10 min. Detection was carried out using a 2996 photodiode array detector (Waters, Milford MA) at 280 nm for the quantification of 7HCS. For quantification based on peak areas at 280 nm, a calibration curve of 7HCS was made using a commercially available standard.

Fractionation of methanolic basil extract

After confirming the SULT inhibitory capacity of the methanolic basil extract, the extract was fractionated into 60 fractions upon injecting 50 μL of a 150 mg/mL basil extract dissolved in methanol/water 1:1 and filtrated through a 0.45 μMFP filter onto an Alltima C18 5 μM column, 150 mm×4.6 mm (Grace Alltech, Breda, The Netherlands). The gradient was made with ultra-pure water containing 0.1% (*v*/*v*) acetic acid and methanol and the flow rate was set to 1 mL/min. A linear gradient was then applied as follows: the methanol percentage was increased from 0% to 100% in 60 min, and kept at 100% for 10 min. Initial conditions were retained in 10 min, and the system was equilibrated at these conditions for 10 min. Every 1 min a single fraction was collected in an Eppendorf tube. Subsequently, methanol was evaporated under a nitrogen atmosphere and fractions were frozen at −80°C to allow freeze drying using a freeze dryer (Christ Alpha RVC, Leerdam, the Netherlands) under the following conditions: 10–20 bar for 3 h. Methanol evaporation and freeze dying had no effect on the SULT inhibitory capacity of

the fractions (data not shown). To have more concentrated fractions, the fractionation was performed five times and the replicates were pooled together in 50 µL methanol following freeze drying. Thereafter, each fraction was examined for its inhibitory potential on SULT activity using the SULT activity assay described above.

LC–ESI–MS/MS analysis

The fraction with the highest inhibitory effect on SULT activity was analyzed on a Finnigan Surveyor HPLC system coupled to an LXQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) fitted with an ESI source. Aliquots of 20 µL (injected volume) were separated on a Gemini C18 5 µm column, 150 mm×4.6 mm (Phenomenex, Torrance, CA). The gradient was made with acetonitrile and ultra-pure water containing 0.1% (*v*/*v*) formic acid and the flow rate was set to 0.1 mL/min. A linear gradient was applied from 0% to 100% acetonitrile over 30 min and was kept at 100% acetonitrile for 2 min. Mass spectrometric analysis was performed in positive ion mode using a spray voltage of 5 kV, a capillary temperature of 275°C, and nitrogen as sheath gas (8 arbitrary units). Initially, full scan data (m/z 125–2000) were obtained to identify the main protonated molecular ion $[M+H]^+$ present in the sample. Subsequently, a collision induced dissociation spectrum was derived at a normalized collision energy of 30% to aid additional structural characterization.

^{1}H–NMR analysis

^{1}H–NMR was performed using a Bruker Avance III 600 MHz (Ettlingen, Germany) with cryoprobe. A Noesygppr1d pulse sequence with 3 s delay, 0.1 s mixing time and a 1.8 s acquisition time were used (18,028 Hz sweep width, 64 K data points). Spectra were obtained at 25°C. Resonances were reported relative to methanol-*d*4 at 3.34 ppm.

Cytotoxicity assay

Cytotoxicity of 1′-hydroxyestragole and nevadensin towards isolated rat hepatocytes was determined by measuring the amount of lactate dehydrogenase (LDH) leakage into the medium after 4 h incubation with the tested concentrations. The concentration of 1′-hydroxyestragole of 50 µM was selected because it represents a concentration at which no cytotoxicity was reported in the HepG2 hepatoma cell line but at which the quantification of the resulting DNA adducts was feasible (Jeurissen *et al.*, 2008). LDH activity was measured by analyzing the appearance of lactate at 340 nm using a spectrophotometer Cobas Mira Plus analyzer (Minnesota, USA). An ENZYLINE™ LDH Optimise 10 kit (BioMerieux® SA, Lyon, France) was used which provides the substrate pyruvate and the cofactor nicotinamide adenine dinucleotide (reduced NADH).

Cultures of primary rat hepatocytes

Primary rat hepatocytes were obtained by perfusion of the liver of male Sprague–Dawley rats (220–250 g) with a collagenase solution as previously described (Sidhu *et al.*, 1993; Cavin *et al.*, 2001). Cell viability, estimated by trypan blue exclusion, was found to range from 90% to 95%. The cells were seeded at a density of 3.5×10^6

cells/cm^2 per 60 mm plastic tissue culture dishes in 2.5 mL of William's E medium supplemented with 2 mM L-glutamine, 10 mM hepes pH 7.4, 1% ITS+, 15,000 U penicillin/streptomycin, 100 nM dexamethasone and 5% fetal bovine serum (Hi-clone). Hepatocytes were allowed to attach for 2 h and then washed with Earle's balanced salt solution (EBSS) to remove debris and unattached cells. Fresh serum-free medium containing 25 nM of dexamethasone was added and an overlay of matrigel (233 µg/mL) was then applied. 1′-Hydroxyestragole and nevadensin were added to the culture media 24 h after cell seeding at the required concentrations (as described below).

Treatment of primary rat hepatocytes

Primary rat hepatocytes were treated with 50 µM 1′-hydroxyestragole (added from a 1000 times concentrated stock solution in DMSO) in William's E exposure medium supplemented with 2 mM L-glutamine, 10 mM hepes pH 7.4, ITS+, 15,000 U penicillin/streptomycin, 25 nM dexamethasone. In addition, increasing concentrations of the identified SULT inhibitor (0 (control), 0.02, 0.2, and 4 µM, added from 200 times concentrated stock solutions in DMSO) were added. The cells were exposed for 4 h at 37°C in a humidified atmosphere containing 5% CO_2 and 95% normal air. After exposure, cells were scraped in EBSS, and cells from three dishes were pooled in an Eppendorf vial and centrifuged at 6,000 rpm for 5 min (Eppendorf centrifuge, type 5415C, Hamburg, Germany) to obtain pellets. Thereafter, pellets were used for DNA extraction and digestion.

Extraction and digestion of E-3′-*N*^{2}-dGuo formed in primary rat hepatocytes

DNA was isolated from rat hepatocyte pellets using the Get pure DNA Kit-Cell, Tissue kit (Dojindo Molecular Technology Inc., Kumamoto, Japan), according to the accompanying protocol. DNA pellets were dissolved in 100 µL ultra-pure water and stored at 4°C until DNA digestion. To this end, 12 µL of phosphodiesterase II solution (0.0004 U/µL), 10 µL of nuclease P1 solution (0.5 µg/µL in water), and 20 µL buffer (300 mM sodium acetate, 1 mM zinc sulfate, pH 5.3) were added to the DNA solution. The samples were incubated for 4 h at 37°C. Thereafter, 6 µL of a 1 U/µL alkaline phosphatase solution (1.3 U), 13 µL of phosphodiesterase I solution (0.00026 U/µL in water), and 20 µL buffer (500 mM Tris, 1 mM EDTA, pH 8) were added (Delatour *et al.*, 2008). The samples were incubated for another 2 h at 37°C. Then, samples were filtered using Eppendorf tubes with a cut off membrane of 5,000 NMWL (Millipore). The hydrolyzed samples were evaporated to dryness using a speed vacuum Concentrator 5301 (Westbury, NY, USA) and reconstituted in 50 µL water.

Quantification of E-3′-*N*^{2}-dGuo by isotope dilution LC–ESI–MS/MS

Quantification was done using a sensitive method by isotope dilution, the LC–ESI–MS/MS method was adapted from our previous study (Punt *et al.*, 2007). Detailed explanation of the LC–ESI–MS/MS instrument method, sample preparation, and synthesis of the estragole nucleoside ($^{15}N_5$) E-3′-*N*^{2}-dGuo and E-3′-*N*^{2}-dGuo are described previously

(Paini *et al.*, 2010). Briefly, LC–ESI–MS/MS analysis was performed on an Agilent 1100 series liquid chromatography system (Waldbronn, Germany) coupled to a TSQ Quantum triple quadruple mass spectrometer (Thermo Scientific, Sweden). 50 µL of the digested sample were mixed with 10 µL of the internal standard ($^{15}N_5$) E-3′-N^2-dGuo (0.012 nmol). Aliquots of 10 µL were injected onto an Agilent Zorbax Extend C18 column, 2.1×50 mm, 3.5 Micron 80 Å (Basel, Switzerland), with a Zorbax guard column. Sample analysis was carried out by the selected reaction monitoring (SRM). Mass spectrometric analysis was done in a positive ion mode. The ion at m/z 414 corresponds to the protonated molecule and the major product ion at m/z=298 is formed by the loss of the 2′-deoxyribose moiety (MW=116 Da); corresponding to the adducted base guanine (MW=298 Da). The internal standard ($^{15}N_5$) E-3′-N^2- dGuo and the E-3′-N^2-dGuo were synthesized from a reaction between 1′-acetoxyestragole and 2′-deoxyguanosine following the protocol previously described (Phillips *et al.*, 1981). The adduct E-3′-N^2-dGuo detected in the samples was adjusted to the total DNA amount detected in the sample and corrected for the MW of dGuo 347.2 g/mol in order to quantify the number of adducts formed per 1000 dGuo.

Determination of IC_{50}

To determine the The half maximal inhibitory concentration (IC_{50}) of the SULT inhibition by the identified SULT inhibitor of basil extract, increasing concentrations (0–4 µM) of the commercially available identified SULT inhibitor were incubated with pooled rat or human liver S9 fractions in the presence of 25 µM 7HC. The incubation conditions were as described above for the SULT activity assay, except for the final volume of the mixture which was increased to 200 µL. Moreover, the inhibitor was dissolved in DMSO instead of methanol to avoid evaporation and maintaining the initial concentrations. The inhibitor was added from 200 times concentrated stocks and the control incubations were performed without the SULT inhibitor but in the presence of 0.5% DMSO. The IC_{50} of the DNA adduct inhibition by the identified SULT inhibitor was calculated from the data obtained in primary rat hepatocytes using 1′-hydroxyestragole as a substrate (as described above) Thereafter, the IC_{50} was derived as described in the data analysis section.

Determination of the inhibition constant (*K*i)

The *K*i for SULT inhibition by the identified SULT inhibitor was determined by performing incubations for SULT activity by pooled rat and human liver S9 fractions, performed as described above but in the presence of increasing concentrations of 7HC (0–25 µM) in the absence or presence of 2 nM of the identified SULT inhibitor (a concentration that is close to the identified IC_{50} in the two S9 fractions) (see Results section). The apparent maximum velocity (V_{max}(app)), apparent Michaelis–Menten constant (K_m(app)) and the inhibition constant (*K*i) for the SULT activity were determined by fitting the obtained data from triplicate independent measurements to the standard Michaelis–Menten equations as described below (see Data analysis section).

Effect of the identified inhibitor on 1′-hydroxyestragole glucuronidation and oxidation

The effect of the identified SULT inhibitor on 1′-hydroxyestragole oxidation was examined with pooled human and male rat liver microsomal fractions whereas the effect on 1′-hydroxyestragole glucuronidation was examined with pooled human liver microsomal fractions and male rat S9 fractions. The incubations were performed as described by our group previously (Punt *et al.*, 2008; Punt *et al.*, 2009) in the absence or presence of 20 μM of the identified SULT inhibitor added from a 200 times concentrated stock solution in DMSO. The choice of the liver fractions was according to our previous studies (Punt *et al.*, 2008; Punt *et al.*, 2009) where the kinetic parameters for these reactions were determined.

Data analysis

The apparent maximum velocity (V_{max}(app)) and apparent Michaelis–Menten constant (K_m(app)) for the formation of phase II metabolites of estragole were determined by fitting the data to the standard Michaelis–Menten equation $v=V_{max}/(1+(K_m/[S]))$,with [S] being the substrate concentration, using the Life Science Workbench (LSW) data analysis toolbox (version 1.1.1,MDL information Systems, Inc.). The IC_{50} was obtained by fitting the data to the standard hyperbolic equation $Y=V_{max}\ (1-(X/(IC_{50}+X)))+Y2$, with Y representing the percentage of the inhibited 7HCS compared to control, and Y2 representing the lowest value of Y. Analysis was performed using the LSW data analysis toolbox. The inhibition constant (*K*i) was calculated by fitting the V_{max} for the inhibited as well as the uninhibited sulfonation to the Michaelis–Menten equation for non-competitive inhibition ($V'_{max}=V_{max}/(1+([I]/Ki))$), with V'_{max} being the inhibited V_{max} and [I] being the inhibitor concentration (μM). To test whether the change in the V_{max} and K_m in the presence of nevadensin was significant, a two sample t-test (one sided) was performed after determining variances equality by the Levene's version of the F test with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Estragole PBBK model

The rat and human PBBK models in our previous work to describe the dose-dependent bioactivation and detoxification of estragole in male rat liver (Punt *et al.*, 2008) and human liver (Punt *et al.*, 2009) were used in the present study to model the possible consequences of SULT inhibition for estragole bioactivation and detoxification. The models include separate compartments for liver, lung and kidney, which were found to be involved in the metabolism of estragole. Furthermore, a separate compartment for fat tissue was included in order to take into account the relatively higher partition coefficient of estragole in fat tissue. All other tissues were lumped into a rapidly perfused tissue group or a slowly perfused tissue group. The kinetic constants for phase I metabolism as well as for sulfonation of 1′- hydroxyestragole were according to our previous studies (Punt *et al.*, 2007; Punt *et al.*, 2008; Punt *et al.*, 2009). The kinetic constants for glucuronidation and oxidation of 1′-hydroxyestragole were obtained in

the present study using similar incubation conditions as previously described by us (Punt *et al.*, 2008, 2009) (see Results section). The values of the apparent maximum velocity (V_{max}(app)) for the different phase I and II pathways were scaled to the liver assuming an S9 and microsomal protein yield of 143 and 32 mg per g liver respectively based on our previous work (Punt *et al.*, 2008, 2009). The rate of estragole metabolism was calculated using Michaelis–Menten equations representing the various phase I and II reactions. Based on our previous work, estragole uptake from the gastro intestinal tract was assumed to be efficient and following first order kinetics in rat (Punt *et al.*, 2008) and human (Punt *et al.*, 2009). This is in line with studies reported in the literature (Sangster *et al.*, 1987) where it was demonstrated that estragole was readily absorbed from the gastrointestinal tract when administered to human volunteers at a dose level close to the ones encountered in the diet (100 µg 14C-estragole).

In the present study the inhibition of SULT-mediated conversion by the identified inhibitor from basil extract was integrated in the model. Since the inhibitor nevadensin was shown to inhibit the SULT catalyzed conversion of 7HC to 7HCS in a non-competitive way, reducing V_{max} and not affecting K_m, a modulation factor (1+([I]/*K*i)) for the apparent V_{max} value was included in the Michaelis–Menten equation representing the sulfonation pathway as follows:

$$dAMHES/dt = V_{max,L\text{-}HES}/(1+([I]/Ki))\times CL_{HE}/PL_{HE}/(K_{m,L\text{-}HES}+(CL_{HE}/PL_{HE}))$$

where AMHES is the amount of 1′-sulfooxyestragole (µmol), $V_{max,L\ HES}$ is the maximum rate of formation of 1′-sulfooxyestragole, [I] is the concentration of the identified SULT inhibitor nevadensin in the liver (µmol/L), *K*i is the inhibition constant for inhibition of the sulfonation by nevadensin (µmol/L), CL_{HE} is the concentration of 1′-hydroxyestragole in the liver (µmol/L), PL_{HE} is the liver/blood partition coefficient, and K_m is the Michaelis–Menten constant for the formation of 1′-sulfooxyestragole (µmol/L). The inhibition constant (*K*i) for inhibition of the conversion of 7HC to 7HCS by the identified inhibitor was used in the model of estragole assuming equal *K*i values for the two substrates (1′-hydroxyestragole and 7HC) and this was based on the fact that the type of inhibition was found to be non-competitive and, thus, not occurring at the substrate binding site of SULT.

Using our PBBK models for estragole bioactivation and detoxification (Punt *et al.*, 2008, 2009), the time dependent concentration of estragole (CL_E) in the liver of rat and human following an exposure to different doses of estragole can be predicted over a period of 24 h. As a first approximation, the molar ratio (f) between estragole and nevadensin was kept at a constant value during the modeling time. To this end, nevadensin was assumed to follow a similar time dependent concentration curve in the liver as estragole and this was represented in the model by using the following equation $[I]=CL_E\times f$ where CL_E is the concentration of estragole in the liver (µmol/L) and f represents the molar ratio between the nevadensin and estragole concentrations. To obtain some insight into the effects resulting from possible differences in kinetics and bioavailability of the two compounds, the modeling was performed at different molar ratios (f). Thus the molar ratio (f) was varied

from 0 (no nevadensin) to 2 (representing a nevadensin concentration that is twice as high as estragole). Accordingly, the effect of varying doses of nevadensin on the formation of 1′-sulfooxyestragole, 1′-hydroxyestragole glucuronide, and 1′-oxoestragole after 24 h could be predicted at different estragole doses.

The doses of estragole chosen for the modeling were 0.07 mg/kg bw, the dose level considered relevant for human dietary exposure from all food sources (SCF, 2001a) and 50 mg/kg bw representing a dose level at which different alkenylbenzenes were demonstrated to induce liver DNA adducts and/or hepatomas in rodents (Hagan *et al.*, 1965; Boberg *et al.*, 1983; Miller *et al.*, 1983; NTP, 2000; Ellis, 2007; Rietjens *et al.*, 2010).

RESULTS

Inhibition of SULT activity by a methanolic basil extract

Increasing concentrations of the methanolic basil extract inhibited SULT enzyme activity in a dose-dependent manner (Figure 2A). SULT activity was completely blocked at 100 µg basil extract/mL similar to the effect induced by 25 µM of the SULT inhibitor PCP. Blanc incubations, which lacked the cofactor PAPS or which lacked the rat or human S9 fractions, did not show any formation of 7HCS (data not shown) indicating that the 7HCS formation that was inhibited by the basil extract was totally dependent on the presence of PAPS and the SULT content of S9 fractions.

SULT inhibition by different fractions of basil extract

The methanolic basil extract was fractionated into 60 fractions using HPLC. The potency of each fraction in inhibiting SULT activity was examined. As shown in Figure 2B, different basil fractions were able to inhibit SULT activity to a different extent. The fraction with the highest inhibition potency was fraction 50 (Figure 2B) and when re-analyzed on HPLC-UV this fraction appeared to contain only one major constituent (data not shown).

Identification of a potent SULT inhibitor in basil extract

LC–ESI–MS/MS analysis of fraction 50 revealed the presence of a single peak eluting at 29.6 min, corresponding to a compound with a quasi-molecular ion at m/z 345. Fragmentation of this ion at m/z 345 gave rise to three major fragment ions at m/z 330 (100%), 315 (30%), and 312 (20%). Both the observed molecular ion and its fragmentation pattern appear to correspond to those reported for the compound nevadensin (5,7-dihydroxy-6,8,4′-trimethoxy flavone) (Grayer *et al.*, 1996) (Figure 3). The UV spectrum with λ_{max}^{MeOH} values observed at 283 and 330 nm was also in accordance to what has been described for nevadensin (Grayer *et al.*, 1996) and similar to the UV spectrum of a commercially available standard of nevadensin. ^{1}H–NMR analysis of the compound and comparison of the ^{1}H–NMR characteristics to those of a commercially available standard, also confirmed the compound in fraction 50 to be nevadensin. ^{1}H–NMR analysis of the compound and comparison of the ^{1}H–NMR characteristics to those of a commercially available standard, also confirmed the

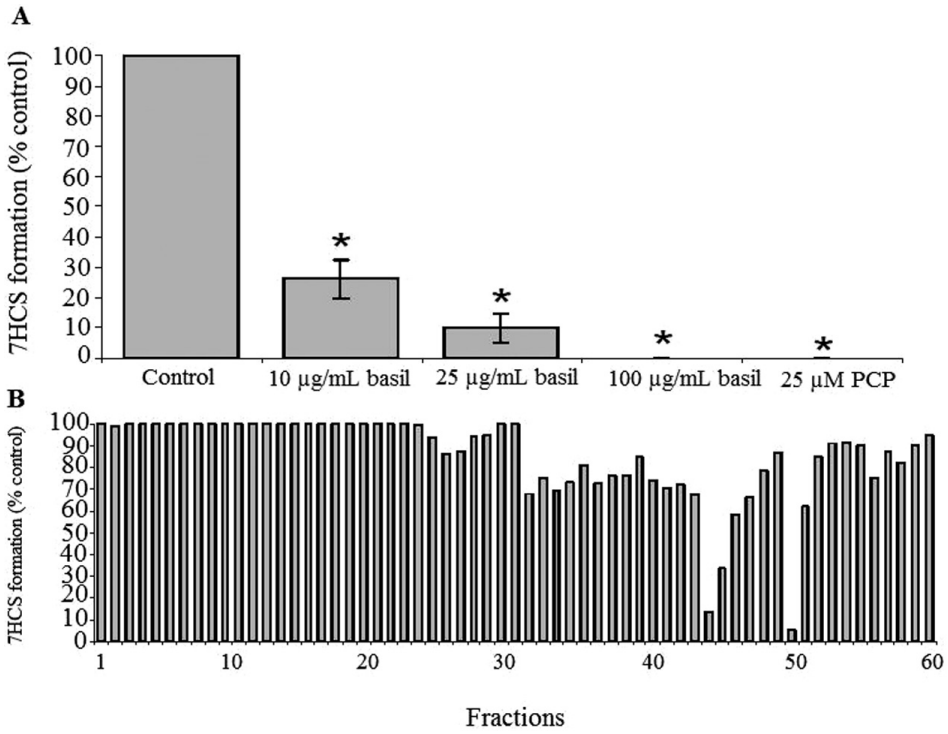


Figure 2. Inhibition of SULT catalyzed conversion of 7HC to 7HCS by (A) pooled rat liver S9 homogenates in the presence of increasing concentrations of crude basil extracts or 25 µM PCP, and (B) HPLC collected basil fractions. 100% 7HCS formation is equal to 1.8 ± 0.68 nmol/min/mg S9 protein. Data points represent mean (±SD) of triplicate measurements obtained in independent experiments. An asterisk (*) indicates a significant inhibition compared to the incubation without inhibitor ($P < 0.05$).

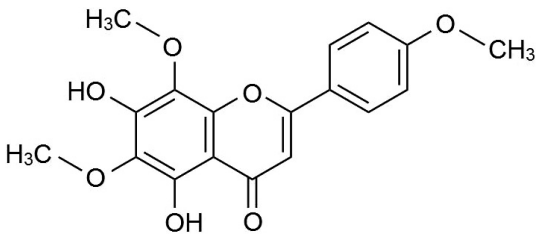


Figure 3. Structural formula of nevadensin; the SULT inhibitor from basil identified in the present study.

compound in fraction 50 to be nevadensin (Grayer *et al.*, 1996; Liu *et al.*, 1996). ^{1}H–NMR analysis revealed the following characteristics: Nevadensin ($C_{18}O_7H_{16}$); ^{1}H–NMR (CD3OD, δ ppm): 8.02 (2H, d, J=8.9 Hz, H-2′,6′), 7.15 (2H, d, J=8.9 Hz, H-3′,5′), 6.67 (1H, s, H-3), 3.97, 3.93, 3.91 (3*3H, s, OMe-6,8,4′).

Inhibition of E-3′-N^2-dGuo formation by nevadensin in primary rat hepatocytes exposed to 1′-hydroxyestragole

To investigate whether the inhibition of SULT activity by nevadensin in a subcellular system such as S9 is maintained in an intact cell system, the effect of nevadensin on E-3′-N^2-dGuo adduct formation in primary rat hepatocytes exposed to 1′-hydroxyestragole was quantified. To this end, primary rat hepatocytes were exposed to 50 μM 1′-hydroxyestragole in the absence or presence of increasing nevadensin concentrations (0–4 μM) and the major adduct E-3′-N^2-dGuo was quantified by LC–ESI–MS/MS. The compounds were not cytotoxic to primary rat hepatocytes under the conditions used in these experiments as indicated by LDH activity measurements (data not shown). The results obtained revealed that the inhibition of E-3′-N^2-dGuo was dose-dependent with an IC_{50} of 0.16±0.09 μM nevadensin (Figure 4), suggesting that the flavonoid nevadensin is able to pass through the cell membrane and exert an intracellular effect.

Effect of nevadensin on the kinetics of SULT activity

The addition of increasing concentrations (0–4 μM) of nevadensin to SULT incubations with rat and human pooled liver S9 fractions, PAPS and 25 μM 7HC as the substrate revealed a dose-dependent inhibition of SULT activity with IC_{50} values of 4 nM and 3 nM in incubations with male rat and human liver S9 fractions respectively (Figure 5).

Plots of 7HCS formation by pooled liver S9 fractions of the two species versus the substrate concentration in the presence or absence of 2 nM of nevadensin are shown in Figure 6. The apparent K_m and V_{max} values derived from these curves for 7HCS formation by male rat and human liver S9 fractions in the absence and presence of 2 nM nevadensin are presented in Table 1. The catalytic efficiency for the SULT-

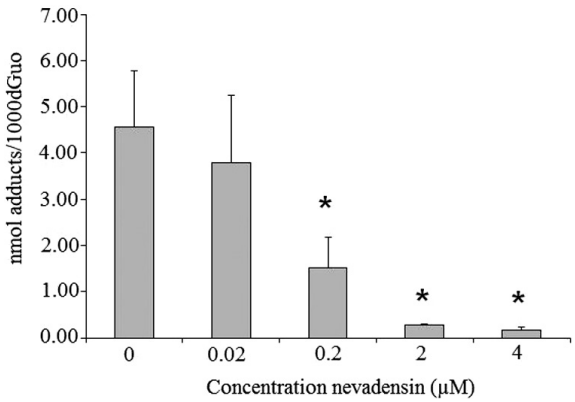


Figure 4. E-3′-N^2-dGuo formation in primary rat hepatocytes exposed for 4 h to 50 μM 1′-hydroxyestragole in the absence or presence of increasing concentrations (0-4 μM) of nevadensin. Data points represent mean (±SD) of triplicate measurements obtained in independent experiments. An asterisk (*) indicates a significant inhibition compared to the incubation without inhibitor ($P < 0.05$).

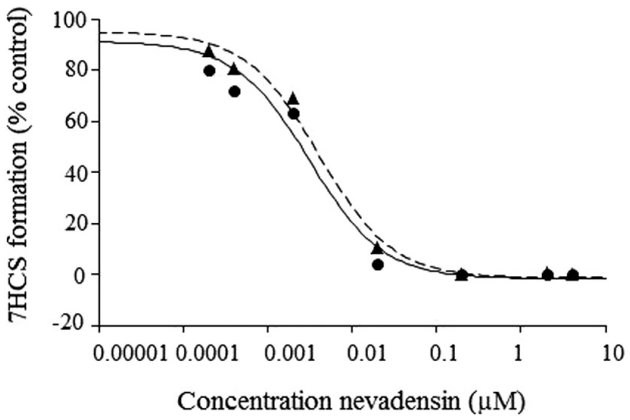


Figure 5. Inhibition of SULT activity in incubations with male rat S9 (dotted line) and pooled human liver S9 (solid line) by increasing concentrations of nevadensin (0-4 µM).

Table 1. Kinetic parameters of SULT-dependent formation of 7HCS by rat and pooled human liver S9 in the absence or presence of 2 nM nevadensin.

	Without nevadensin		with 2 nM nevadensin		
Species	apparent V_{max} (pmol/min/ mg S9 protein)	apparent K_m (µM)	apparent V_{max} (pmol/min/ mg S9 protein)	apparent K_m (µM)	*K*i (µM)
Rat	1901±252	3.7±0.6	1251±173	3.1±0.2	0.004
Human	884±102	2.6±0.3	606±73	2.4±0.3	0.004

dependent formation of 7HCS, calculated as the apparent V_{max}/K_m, was about 1.5 fold higher for male rat liver S9 than for human liver S9 fractions. The apparent V_{max} for sulfonation was lowered 1.5 times for human as well as rat S9 fractions in the presence of 2 nM of nevadensin while the apparent K_m values remained unaffected. These results point at a non-competitive type of inhibition for both rat and human SULT activity in the liver by nevadensin. The inhibition constant (*K*i) obtained from the data amounted to 4 nM for both rat and human S9 fractions.

Effect of nevadensin on 1′-hydroxyestragole glucuronidation and oxidation

The kinetics of 1′-hydroxyestragole glucuronidation and oxidation in the absence and presence of 20 µM nevadensin were investigated for male rat as well as for pooled human liver S9/microsomal fractions (Table 2). The apparent V_{max} and K_m values for 1′-hydroxyestragole oxidation in the two fractions and for 1′-hydroxyestragole glucuronidation in pooled human microsomes (Table 2) in the absence of nevadensin were in agreement to what we have reported previously (Punt *et al.*, 2008, 2009).

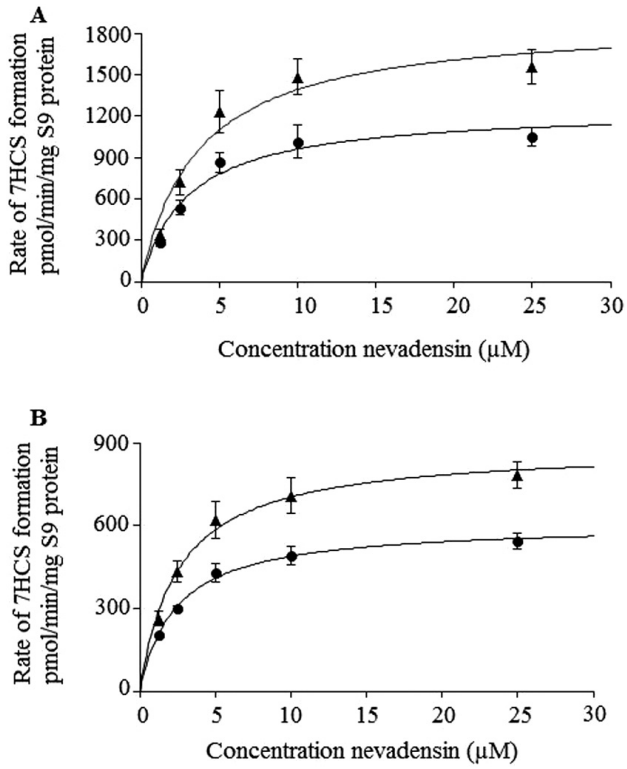


Figure 6. SULT dependent formation of 7HCS in incubations with male rat liver S9 (A) and pooled human liver microsomal fractions (B) in the absence (▲) or presence (●) of 2 nM nevadensin and increasing concentrations (0-25 μM) 7HC. Data points represent mean (±SD) of triplicate measurements obtained in independent experiments.

Table 2. Kinetic parameters for conversion of 1′-hydroxyestragole to 1′-oxoestragole in incubations with male rat and pooled human liver microsomal fractions and for 1′-hydroxyestragole glucuronide formation in male rat liver S9 and pooled human liver microsomal fractions in the absence or presence of 20 μM nevadensin.

	Without nevadensin		with 20 μM nevadensin	
Species	apparent V_{max} (pmol/min/ mg S9 protein)	apparent K_m (μM)	apparent V_{max} (pmol/min/ mg S9 protein)	apparent K_m (μM)
		Oxidation		
Rat	15±0.6	2890±286	11±0.8	1937±169
Human	13±1.7	830±114	10.7±5	1089±664
		Glucuronidation		
Rat	151±20	241±40	138±23	209±16
Human	1.3±0.2	1708±405	1.3±0.2	1721±393

For 1′-hydroxyestragole glucuronide formation in male rat liver S9 fractions, the apparent V_{max} was 151± 20 nmol/min/mg S9 protein which is 21 fold higher than reported in our previous work (Punt *et al.*, 2009) probably reflecting intra-species variation. However the obtained K_m of 241±41 µM was in the same order of magnitude. The addition of 20 µM of nevadensin to the incubations had no effect on the glucuronidation of 1′-hydroxyestragole in both rat S9 as well as pooled human microsomal fractions since the decrease in the apparent V_{max} and K_m values was not significant compared with the values of the uninhibited reaction (without nevadensin) (Table 2). Similarly, the addition of 20 µM nevadensin did not have an effect on the kinetics for oxidation of 1′-hydroxyestragole to 1′-oxoestragole by the human liver microsomal fractions (Table 2). In contrast, when using the male rat liver microsomal fractions of rat, the addition of 20 µM nevadensin resulted in a small but significant (P <0.01, obtained with a Student t-test in SPSS) decrease in the apparent V_{max} and K_m values for 1′-hydroxyestragole oxidation suggesting an un-competitive type of inhibition (Figure 7; Table 2). In this type of inhibition, the relative decrease in the apparent V_{max} and K_m values is similar (about 1.36) and thus the Ki value can be calculated either by fitting the kinetic parameters to the equation ($V'_{max}=V_{max}/(1+([I]/Ki))$) or ($K'_m=K_m/(1+([I]/Ki))$). Characteristic for this type of inhibition is that the inhibition becomes only significant at a relatively high substrate concentration which in this case was found to be higher than 1000 µM 1′-hydroxyestragole (Figure 7). Based on our previous PBBK data (Punt *et al.*, 2008; Punt *et al.*, 2009), this is not in the range of 1′- hydroxyestragole concentrations expected in the in vivo situations.

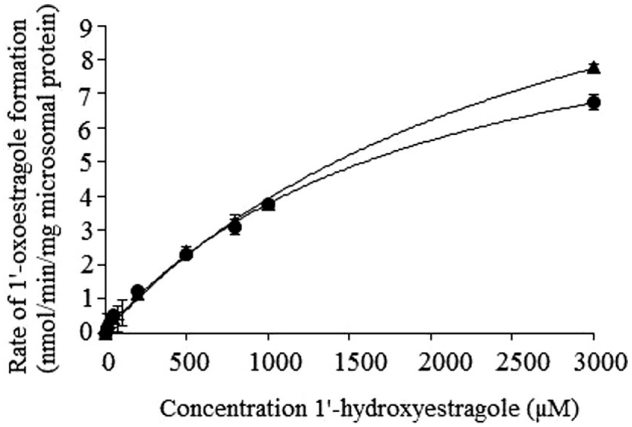


Figure 7. Rate of 1′-oxoestragole formation in incubations with male rat microsomes containing increasing concentrations of 1′-hydroxyestragole in the absence (▲) or presence (●) of 20 µM nevadensin. Data points represent mean (±SD) of triplicate measurements obtained in independent experiments.

PBBK based predictions for estragole phase II metabolism in the presence of nevadensin

Figure 8 illustrates the PBBK predicted effect of an increasing dose of nevadensin (expressed as molar ratio to estragole) on the formation of 1′-sulfooxyestragole in rat and human liver. The data calculated for varying molar ratios of nevadensin to estragole also reflect what would happen if the uptake of nevadensin would be lower than 100% (Han *et al.*, 1981). As predicted by the PBBK model, the co-administration of a dose of estragole of 0.07 mg/kg bw (SCF, 2001a) together with an increasing dose of nevadensin will lead to a dose dependent decrease in the formation of the ultimate carcinogen 1′-sulfooxyestragole, whereas the levels of 1′-hydroxyestragole glucuronide and 1′-oxoestragole formation remain unaffected (data not shown). Assuming 100% uptake of nevadensin and metabolic conversion similar to that of estragole, the PBBK model predicts that at a molar ratio between estragole and nevadensin of 1, a 91% and 96% inhibition of the formation of 1′-sulfooxyestragole in the liver of male rat and human can be expected (Figure 8A).

Assuming a 1% instead of a 100% uptake of nevadensin (theoretically similar to a nevadensin: estragole molar ratio of 0.01), the model still predicts about 17% and 43% inhibition of 1′-sulfooxyestragole formation as compared to control in rat and human respectively (Figure 8A). Figure 8B presents the results obtained when the same PBBK modeling was performed at a dose of estragole of 50 mg/kg bw representing a dose level at which different alkenylbenzenes were shown to induce hepatic tumours and/or DNA adducts in vivo (Hagan *et al.*, 1965; Boberg *et al.*, 1983; Miller *et al.*, 1983; NTP, 2000; Ellis, 2007; Rietjens *et al.*, 2010). At a molar ratio of nevadensin to estragole of 0.06, at which the two compounds are expected to be present in basil (as characterized in the present study by HPLC by means of a calibration curve), the model predicts an almost complete inhibition of 1′-sulfooxyestragole formation in the liver of male rat and human when assuming 100% uptake of nevadensin (Figure 8B). Assuming 1% uptake of nevadensin (theoretically similar to a nevadensin: estragole ratio of 6×10^{-4}), the PBBK model still predicts 84% and 88% inhibition of 1′-sulfooxyestragole formation in the two species respectively (Figure 8B).

DISCUSSION

The present study identifies nevadensin (5,7-dihydroxy-6,8,4′-trimethoxyflavone) as a major SULT inhibiting ingredient in basil. Nevadensin is a partially methylated flavone aglycone. The presence of three methoxy groups explains its elution at a high methanol concentration (~83%) due to an increased lipophilicity compared to non-methylated flavones (Grayer *et al.*, 1996; Vieira *et al.*, 2003) nevadensin has been reported as a major flavone constituent in the Lamiaceae family from the genus *Ocimum basilicum* and has been reported to amount to 5.6–58.4% of the total flavones present (Grayer *et al.*, 1996). In addition to nevadensin, basil may contain other flavone aglycones, including, for example, apigenin, cirsilineol, eupatorin, and salvigenin (Grayer *et al.*, 1996). However,

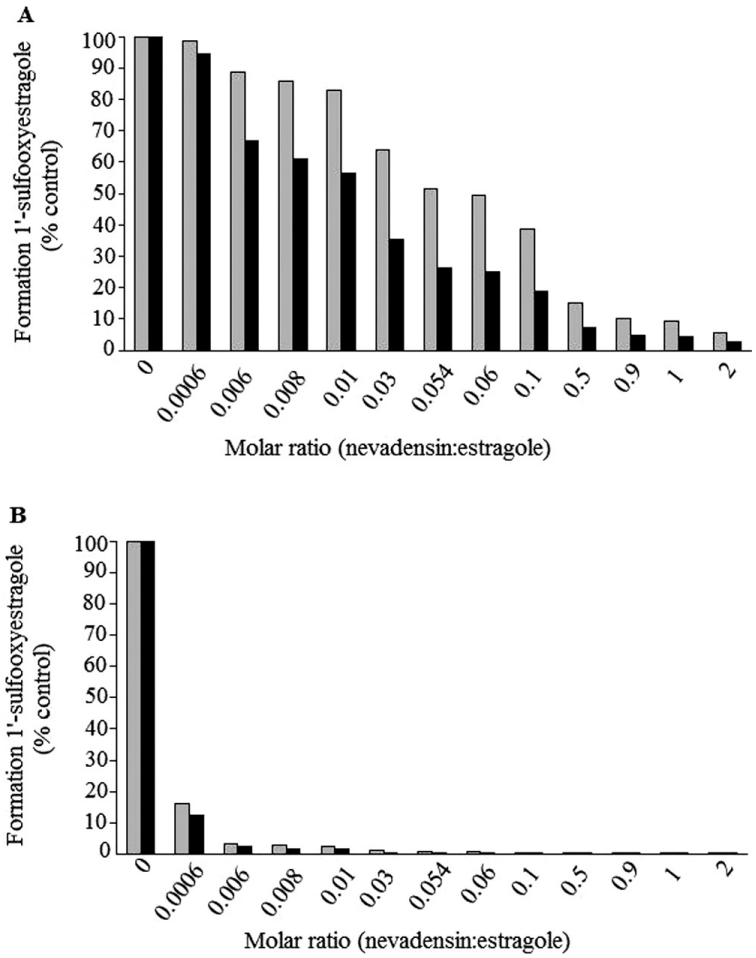


Figure 8. PBBK-based predictions for the dose dependent effect of nevadensin on 1′-sulfooxyestragole formation in the liver of rat (gray bar) and human (black bar) at (A) 0.07 mg/kg bw and (B) 50 mg/ kg bw of estragole.

most of these flavones are present in relatively lower quantities than nevadensin or may be absent in some varieties of basil, which, together with the data of the present study, leads to the conclusion that nevadensin is the major flavone type SULT inhibitor present in basil.

Several other studies already reported that some flavones exert an inhibitory effect on SULT activity (Surh, 1983; Gilissen *et al.*, 1994; Kato and Yamazoe, 1994; Miller, 1994; Morimitsu *et al.*, 2004). The study of Morimitsu *et al.* (2004) even revealed a structure activity relationship for the inhibitory action of flavonoids on sulfonation. In agreement with the present study, nevadensin was found to possess the structural requirements for effective inhibitory activity linked to the presence of C5 and C7 hydroxyl substituents on the A ring and also a C2–3 double bond in conjunction with

the C4 carbonyl group on the C-ring. Salvigenin, a major flavone constituent in basil (Grayer *et al.*, 1996) is not expected to exert an effect on sulfonation because it lacks these structural requirements. Apigenin on the other hand which is a minor flavone constituent in basil (Grayer *et al.*, 1996) is reported to inhibit sulfonation formation in cultured hepatocytes (Morimitsu *et al.*, 2004) and also possesses the structural features for SULT inhibition mentioned above.

Since the composition and levels of flavonoids and other active ingredients like alkenylbenzenes in a herb or spice can vary significantly based on different factors such as plant maturity at harvest, harvesting techniques, storage conditions, processing (e.g. drying), and method of measurement (e.g. extraction), present state-of-the-art intake estimates for ingredients from botanicals and botanical preparations are not refined to such an extent that the actual human exposure levels of these two chemicals can be determined in an accurate way. However to get an impression about the effect of nevadensin on estragole bioactivation at different levels of intake and bioavailability/uptake, PBBK modeling was performed at various molar ratios of the two compounds.

The Michaelis–Menten constants obtained in the present study for 7HCS formation in incubations with S9 fractions from male rat and human liver were in agreement with our previous work and literature (Wang *et al.*, 2006; Punt *et al.*, 2007). The kinetics for the inhibition of SULT activity by nevadensin obtained in the present study indicate a non-competitive inhibition since the addition of 2 nM of nevadensin resulted in lowering the apparent V_{max} values without an effect on the apparent K_m value. It is not unexpected to observe a non-competitive inhibition type as there were a few reports showing that several dietary flavonoids such as quercetin, kaempferol, genistein, and diadzein were found to inhibit the sulfonation of estradiol by SULT1A1 non-competitively with *K*i values ranging from 100 to 700 nM (Walle *et al.*, 1995; Eaton *et al.*, 1996; Ghazali and Waring, 1999; Mesía -Vela and Kauffman, 2003). In comparison, the *K*i value for nevadensin dependent SULT inhibition (4 nM) for both male rat and human liver S9 fractions was two orders of magnitude lower, indicating that nevadensin is a potent SULT inhibitor.

In addition to the effect of nevadensin on SULT activity, also its possible effect on other phase II pathways contributing to 1′-hydroxyestragole conversion in rat and human liver, namely, the glucuronidation and oxidation of 1′-hydroxyestragole, was characterized in the present study. The relative inhibition of SULT activity as compared to 1′-hydroxyestragole detoxification through glucuronidation and oxidation can be expected to ultimately determine whether metabolism of 1′-hydroxyestragole will be shifted toward detoxification at the expense of sulfonation. In sharp contrast to the effect of nevadensin on sulfonation, nevadensin up to a concentration of 20 μM did not inhibit detoxification via glucuronidation and oxidation of 1′-hydroxyestragole to any significant extent at physiologically relevant doses of the ultimate carcinogen 1′-hydroxyestragole in S9 and microsomal liver fractions.

Due to the practical constraints when using 1′-hydroxyestragole as substrate for the SULT assay, because of the instability of its sulfonated metabolite, conversion of 7HC to 7HCS was used in the present study to characterize the kinetics for nevadensin

SULT-mediated inhibition. This raises the question regarding the relevance of the *K*i obtained for sulfonation when using 7HC instead of 1′-hydroxyestragole as a substrate. This is mainly because the catalytic efficiency of the SULT-mediated conversion is dramatically different for the two substrates, amounting to 559 and 0.3 pmol/min/mg S9 protein in male rat and 353.6 and 0.01 pmol/min/mg S9 protein in human liver S9 fractions for 1′-hydroxyestragole and 7HC respectively. However, the type of inhibition by nevadensin was found to be non-competitive, meaning that only the apparent V_{max} and not the apparent K_m of the enzyme was found to be affected, and this corroborates the assumption that nevadensin does not interfere with the substrate binding site and exerts its inhibiting effect by interaction with another site on the enzyme that is not involved in substrate binding (Simmons, 1996). Therefore, the *K*i is not expected to be significantly different when using 1′-hydroxyestragole or 7HC as the substrate. This would be in line with the results reported by Eaton *et al*. (1996) showing that the *K*i values for the quercetin-mediated inhibition of sulfonation for the substrates acetaminophen, minoxidil, and p-nitrophenol, each showing a vastly different K_m value, were similar and all SULT conversions were inhibited in a non-competitive way by this other flavonoid.

It is also of importance to note that flavonoid glucuronides and sulfonates are reported to be the predominant circulating chemical species and this may hold for nevadensin as well. However, studies in which cells in vitro were exposed to glucuronidated and sulfonated conjugates of a related flavonoid, quercetin, demonstrated that within the cells the flavonoid was no longer conjugated, due to efficient extra- and/or intracellular deconjugation (Lee-Hilz *et al*., 2008).

The actual SULT isoenzyme responsible for 1′-hydroxyestragole sulfonation has not yet been elucidated, and also identification of the SULT enzyme inhibited by nevadensin remains a subject for future research.

In a next step of the present study the inhibition of SULT by nevadensin was incorporated into our physiologically based biokinetic (PBBK) models for estragole bioactivation and detoxification in rat and human (Punt *et al*., 2008, 2009). These modified PBBK models allow evaluation of the bioactivation and detoxification of estragole in the liver of male rat and human when exposure to estragole is combined with exposure to nevadensin. This approach illustrates the conclusions reported before that PBBK modeling facilitates the extrapolation of the occurrence and magnitude of interactions of binary mixtures from the in vitro to the in vivo situation (Krishnan *et al*., 2002). The models thus obtained in the present study allowed prediction of the dose-dependent inhibition of the formation of the ultimate carcinogenic metabolite 1′-sulfooxyestragole in the liver of male rat and human by co-administered dose levels of nevadensin and estragole. The models also predict the nevadensin-mediated changes in the overall formation of the metabolites that reflect detoxification by glucuronidation and oxidation of 1′-hydroxyestragole. Since the actual kinetic parameters for nevadensin were not available and because the quantification of these parameters was beyond the scope of the present paper, in a first approximation, both estragole and nevadensin were assumed to follow a similar time dependent

concentration curve. Different molar ratios of the two compounds were modelled to provide some insight in the effects of possible differences in kinetics and uptake of the two compounds. The PBBK models developed predict that at an estragole dose level of 50 mg/kg bw and a nevadensin: estragole ratio of 0.06, the estimated ratio between the two compounds in basil, nevadensin would inhibit 1′-sulfooxyestragole formation in male rat and human liver by respectively 99.7% and 99.5% or 84% and 88% assuming 100 or 1% nevadensin uptake. Thus formation of 1′-sulfooxyestragole is expected to be significantly, though not completely blocked.

Other flavonoids that are part of the human diet possess a potent SULT inhibitory effect as well (Gibb *et al.*, 1987; Marchetti *et al.*, 2001; De Santi *et al.*, 2002; Morimitsu *et al.*, 2004) implying that the formation of 1′-sulfooxyestragole could be even lower due to the combination of estragole exposure with other food borne SULT inhibitors together with nevadensin from basil. Moreover, not only can the bioactivation of alkenylbenzenes be affected by the food matrix but also their bioavailability might be reduced (Schilter *et al.*, 2003) due to reduced absorption from the gastrointestinal tract which can be attributed to slow or inadequate release of alkenylbenzenes from the complex food matrix. Another point of interest is that the modeling also reveals that the effect of a given ratio of nevadensin to estragole on 1′-sulfooxyestragole formation varies with the dose of estragole. Thus at a molar ratio of nevadensin to estragole of 0.06, the percentage of inhibition decreases going from 99.7 and 99.5% at an estragole dose of 50 mg/kg bw to 51% and 75% at an estragole dose of 0.07 mg/kgbw in the liver of rat and human respectively. This can be explained by the fact that the concentration of the inhibitor nevadensin is higher in the former situation (at 50 mg/kg bw) than in the latter situation (at 0.07 mg/kg bw) resulting in more efficient inhibition given the *K*i of 4 nm in the two species. This is a well-known characteristic of non-competitive inhibition (Simmons, 1996). Altogether, the PBBK modeling reveals that the inhibition of SULT-mediated bioactivation to 1′-sulfooxyestragole upon combined exposure to estragole and nevadensin may be significant even at realistic low dose human exposure levels and even when the uptake of ND would be significantly lower than 100%.

Furthermore it may be of interest to note that many flavonoids have been shown to interact with efflux transporters such as ABCB1, MRP1, MRP2, and BCRP in vitro as well as in vivo (Brand *et al.*, 2006; Morris and Zhang, 2006) but no such studies were found for nevadensin in particular. Thus to predict how these effects would influence the results and conclusions is difficult to quantify. In a qualitative way inhibition of apical ABC transporters in intestinal cells will increase bioavailability whereas inhibition of basolateral transporters may oppose bioavailability. Thus, one can assume for example that bioavailability of both compounds will be affected in a similar way and this is actually what has been done in the present modeling approach assuming toxico kinetics for nevadensin to mimic those of estragole keeping the ratio between the two compounds constant in time.

Nevadensin was also shown to inhibit the DNA adduct formation in 1′-hydroxyestragole exposed primary rat hepatocytes with an IC_{50} value of 0.16±0.09 µM and thus one order of magnitude higher than the IC_{50} required for in vitro inhibition

of SULT-mediated conversion of 7HC in S9 fractions. Taking into account that the DNA adduct inhibition occurs at the SULT enzyme level, the IC_{50} for the inhibition of DNA adduct formation can be compared to that obtained for the SULT inhibition in liver S9 fractions. The difference in IC_{50} values might be attributable to high serum protein binding, poor plasma membrane permeability, as well as degradation to inactive metabolites (Griffiths and Smith, 1972; Kühnau, 1976). These findings are in line with those of Morimitsu *et al*. (2004) where the IC_{50} for the inhibited sulfonation by a series of flavonoids in primary rat hepatocytes was one order of magnitude higher than in liver cytosolic fractions. The efficiency of nevadensin in inhibiting the sulfonation of 1′-hydroxyestragole and thus its potential protective effect against bioactivation of estragole to the proximate carcinogen still needs to be established in in vivo experiments where other factors such as its bioavailability and metabolic stability should play a crucial role. Of interest in this respect is that methylated flavones such as nevadensin have been demonstrated to be much more metabolically stable than their unmethylated analogues which are reflected by their resistance to hepatic metabolism and stability in the intestinal epithelial cell layer. Furthermore, they also have higher intestinal absorption through Caco-2 cell monolayers. Both factors should greatly increase their oral bioavailability (Wen and Walle, 2006; Walle, 2007).

According to our previous studies, it is also important to stress that species dependent differences in the in vivo metabolism of estragole may exist (Sangster *et al.*, 1987; Punt *et al.*, 2009; Rietjens *et al.*, 2010). For example, detoxification of 1′ -hydroxyestragole in rat mainly proceeds through glucuronidation whereas in human it mainly proceeds by oxidation to 1′-oxoestragole (Punt *et al.*, 2009). Clearly, the PBBK models will be of use to investigate also the possible species differences in the effects of the inhibitor nevadensin on the in vivo metabolic pathways of estragole. The initial modeling results presented in Figure 8 for example already suggest that the inhibiting effect of nevadensin on 1′-sulfooxyestragole formation levels may be somewhat more pronounced in human than in rat.

In conclusion, the present study identifies the potent SULT inhibitor present in basil to be nevadensin, an important and major flavonoid constituent of basil. This also points at a strong matrix effect of combined exposure to estragole and nevadensin. The SULT inhibition was shown to lead to the inhibition of the formation of the ultimate carcinogenic metabolite, without reducing the rate of detoxification of the proximate carcinogen 1′-hydroxyestragole via glucuronidation or oxidation. If the PBBK predicted data can be validated in vivo, which is an important issue for future research, this would imply that the consumption of estragole within a matrix of sweet basil or other food items containing SULT inhibitors would not pose a significant risk if any. Dietary SULT inhibitors include a wide variety of compounds such as for example flavonoids like nevadensin, which was identified in the present paper to be a highly efficient SULT inhibitor, but also epicatechin gallate, epigallocatechin gallate, gallocatechin gallate (Coughtrie and Johnston, 2001), quercetin (Walle *et al.*, 1995), and also including some food additives such as carmoisine, octyl gallate, tartrazine and vanillin (Bamforth

et al., 1993). The safety of alkenylbenzene-containing botanicals could consequently be judged and regulated on the basis of the estimated daily intakes of the different alkenylbenzenes compared to the estimated daily intakes of the different respective SULT inhibitors that are present in the diet. Further research investigations are directed at screening estragole-containing botanicals for the presence of SULT inhibitors, quantifying their inhibiting efficiencies as potential chemopreventive compounds, reducing DNA adduct formation and ultimately carcinogenic risk in vivo.

ACKNOWLEDGMENT

The authors would like to acknowledge Dr. Yiannis Fiamegos and Florian Viton for the synthesis of 1′-hydroxyestragole. They would like also to thank Biqualys (Wageningen, The Netherlands) for providing possibilities for performing the ^{1}H–NMR analysis of nevadensin. This work was supported in part by a grant from the International Organization of the Flavour Industry (IOFI) and by the Nestlé Research Centre (Switzerland).

REFERENCES

1. Bamforth, K. J., Jones, A. L., Roberts, R. C., and Coughtrie, M. W. H. (1993). Common food additives are potent inhibitors of human liver 17α-ethinyloestradiol and dopamine sulphotransferases. *Biochemical Pharmacology* **46**, 1713-1720.
2. Boberg, E. W., Miller, E. C., and Miller, J. A. (1983). Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1′-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1′-hydroxysafrole in mouse liver. *Cancer Research* **43**, 5163-5173.
3. Borchert, P., Wislocki, P. G., Miller, J. A., and Miller, E. C. (1973). The metabolism of the naturally occurring hepatocarcinogen safrole to 1′-hydroxysafrole and the electrophilic reactivity of 1′-acetoxysafrole. *Cancer Research* **33**, 575-589.
4. Brand, W., Schutte, M. E., Williamson, G., van Zanden, J. J., Cnubben, N. H. P., Groten, J. P., van Bladeren, P. J., and Rietjens, I. M. C. M. (2006). Flavonoid-mediated inhibition of intestinal ABC transporters may affect the oral bioavailability of drugs, food-borne toxic compounds and bioactive ingredients. *Biomedicine and Pharmacotherapy* **60**, 508-519.
5. Cavin, C., Mace, K., Offord, E. A., and Schilter, B. (2001). Protective effects of coffee diterpenes against aflatoxin B1-induced genotoxicity: Mechanisms in rat and human cells. *Food and Chemical Toxicology* **39**, 549-556.
6. Coughtrie, M. W. H., and Johnston, L. E. (2001). Interactions between dietary chemicals and human sulfotransferases - Molecular mechanisms and clinical significance. *Drug Metabolism and Disposition* **29**, 522-528.
7. De Santi, C., Pietrabissa, A., Mosca, F., Rane, A., and Pacifici, G. M. (2002). Inhibition of phenol sulfotransferase (SULT1A1) by quercetin in human adult and foetal livers. *Xenobiotica* **32**, 363-368.
8. De Vincenzi, M., Silano, M., Maialetti, F., and Scazzocchio, B. (2000). Constituents of aromatic plants: II. Estragole. *Fitoterapia* **71**, 725-729.
9. Delatour, T., Mally, A., Richoz, J., Ã–zden, S., Dekant, W., Ihmels, H., Otto, D., Gasparutto, D., Marin-Kuan, M., Schilter, B., and Cavin, C. (2008). Absence of 2′-deoxyguanosine-carbon 8-bound ochratoxin A adduct in rat kidney DNA monitored by isotope dilution LC-MS/MS. *Molecular Nutrition and Food Research* **52**, 472-482.
10. Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-Allyl-4-methoxybenzene) and 1′-Hydroxyestragole in the mouse and mutagenicity of 1′-acetoxyestragole

in bacteria. *Journal of the National Cancer Institute* **57**, 1323-1331.

11. Eaton, E. A., Walle, U. K., Lewis, A. J., Hudson, T., Wilson, A. A., and Walle, T. (1996). Flavonoids, potent inhibitors of the human P-form phenolsulfotransferase: Potential role in drug metabolism and chemoprevention. *Drug Metabolism and Disposition* **24**, 232-237.
12. EFSA (2005). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. *EFSA Journal* **282**, 1-31.
13. Ellis, J. K. (2007). Toxicological assessment of low dose exposure to the flavour methyl eugenol. *Interim report by imperial college london for the Flavor and Extract Manufaturers Association*.
14. EMEA (2005). Committee on herbal medicinal products (HMPC) Public statement on the use of herbal medicinal products containing estragole. London 23 November 2005. Doc ref: EMEA/HMPC/137212/2005.
15. Fennell, T. R., Wiseman, R. W., Miller, J. A., and Miller, E. C. (1985). Major role of hepatic sulfotransferase activity in the metabolic activation, DNA adduct formation, and carcinogenicity of 1'-hydroxy-2',3'-dehydroestragole in infant male C57BL/6J x C3H/HeJ F1 mice. *Cancer Research* **45**, 5310-5320.
16. Ghazali, R. A., and Waring, R. H. (1999). The effects of flavonoids on human phenolsulphotransferases: Potential in drug metabolism and chemoprevention. *Life Sciences* **65**, 1625-1632.
17. Gibb, C., Glover, V., and Sandler, M. (1987). In vitro inhibition of phenolsulphotranferase by food and drink constituents. *Biochemical Pharmacology* **36**, 2325-2330.
18. Gilissen, R. A. H. J., Hume, R., Meerman, J. H. N., and Coughtrie, M. W. H. (1994). Sulphation of N-hydroxy-4-aminobiphenyl and N-hydroxy-4-acetylaminobiphenyl by human foetal and neonatal sulphotransferase. *Biochemical Pharmacology* **48**, 837-840.
19. Grayer, R. J., Bryan, S. E., Veitch, N. C., Goldstone, F. J., Paton, A., and Wollenweber, E. (1996). External flavones in sweet basil, Ocimum basilicum, and related taxa. *Phytochemistry* **43**, 1041-1048.
20. Griffiths, L. A., and Smith, G. E. (1972). Metabolism of apigenin and related compounds in the rat. Metabolite formation in vivo and by the intestinal microflora in vitro. *Biochemical Journal* **128**, 901-911.
21. Hagan, E. C., Jenner, P. M., Jones, W. I., Fitzhugh, O. G., Long, E. L., Brouwer, J. G., and Webb, W. K. (1965). Toxic properties of compounds related to safrole. *Toxicology and Applied Pharmacology* **7**, 18-24.
22. Han, G. Z., Su, C. Y., and Zhang, Y. (1981). Determination of nevadensin in biological specimens and its pharmacokinetic study. *Acta Pharmacologica Sinica* **2**, 182-185.
23. JECFA (2008). Sixty-ninth Meeting, Rome, Italy, 17–26 June 2008. (JECFA, Ed.), (http://www.who.int/entity/ipcs/food/jecfa/summaries/summary69.pdf).
24. Jeurissen, S. M. F., Punt, A., Delatour, T., and Rietjens, I. M. C. M. (2008). Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1 '-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. *Food and Chemical Toxicology* **46**, 2296-2302.
25. Kato, R., and Yamazoe, Y. (1994). Metabolic activation of N-hydroxylated metabolites of carcinogenic and mutagenic arylamines and arylamides by esterification. *Drug Metabolism Reviews* **26**, 413-430.
26. Krishnan, K., Haddad, S., Béliveau, M., and Tardif, R. (2002). Physiological modeling and extrapolation of pharmacokinetic interactions from binary to more complex chemical mixtures. *Environmental Health Perspectives* **110**, 989-994.
27. Kühnau, J. (1976). The flavonoids. A class of semi-essential food components: their role in human nutrition. *World review of nutrition and dietetics* **24**, 117-191.
28. Lawrence, B. M. (1992). Chemical components of Labiatae oils and their exploitation. *Advances in Labiatae Science*, 399-436.
29. Lee-Hilz, Y. Y., Stolaki, M., van Berkel, W. J. H., Aarts, J. M. M. J. G., and Rietjens, I. M. C. M. (2008). Activation of EpRE-mediated gene transcription by quercetin glucuronides depends on their deconjugation. *Food and Chemical Toxicology* **46**, 2128-2134.

30. Liu, Y., Wagner, H., and Bauer, R. (1996). Nevadensin glycosides from Lysionotus pauciflorus. *Phytochemistry* **42**, 1203-1205.
31. Marchetti, F., De Santi, C., Vietri, M., Pietrabissa, A., Spisni, R., Mosca, F., and Pacifici, G. M. (2001). Differential inhibition of human liver and duodenum sulphotransferase activities by quercetin, a flavonoid present in vegetables, fruit and wine. *Xenobiotica* **31**, 841-847.
32. Mesía -Vela, S., and Kauffman, F. C. (2003). Inhibition of rat liver sulfotransferases SULT1A1 and SULT2A1 and glucuronosyltransferase by dietary flavonoids. *Xenobiotica* **33**, 1211-1220.
33. Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Research* **43**, 1124-1134.
34. Miller, J. A. (1994). Sulfonation in chemical carcinogenesis - History and present status. *Chemico-Biological Interactions* **92**, 329-341.
35. Morimitsu, Y., Sugihara, N., and Furuno, K. (2004). Inhibitory effect of flavonoids on sulfo- and glucurono-conjugation of acetaminophen in rat cultured hepatocytes and liver subcellular preparations. *Biological and Pharmaceutical Bulletin* **27**, 714-717.
36. Morris, M. E., and Zhang, S. (2006). Flavonoid-drug interactions: Effects of flavonoids on ABC transporters. *Life Sciences* **78**, 2116-2130.
37. NTP (2000). National Toxicology Program on toxicology and carcinogenesis studies of Methyleugenol (CAS NO. 93-15-12) in F344/N rats and B6C3F1 mice (Gavage Studies). DRAFT NTP-TR-491; NIH Publication No. 98-3950., 1-412.
38. Otake, Y., Hsieh, F., and Walle, T. (2002). Glucuronidation versus oxidation of the flavonoid galangin by human liver microsomes and hepatocytes. *Drug Metabolism and Disposition* **30**, 576-581.
39. Paini, A., Punt, A., Viton, F., Scholz, G., Delatour, T., Marin-Kuan, M., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). A physiologically based biodynamic (PBBD) model for estragole DNA binding in rat liver based on in vitro kinetic data and estragole DNA adduct formation in primary hepatocytes. *Toxicology and Applied Pharmacology* **245**, 57-66.
40. Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. *Cancer Research* **41**, 176-186.
41. Phillips, D. H., Reddy, M. V., and Randerath, K. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. *Carcinogenesis* **5**, 1623-1628.
42. Punt, A., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2007). Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1'-hydroxyestragole. *Chemical Research in Toxicology* **20**, 991-998.
43. Punt, A., Freidig, A. P., Delatour, T., Scholz, G., Boersma, M. G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2008). A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat. *Toxicology and Applied Pharmacology* **231**, 248-259.
44. Punt, A., Paini, A., Boersma, M. G., Freidig, A. P., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2009). Use of physiologically based biokinetic (PBBK) modeling to study estragole bioactivation and detoxification in humans as compared with male rats. *Toxicological Sciences* **110**, 255-269.
45. Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis* **5**, 1613-1622.
46. Rietjens, I. M. C. M., Punt, A., Schilter, B., Scholz, G., Delatour, T., and van Bladeren, P. J. (2010). In silico methods for physiologically based biokinetic

models describing bioactivation and detoxification of coumarin and estragole: Implications for risk assessment. *Molecular Nutrition and Food Research* **54**, 195-207.

47. Sangster, S. A., Caldwell, J., and Hutt, A. J. (1987). The metabolic disposition of [methoxy-^{14}C]-labelled *trans*-anethole, estragole and *p*-propylanisole in human volunteers. *Xenobiotica* **17**, 1223-1232.
48. SCF (2001a). Opinion of the Scientific Committee on Food on Estragole (1-allyl-4-methoxybenzene). European Commission, Health and Consumer Protection Directorate. General, Report Series 10, Directorate C, Scientific Opinions, Brussels, Belgium. Obtained January 10, 2008, at http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.
49. SCF (2001b). Opinion of the Scientific Committee on Food on methyleugenol (4-allyl-1,2-dimethoxybenzene.
50. SCF (2001c). Opinion of the Scientific Committee on Food on the safety of the presence of safrole (1-allyl-3,4-methylene dioxy benzene) in flavourings and other food ingredients with flavouring properties.
51. Schilter, B., Andersson, C., Anton, R., Constable, A., Kleiner, J., O'Brien, J., Renwick, A. G., Korver, O., Smit, F., and Walker, R. (2003). Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements. *Food and Chemical Toxicology* **41**, 1625-1649.
52. Sidhu, J. S., Farin, F. M., and Omiecinski, C. J. (1993). Influence of extracellular matrix overlay on phenobarbital-mediated induction of CYP2B1, 2B2, and 3A1 genes in primary adult rat hepatocyte culture. *Archives of Biochemistry and Biophysics* **301**, 103-113.
53. Simmons, J. E. (1996). Application of physiologically based pharmacokinetic modelling to combination toxicology. *Food and Chemical Toxicology* **34**, 1067-1073.
54. Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - Methyl eugenol and estragole. *Food and Chemical Toxicology* **40**, 851-870.
55. Surh, Y. J., Tannenbaum, S. R. (1983). Activation of the maillard reaction product 5-(hydroxymethyl)furfural to strong mutagens via allylic sulfonation and chlorination. *Chemical Research in Toxicology* **7**, 313-318.
56. Vieira, R. F., Grayer, R. J., and Paton, A. J. (2003). Chemical profiling of Ocimum americanum using external flavonoids. *Phytochemistry* **63**, 555-567.
57. Walle, T. (2007). Methylation of dietary flavones greatly improves their hepatic metabolic stability and intestinal absorption. *Molecular Pharmaceutics* **4**, 826-832.
58. Walle, T., Eaton, E. A., and Walle, U. K. (1995). Quercetin, a potent and specific inhibitor of the human P-form phenolsulfotransferase. *Biochemical Pharmacology* **50**, 731-734.
59. Wang, Q., Jia, R., Ye, C., Garcia, M., Li, J., and Hidalgo, I. J. (2005). Glucuronidation and sulfation of 7-hydroxycoumarin in liver matrices from human, dog, monkey, rat, and mouse. *In Vitro Cellular and Developmental Biology - Animal* **41**, 97-103.
60. Wang, Q., Ye, C., Jia, R., Owen, A. J., Hidalgo, I. J., and Li, J. (2006). Inter-species comparison of 7-hydroxycoumarin glucuronidation and sulfation in liver S9 fractions. *In Vitro Cellular and Developmental Biology - Animal* **42**, 8-12.
61. Wen, X., and Walle, T. (2006). Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug Metabolism and Disposition* **34**, 1786-1792.
62. Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F1 mice. *Cancer Research* **47**, 2275-2283.

3

Matrix modulation of the bioactivation of estragole by constituents of different alkenylbenzene-containing herbs and spices and physiologically based biokinetic (PBBK) modeling of possible in vivo effects

Based on: Alhusainy W, van den Berg SJPL, Paini A, Campana A, Asselman M, Spenkelink A, Punt A, Scholz G, Schilter B, Adams TB, van Bladeren PJ, and Rietjens IMCM

Toxicological Sciences 129 (2012), 174–187

ABSTRACT

The alkenylbenzene estragole is a constituent of several herbs and spices. It induces hepatomas in rodents at high doses following bioactivation by cytochrome P450s and sulfotransferases (SULTs) giving rise to the ultimate carcinogenic metabolite 1'-sulfooxyestragole which forms DNA adducts. Methanolic extracts from different alkenylbenzene-containing herbs and spices were able to inhibit SULT activity. Flavonoids including quercetin, kaempferol, myricetin, apigenin, and nevadensin were the major constituents responsible for this inhibition with *K*i values in the nano to micromolar range. In human HepG2 cells exposed to the proximate carcinogen 1'-hydroxyestragole, the various flavonoids were able to inhibit estragole DNA adduct formation and shift metabolism in favour of glucuronidation which is a detoxification pathway for 1'-hydroxyestragole. In a next step, the kinetics for SULT inhibition were incorporated in physiologically based biokinetic (PBBK) models for estragole in rat and human to predict the effect of co-exposure to estragole and (mixtures of) the different flavonoids on the bioactivation in vivo. The PBBK model based predictions indicate that the reduction of estragole bioactivation in rat and human by co-administration of the flavonoids is dependent on whether the intracellular liver concentrations of the flavonoids can reach their *K*i values. It is expected that this is most easily achieved for nevadensin which has a *K*i value in the nanomolar range and is, due to its methylation, more metabolically stable than the other flavonoids.

INTRODUCTION

Herbs and spices have a long history of use as food, flavouring and coloring agents throughout the world (Smith *et al.*, 2002; Dearlove *et al.*, 2008). In culinary usage, herbs are most commonly seasonings derived from leaves of plants. Spices, in contrast, are obtained from seeds, berries, fruits, barks, or roots (Dearlove *et al.*, 2008). Alkenylbenzenes such as estragole (1-allyl-4-methoxybenzene), present in the volatile oils, contribute to the characteristic taste and fragrance of many herbs and spices including nutmeg, basil, anise, mace, tarragon, fennel, and pimento (Smith *et al.*, 2002). Oral intake of estragole results primarily from consumption of foods, mainly herbs and spices, and of their essential oils.

Based on rodent studies, estragole was found to be genotoxic and carcinogenic (Drinkwater *et al.*, 1976; Miller *et al.*, 1983). Based on these findings, the addition of estragole as a pure substance in foodstuffs has been prohibited since September 2008 within the European Union (European Commission, 2008). Realistic daily intake levels of estragole have been estimated to range from 0.01 mg/kg bw/day (Smith *et al.*, 2002) to 0.07 mg/kg bw/day (SCF, 2001). Given that at present addition of estragole as a pure compound to individual food categories is no longer allowed, the value of 0.01 mg/kg bw/ day resulting mainly from herbs and spices (Smith *et al.*, 2002) is taken in the present study as the value for current levels of dietary human intake.

Figure 1 presents an overview of the bioactivation and detoxification pathways of estragole. Based on in vivo studies, at low doses (<10 mg/kg bw), *O*-demethylation appears to be more favoured in humans and in rodents and yields phenolic derivatives which are to a large extent excreted as the sulfonate or glucuronic acid conjugate (Anthony *et al.*, 1987; Sangster *et al.*, 1987). Bioactivation of estragole proceeds by 1′-hydroxylation to 1′-hydroxyestragole and subsequent sulfonation to 1′-sulfooxyestragole. The sulfonate conjugate of 1′-hydroxyestragole is unstable and hydrolyzes to form a reactive electrophilic intermediate (carbocation ion) which has been linked to glutathione (GSH) depletion, oxidative stress, protein-, DNA-, and GSH-adduct formation, probably at or near the site of formation (Phillips *et al.*, 1981; Smith *et al.*, 2002). Therefore, the sulfonate conjugate of 1′-hydroxyestragole is considered to be the ultimate hepatotoxic and hepatocarcinogenic agent in rodents (Boberg *et al.*, 1983). This conclusion was corroborated by co-administration of the specific sulfotransferase (SULT) inhibitor pentachlorophenol (PCP), which resulted in a potent inhibition of hepatic tumour induction by long-term dietary administration of the closely related alkenylbenzenes safrole or 1′-hydroxysafrole and a significant decrease of SULT activity and hepatic DNA and RNA adduct formation by 1′-hydroxysafrole in mice (Boberg *et al.*, 1983).

A significant difficulty in evaluating the metabolic, biochemical, and toxicological data for estragole as well as other alkenylbenzenes is that human exposure to these substances results from exposure to a complex mixture of food, spice, and spice oil constituents which may significantly impact the biochemical fate and toxicological risk of the alkenylbenzenes. In this respect, we have previously demonstrated that a methanolic extract from basil, which contained estragole, also contained the flavone nevadensin

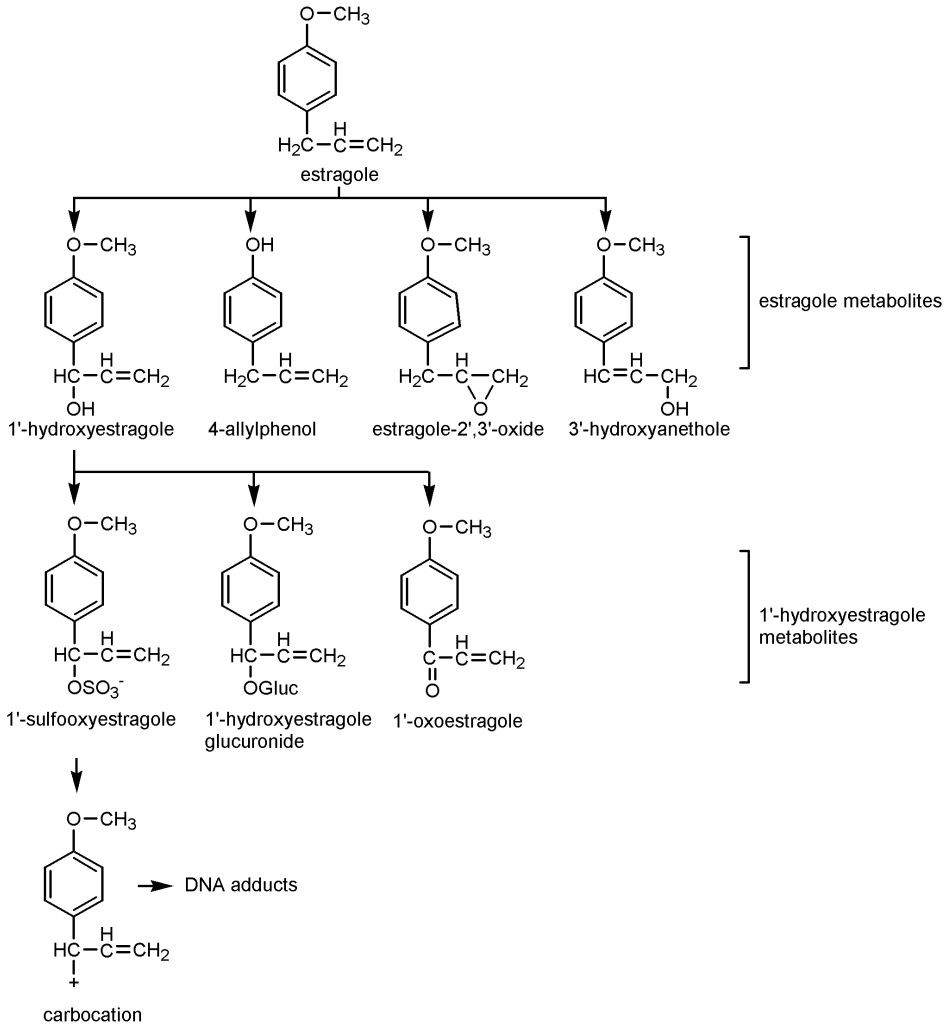


Figure 1. Bioactivation and detoxification pathways of estragole.

(5,7-dihydroxy-6,8,4′-trimethoxyflavone) which was able to inhibit DNA-adduct formation in rat hepatocytes exposed to the proximate carcinogen 1′ hydroxyestragole (Jeurissen *et al.*, 2008; Alhusainy *et al.*, 2010). This inhibition by nevadensin was shown to occur at the level of SULT-mediated bioactivation of 1′-hydroxyestragole (Jeurissen *et al.*, 2008; Alhusainy *et al.*, 2010). Altogether, the results of our previous research point at a reduction of DNA-adduct formation and consequently a potential reduction of cancer risk when estragole exposure occurs within a food matrix containing SULT inhibitors compared with what is observed upon exposure to pure estragole. Given these previous results, the objectives of the present study were i) to identify the presence

and nature of possible SULT inhibitors able to interfere with estragole bioactivation in other alkenylbenzene-containing botanical preparations and ii) to predict the possible effects of combined exposure to a mixture of SULT inhibitors on formation levels of the ultimate carcinogen 1′-sulfooxyestragole at physiologically relevant concentrations by incorporating the kinetics for SULT inhibition by the compounds in the mixture into our recently developed physiologically based biokinetic (PBBK) models for estragole in the liver of rat (Punt *et al.*, 2008) and human (Punt *et al.*, 2009).

MATERIALS AND METHODS

Materials and chemicals

1′-Hydroxyestragole and 1′-acetoxyestragole are genotoxic and carcinogenic compounds and caution should be taken when handling them.

Twelve dried herbs and spices were purchased from local supermarkets: nutmeg and mace (*Myristica fragrans* Houtt.), star anise (*Illicium verum* Hook. f.), parsley (*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill), pimento/allspice (*Pimenta dioica* (L.) Merr.), cinnamon (*Cinnamomum zeylanicum* J. Presl.), dill (*Anethum graveolens* L.), laurel (*Laurus nobilis* L.), tarragon (*Artemisia dracunculus* L.), fennel (*Foeniculum vulgare* Mill.), anise (*Pimpinella anisum* L.), and basil (*Ocimum basilicum* L.). 7-Hydroxycoumarin (7HC; 99%), 7-hydroxycoumarin sulfate (7HCS; 99%), quercetin, kaempferol, myricetin, apigenin, luteolin, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), GSH, uridine 5′-diphosphoglucuronic acid (UDPGA), and fetal bovine serum were obtained from Sigma Aldrich (Steinheim, Germany). Nicotinamide adenine dinucleotide (NAD+) was obtained from Roche Diagnostics (Mannheim, Germany).

Nevadensin was purchased from Apin Chemicals (Milton, U.K.). Trifluoroacetic acid was obtained from Mallinckrodt Baker B.V. (The Netherlands). Methanol (Ultra Liquid Chromatography/Mass Spectrometry, ULC/MS) and acetonitrile (ULC/MS) were obtained from Biosolve (Valkenswaard, The Netherlands), and pro-analysis grade acetic acid was purchased from VWR (Darmstadt, Germany). Hanks Balanced Salt Solution (HBSS), PBS, and gentamicin were purchased from Gibco (Paisley, U.K.). DMEM-F12 L-glutamine medium, tris-hydroxymethylaminomethane (tris), and trypsin were obtained from Invitrogen (Breda, The Netherlands). Dimethyl sulfoxide (DMSO), spectroscopic grade (99.9%) was supplied by Acros Organics (Geel, Belgium).

Pooled male rat (Sprague–Dawley), pooled human liver S9, and microsomal homogenates were obtained from BD Gentest (Woburn, MA), and PCP (98%) was obtained from Sigma-Riedel de Haen (Seelze, Germany). Nano pure water was obtained from a Barnstead Nano pure Type I ultrapure water system.

1′-Hydroxyestragole was synthesized as described previously by Drinkwater *et al.* (1976) based on a method described for the synthesis of 1′-hydroxysafrole (Borchert *et al.*, 1973). Structural confirmation by Gas Chromatography-Mass Spectrometry (GC–MS) was carried out as described previously (Punt *et al.*, 2007).

Preparation of methanolic herb and spice extracts

Methanolic herb and spice extracts were prepared according to the method described previously (Jeurissen *et al.*, 2008; Alhusainy *et al.*, 2010). In short, herb and spice extracts were prepared by stirring 5 g of dried herb or spice twice for 2 h at room temperature, each time with 100 mL of a mixture of methanol, ultra-pure water, and acetic acid (ratio 80:19:1). The extracts obtained were filtered using a folded filter (Schleicher & Schuell), and the pooled filtrates were evaporated to dryness under vacuum using a Rotavapor apparatus (Heidolph Laborota 4000 efficient). Star anise was first minced using a pair of scissors, and then using a pestle and mortar to obtain an efficient extraction yield. The extraction yields for the different herbs and spices ranged between 18 and 49% (*w/w*). Afterwards, the dried extracts were dissolved in methanol to achieve three concentrated extract stocks (2, 5, and 20 mg/mL) as described previously (Alhusainy *et al.*, 2010).

Measurement of SULT activity

SULT activity and its inhibition by different herb and spice extracts (nutmeg, mace, star anise, parsley, pimento/allspice, cinnamon, dill, laurel, tarragon, fennel, anise, and basil) were quantified using the standard substrate 7-hydroxycoumarine (7HC) in line with our previous work (Alhusainy *et al.*, 2010). Unlike the sulfonated metabolite of 1′-hydroxyestragole, the metabolite resulting from the sulfonation of 7HC is stable in aqueous solution and can be detected and quantified by High Performance Liquid Chromatography-Ultraviolet detection (HPLC–UV) as described previously (Wang *et al.*, 2006; Alhusainy *et al.*, 2010).

Identification of SULT inhibitors in alkenylbenzene-containing herbs and spices

The methanolic herb and spice extracts which resulted in the most potent inhibition of SULT activity were fractionated using HPLC–UV, and the fractions obtained were tested for their effect on SULT activity as described in our previous work (Alhusainy *et al.*, 2010). The compounds in the most active fractions were identified based on comparison of their UV spectra and retention time to the UV spectra and the retention times of commercially available reference compounds. In addition, a literature review was made to screen for major SULT inhibitors present in these alkenylbenzene-containing herbs and spices.

Kinetics for inhibition of SULT-catalyzed conversion of 7HC into 7HCS and of oxidation and glucuronidation of 1′-hydroxyestragole into 1′-oxoestragole and 1′-hydroxyestragole glucuronide by selected flavonoids

The *K*i for SULT inhibition was determined by performing incubations for SULT activity with pooled male rat liver S9 fractions in the presence of increasing concentrations of 7HC (0–100 μM) in the absence or presence of 0.75 μM quercetin, 0.3 μM kaempferol, 5 μM myricetin, or 0.35 μM apigenin. These flavonoid concentrations were selected because they were close to the identified inhibitory concentration 50% (IC_{50}) values

determined with pooled male rat liver S9 fractions (data not shown). Incubations were performed as described previously (Alhusainy *et al.*, 2010).

The possible effects of the identified SULT inhibitors on the detoxification of 1′-hydroxyestragole by glucuronidation and oxidation (Figure 1) were quantified in vitro as well. The *K*i for the inhibition of 1′-hydroxyestragole oxidation was determined by performing incubations with pooled human liver microsomes in the presence of increasing concentrations of 1′-hydroxyestragole (0–1000 μM) in the absence or presence of 5 μM quercetin, 5 μM kaempferol, 10 μM myricetin, and 10 μM apigenin. These flavonoid concentrations were selected because they were close to the identified IC_{50} values determined with pooled human liver microsomal fractions (data not shown). Incubations were performed as previously described (Alhusainy *et al.*, 2010). Subsequently, the maximum velocity (V_{max}), the Michaelis–Menten constant (K_m), and the inhibition constant (*K*i) for the formation of 7HCS and 1′-oxoestragole were determined as described below (Data Analysis).

Incubations for testing the effect of the identified SULT inhibitors on 1′-hydroxyestragole glucuronidation were also performed. In short, the effect of the identified botanical constituents quercetin, kaempferol, myricetin, and apigenin on 1′-hydroxyestragole glucuronidation was examined with pooled human liver microsomes. The incubations were performed as described previously (Punt *et al.*, 2009; Alhusainy *et al.*, 2010) in the absence or presence of each respective flavonoid up to a concentration of 80 μM and at 1000 μM of 1′ hydroxyestragole (a concentration close to the K_m value determined by Punt *et al.* (2009) for the formation of 1′-hydroxyestragole glucuronide by pooled human liver microsomes, both added from 200 times concentrated stock solutions in DMSO.

Effect of flavonoid mixtures on SULT activity and on oxidation of 1′-hydroxyestragole

Incubations for testing the SULT inhibition by a mixture of quercetin, kaempferol, apigenin, and nevadensin (each flavonoid added from a 400 times concentrated stock solution in DMSO) were performed using pooled male rat liver S9 fractions at concentrations equal to 0*K*i (DMSO control), 0.2*K*i, 0.5*K*i, 1*K*i, 2*K*i, 5*K*i, or 10*K*i of each flavonoid together with 25 μM of the substrate 7HC with incubation conditions similar to what was described previously (Alhusainy *et al.*, 2010).

Incubations for testing inhibition of conversion of 1′ hydroxyestragole to 1′-oxoestragole by a mixture of quercetin, kaempferol, myricetin, and apigenin were performed using pooled human liver microsomes. These flavonoids were added to the incubation mixtures from 400 times concentrated stock solutions in DMSO at final concentrations equal to 0*K*i (DMSO control), 0.1*K*i, 0.2*K*i, 0.5*K*i, 1*K*i, or 2*K*i of each flavonoid together with 400 μM of the substrate (a concentration close to the K_m value determined by Punt *et al.* (2009) for the conversion of 1′-hydroxyestragole to 1′-oxoestragole by pooled human liver microsomes) using incubation conditions similar to what was described previously (Alhusainy *et al.*, 2010).

Cultivation and treatments of HepG2 cells

A total of approximately 1×10^6 HepG2 cells/well were seeded in a six-well plate with each well containing DMEM/F12 medium containing GlutaMax, 10% fetal bovine serum, and 50 µg/mL gentamycin and incubated in a humidified incubator, 5% CO_2 and 95% air at 37°C until confluent monolayers were obtained. Then, cells were washed with HBSS. Cytotoxicity of the test compounds was evaluated using the MTT test (Hussain *et al.*, 1993).

For testing detoxification and bioactivation of 1′-hydroxyestragole in response to different flavonoid treatments, cells were exposed to 50 µM 1′-hydroxyestragole, added from a 1000 times concentrated stock solution in DMSO, in the absence or presence of the flavonoid mixtures. All flavonoids when tested individually or in a mixture (Table 4 in the Results section) were added individually from 800 times concentrated stock solutions in DMSO to DMEM/F12 containing GlutaMax and 50 µg/mL gentamicin and incubations were performed for 22 h at 37°C in a humidified atmosphere.

To measure the effect of the different flavonoids or their mixtures on the formation of the major DNA adduct N^2-(*trans*-isoestragol-3′-y1)-2′-deoxyguanosine (E-3′-N^2-dGuo), cells were scraped in PBS and the cells of six wells were pooled in an Eppendorf vial and centrifuged at 6,000 rpm for 5 min (Eppendorf centrifuge, type 5415C, Hamburg, Germany) to obtain pellets. The pellets were stored at −20°C until DNA isolation using a Dojindo Get pure DNA Kit Cell, Tissue kit (Dojindo, Amsterdam, The Netherlands), according to the accompanying protocol. Extraction, digestion, and quantification of E-3′-N^2-dGuo were performed as previously described (Paini *et al.*, 2010).

Identification, analysis, and quantification of metabolites

Identification and analysis of 7HCS, 1′-hydroxyestragole glucuronide, and 1′-oxoestragole by HPLC–UV and their quantification by calibration curves using available standards was performed as described in our previous work (Punt *et al.*, 2008, 2009; Alhusainy *et al.*, 2010). Detection and quantification of the major DNA adduct N^2-(*trans*-isoestragol-3′-y1)-2′-deoxyguanosine (E-3′-N^2-dGuo) by Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC–ESI–MS/MS) was performed as previously described (Paini *et al.*, 2010).

Data analysis

The maximum velocity (V_{max}) and Michaelis–Menten constant (K_m) for the formation of 7HCS and 1′-oxoestragole were determined by fitting the data to the standard Michaelis–Menten equation $V = V_{max}[S]/(K_m+[S])$, with [S] being the substrate concentration (µM), using the Life Science Workbench data analysis toolbox (version 1.1.1, MDL information Systems, Inc.). The inhibition constant (Ki) for each of the selected flavonoids was calculated using the Michaelis–Menten equation for non-competitive inhibition ($V^{app}_{max}=V_{max}/(1+([I]/Ki))$, with V^{app}_{max} being the apparent V_{max} for the reaction in the presence of inhibitor(s) (nmol/min/mg S9 or microsomal protein), V_{max} being V_{max} for the reaction in the absence of inhibitor(s) (nmol/min/mg S9 or microsomal protein), and [I] being the inhibitor concentration (µM).

The Michaelis–Menten equation representing an additive effect for a group of non-competitive inhibitors was derived as follows:

$$V = V_{max}/(1+([I_1]/Ki_1) +([I_2]/Ki_2)\ldots +([I_n]/Ki_n))*[S]/(K_m+[S])$$

The derivation is based on the assumption that each enzyme molecule can combine with no more than one of the inhibitors at a time. To test whether the change in the V_{max} and K_m in the presence of the different inhibitors or whether the SULT inhibition by the methanolic herb and spice extracts as compared with control was significant, a two-sample *t*-test (one sided) was performed after determining variances equality by the Levene's version of the F test with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL).

Estragole PBBK model

To obtain insight in the quantitative dose- and species-dependent effects of the flavonoid inhibitors on the formation of the ultimate carcinogenic metabolite 1′-sulfooxyestragole, in the target organ, the liver, we modified our previously developed PBBK models to take the inhibition into account. These models were originally developed to simulate the bioactivation, and detoxification of estragole in both rat (Punt *et al.*, 2008) and human (Punt *et al.*, 2009). The original models were described in detail previously (Punt *et al.*, 2008, 2009) and include separate compartments for liver, lung, and kidney, representing organs which were found to be involved in the metabolism of estragole. A separate compartment for fat tissue was included in order to take into account the relatively higher partition coefficient of estragole in fat tissue. All other tissues were lumped into either a rapidly perfused or slowly perfused tissue group (Ramsey and Andersen, 1984). The physiological parameters were obtained from literature (Brown *et al.*, 1997). The partition coefficients were estimated from the log Kow based on a published method (DeJongh *et al.*, 1997). Log Kow values were estimated using the software package ClogP version 4.0 (Biobyte, Claremont, CA) and amounted to 3.1 for estragole and 1.6 for 1′-hydroxyestragole. The apparent V_{max} values for the different phase I and II reactions, expressed as nmol/min/mg microsomal or S9 protein were determined in vitro and were scaled to the liver using a microsomal protein yield of 32 mg/g liver (Barter *et al.*, 2007) or using an S9 protein yield of 143 mg/g liver (Medinsky *et al.*, 1994). The apparent K_m values were also determined in vitro and were assumed to correspond to the apparent in vivo K_m values. The absorption rate constant (Ka) was set to 1.0/h, resulting in a rapid absorption of estragole from the gastrointestinal tract with an absorption half-life of 0.7 h (Anthony *et al.*, 1987).

Mass balance equations were applied to describe the Absorption, Distribution, Metabolism and Excretion (ADME) of estragole in the different tissue compartments, and the kinetic parameters for the different bioactivation and detoxification reactions of estragole were based on in vitro kinetic data obtained using relevant rat and human tissue fractions. A full description of the mass balance equations can be found in our previous papers (Punt *et al.*, 2008, 2009). Model equations were coded and numerically integrated in Berkeley Madonna 8.0.1 (Macey and Oster, UC Berkeley, CA, USA) using the rosenbrock's algorithm for stiff systems. The predictions of the PBBK model for

male rat (Punt *et al.*, 2008) for the formation of 4-allylphenol and 1′-hydroxyestragole glucuronide were in good agreement with observations in the literature in female Wistar rats in vivo (Anthony *et al.*, 1987), and the PBBK model for human (Punt *et al.*, 2009) could predict levels of the same metabolites within the same order of magnitude compared with the reported levels in vivo in two human volunteers (Sangster *et al.*, 1987).

The uptake of estragole from the gastrointestinal tract in the PBBK models is described by a first-order process, assuming direct entry from the intestine to the liver compartment; thus, uptake of estragole into the liver is set at 100% in line with what is described in literature for estragole (Sangster *et al.*, 1987; Punt *et al.*, 2008, 2009).

In the present study, the PBBK models developed by Punt *et al.* (2008, 2009) for rat and human were modified as described below to take the flavonoid inhibition into account. The modified models were subsequently used to predict the effect of three different flavonoid scenarios (Table 5 in the Results section), representing relevant dietary flavonoid intakes, on the formation of the ultimate carcinogenic metabolite, 1′-sulfooxyestragole, using an estragole dose of 0.01 mg/kg bw/day, representing the estragole dose considered relevant for human dietary exposure from herbs and spices mainly (Smith *et al.*, 2002). Only flavonoids which had an effect on SULT activity, as well as on oxidation of 1′-hydroxyestragole, were included in the first two scenarios (Table 5 in the Results section). The modified models did not include sub-models for ADME characteristics of each flavonoid inhibitor. Rather, as a first approximation, each flavonoid included was assumed to follow a similar time-dependent concentration curve in the liver of rat and human as that of estragole. To make the link between the external dose of compounds and the internal dose in the liver, the molar ratio (L) between estragole at a dose of 0.01 mg/kg bw/day and each SULT inhibitor was calculated based on the relevant intake scenarios in Table 5 and assumed to be maintained in the liver by keeping the molar ratio (L) between estragole and each flavonoid constant during the modeling time (24 h). This was represented in the model using the following equation: $[I] = CL_E \times L \times f$, where [I] is flavonoid concentration in the liver (μmol/l), CL_E is the concentration of estragole in the liver (μmol/l), L is the molar ratio between estragole and each SULT inhibitor which is calculated based on an estragole dose of 0.01 mg/kg bw/day and the relevant intake scenario of flavonoid(s) (Table 5 in the Results section). To obtain some insight into the effects resulting from differences in kinetics and uptake of estragole on one hand and the flavonoids on the other hand, the product of $CL_E \times L$ was multiplied with the factor (f) which varied from 0 to 1. Thus, when f equals 0, this represents no uptake of the flavonoid into the liver, whereas when f equals 1, this represents a 100% uptake of the flavonoid into the liver. The Michaelis–Menten equation representing the sulfonation or oxidation of 1′-hydroxyestragole in the presence of a single inhibitor was as follows:

$$dAMHES/dt = V_{max,L\text{-}HES}/(1+([I]/Ki)) \times CL_{HE}/PL_{HE}/(K_{m,L\text{-}HES} + (CL_{HE}/PL_{HE}))$$

and

$$dAMHEO/dt = V_{max,L\text{-}HEO}/(1+([I]/Ki)) \times CL_{HE}/PL_{HE}/(K_{m,L\text{-}HEO} + (CL_{HE}/PL_{HE}))$$

where dAMHES/d*t* is the rate of 1′-sulfooxyestragole formation (μmol/h) and dAMHEO/d*t* is the rate of 1′-oxoestragole formed (μmol/h), $V_{max,L\text{-}HES}$ is the maximum rate of formation of 1′-sulfooxyestragole, $V_{max,L\text{-}HEO}$ is the maximum rate of formation of 1′-oxoestragole, [I] is the concentration of quercetin (second scenario, Table 5 in the Results section) or nevadensin (third scenario, Table 5 in the Results section) in the liver (μmol/l). Correspondingly, *K*i is the inhibition constant for the inhibition by the respective flavonoids (μmol/L). $K_{m,L\text{-}HES}$ is the Michaelis–Menten constant for the formation of 1′-sulfooxyestragole (μmol/L), $K_{m,L\text{-}HEO}$ is the Michaelis–Menten constant for the formation of 1′-oxoestragole (μmol/L), CL_{HE} is the concentration of 1′-hydroxyestragole in the liver (μmol/L), PL_{HE} is the liver/blood partition coefficient. The Michaelis–Menten equations representing the sulfonation or oxidation of 1′-hydroxyestragole in the presence of a mixture of non-competitive inhibitors were derived assuming an additive effect and that each enzyme molecule can combine with no more than one of the inhibitors at a time and were as follows:

$$dAMHES/dt = V_{max,L\text{-}HES}/(1+([I_1]/Ki_1)+([I_2]/Ki_2)+([I_3]/Ki_3)+([I_4]/Ki_4))\times CL_{HE}/PL_{HE}/(K_{m,L\text{-}HES}+(CL_{HE}/PL_{HE}))$$

and

$$dAMHEO/dt = V_{max,L\ HEO}/(1+([I_1]/Ki_1)+([I_2]/Ki_2)+([I_3]/Ki_3)+([I_4]/Ki_4))\times CL_{HE}/\ PL_{HE}\ /(K_{m,L\text{-}HEO}+(CL_{HE}/PL_{HE}))$$

where $[I_1]$, $[I_2]$, $[I_3]$, and $[I_4]$ are the concentrations of the inhibitors: quercetin, kaempferol, myricetin, and apigenin in the liver (μmol/L), respectively. Correspondingly, Ki_1, Ki_2, Ki_3, and Ki_4, are the inhibition constants for the inhibition by the respective inhibitors (μmol/L), which were assumed to be equal in liver of human and rat.

RESULTS

Inhibition of SULT activity by methanolic herb and spice extracts

Figure 2 presents the effect of increasing concentrations of a series of methanolic herb and spice extracts on SULT activity. With the exception of fennel, increasing concentrations of all methanolic herb and spice extracts inhibited SULT enzyme activity in a dose-dependent manner. The extract from basil was the most potent followed by the extracts from pimento/ allspice, nutmeg, and mace. Extracts from parsley, star anise and cinnamon displayed only moderate SULT inhibition and the extracts from tarragon and laurel significantly inhibited SULT activity only at the highest concentration tested (100 μg extract/mL). Blank incubations, which lacked the cofactor PAPS or which lacked the pooled male rat liver S9 fraction, did not show any formation of 7HCS (data not shown). This indicates that the inhibition of 7HCS formation by the herb and spice extracts was fully dependent on the presence of PAPS and the S9 fraction containing SULT. Incubations with the SULT-inhibitor PCP inhibited SULT activity completely at 25 μM (data not shown).

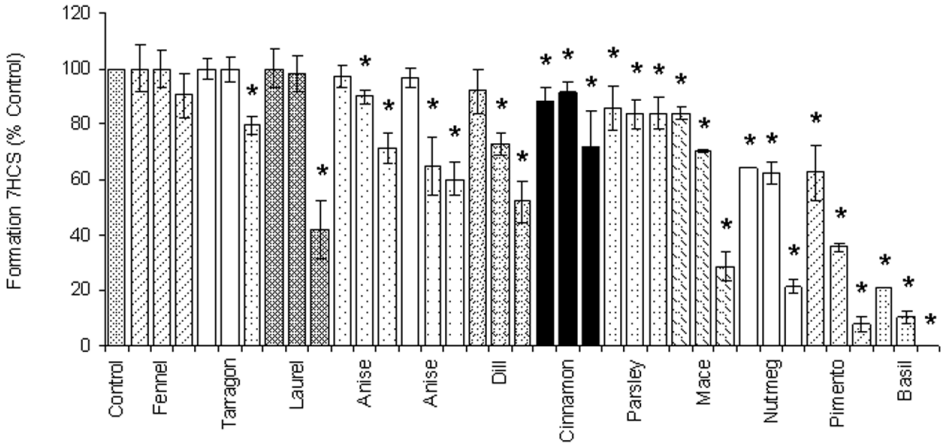


Figure 2. Inhibition of SULT-catalyzed conversion of 7HC into 7HCS by pooled male rat liver S9 fractions in the absence (control) or presence of increasing concentrations (10, 25, and 100 µg/mL) of different methanolic herb and spice extracts. 100% 7HCS formation is equal to 1.3 ± 0.1 nmol/min/mg S9 protein. Data points represent mean (±SD) of triplicate measurements obtained in independent experiments. An asterisk (*) indicates a significant inhibition compared with the incubation without inhibitor ($p < 0.05$).

Identification of SULT inhibitors in the methanolic herb and spice extracts

Based on our previous work (Alhusainy *et al.*, 2010), the flavonoid nevadensin was identified as the major SULT-inhibiting constituent in the methanolic extract from basil. In the present work, based on comparison of HPLC–UV and retention time characteristics to HPLC–UV and retention time characteristics of commercially available reference compounds, apigenin was identified as the second most potent constituent in the same methanolic extract. Among the other most potent methanolic herb and spice extracts, the extract from nutmeg was fractionated and the compounds in the most active fractions were identified as (+) catechin and (−) epicatechin, whereas in the methanolic extract from pimento/allspice, eugenol was identified as the major SULT inhibitor.

Based on the observation that in potent extracts flavonoids appeared to be the major SULT inhibitors identified, a literature review was made to screen for major flavonoids present in alkenylbenzene-containing herbs and spices focusing on flavonoids which have been reported to be SULT inhibitors (Eaton *et al.*, 1996; Morimitsu *et al.*, 2004). Table 1 presents an overview of the flavonoids reported to be present in alkenylbenzene-containing herbs and spices as derived from literature and also reported to be SULT inhibitors. The overview presented reveals that in a series of 15 selected alkenylbenzene-containing herbs and spices, quercetin, kaempferol, apigenin, and luteolin are among the most abundant flavonoids, and of these flavonoids, quercetin and kaempferol are the most widespread in alkenylbenzene-containing herbs and spices (Justesen and Knuthsen, 2001).

Table 1. flavonoids present in some alkenylbenzene-containing herbs and spices based on literature.

Herb	Major flavonoids identified in herbs and spices based on literature and their amounts	References
Basil (*Ocimum basilicum* L.)	Nevadensin (3.78-43.47)[b] Apigenin (0.48-2.14)[b] Luteolin (0.48-0.78)[b] Catechin[c]	(Grayer *et al.*, 2004) (Grayer *et al.*, 2004) (Grayer *et al.*, 2004) (Shan *et al.*, 2005)
Pimento (*Pimenta dioica* (L.) Merr.)	Quercetin and myricetin[c]	(Kikuzaki *et al.*, 2008)
Nutmeg (*Myristica fragrans* Houtt.)[a]	Catechin[c] Quercetin and kaempferol[c]	(Shan *et al.*, 2005) **(Suhaj, 2006)**
Dill (*Anethum graveolens* L.)	Quercetin (48-110)[d] Kaempferol (16-24)[d]	(Justesen and Knuthsen, 2001) **(Suhaj, 2006)**
Tarragon (*Artemisia dranunculus* L.)	Quercetin (10)[d] Kaempferol (11)[d] Luteolin (1)[d]	(Justesen and Knuthsen, 2001) (Justesen and Knuthsen, 2001) (Justesen and Knuthsen, 2001)
Parsley (*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill)	Quercetin (0-1)[d] Apigenin (510-630)[d] Luteolin (0-4)[d]	(Justesen and Knuthsen, 2001) (Justesen and Knuthsen, 2001) (Justesen and Knuthsen, 2001)

[a] Nutmeg is the actual seed of the tree while mace is the dried "lacy" reddish covering or aril of the seed.
[b] Amounts expressed as percentages of total flavones.
[c] Amounts are not reported.
[d] Amounts expressed as (mg/100 g fresh weight).

Selection of SULT-inhibiting flavonoids to be tested in subsequent studies

Given the identification of flavonoids as an important category of food-borne SULT inhibitors, and the fact that these flavonoids will not only be present in the herbs and spices analyzed in this study, the selection of the flavonoids to be used in the subsequent studies was based on their abundance in the diet as a whole. Generally, estimation of the daily dietary flavonoid intake is based on the intake of three quantitatively important dietary flavonols (quercetin, kaempferol, and myricetin) and two quantitatively important dietary flavones (apigenin and luteolin) (Hertog *et al.*, 1993a,b). These flavonoids are all present in alkenylbenzene-containing herbs and spices, as well as throughout the diet, they are all reported to have SULT inhibiting potency; the total estimated daily intake (EDI) levels of these flavonoids as recorded by different studies varies between 18.3 ± 3.4 and 25.9 ± 14.5 mg/day (Hertog *et al.*, 1993a; Hertog *et al.*, 1993b; Lugasi *et al.*, 2003; Mullie *et al.*, 2008).

Given these observations, the flavonoids quercetin, kaempferol, myricetin, apigenin, luteolin, and nevadensin, the latter being the major flavone constituent in basil (Grayer *et al.*, 2004) were used in our subsequent experiments to examine the influence of flavonoids in mixtures or individually on the bioactivation of 1′-hydroxyestragole by SULT and on the detoxification of 1′-hydroxyestragole via glucuronidation and

oxidation using relevant tissue fractions or an intact cell model. Figure 3 presents the structure of the flavonoid inhibitors included in the study.

Inhibition of SULT-catalyzed conversion of 7HC into 7HCS and of oxidation and glucuronidation of 1′-hydroxyestragole into 1′-oxoestragole and 1′-hydroxyestragole glucuronide by the selected flavonoids

After identification of major SULT inhibitors present in the selected alkenylbenzene-containing herbs and spices (nevadensin, quercetin, kaempferol, myricetin, and apigenin), initial studies testing the effect of the selected flavonoids quercetin, kaempferol, myricetin and apigenin on SULT-mediated conversion of 7HC into 7HCS and on oxidation or glucuronidation of 1′-hydroxyestragole revealed that only SULT and oxidation activity appeared to be inhibited. Nevadensin was not included in these inhibition studies because its effect on sulfonation, glucuronidation, and oxidation was already determined in our previous work (Alhusainy *et al.*, 2010). Oxidation and glucuronidation of 1′-hydroxyestragole were not inhibited by nevadensin to any significant extent even at nevadensin concentrations up to 20 μM (Alhusainy *et al.*, 2010).

The effect of the other selected flavonoids quercetin, kaempferol, myricetin, and apigenin on glucuronidation of 1′-hydroxyestragole was examined by adding each flavonoid up to 80 μM together with 1000 μM of the substrate 1′-hydroxyestragole in incubations with pooled human liver microsomes and the cofactor for glucuronidation (UDPGA). None of the flavonoids tested resulted in any significant effect on the formation of 1′-hydroxyestragole glucuronide.

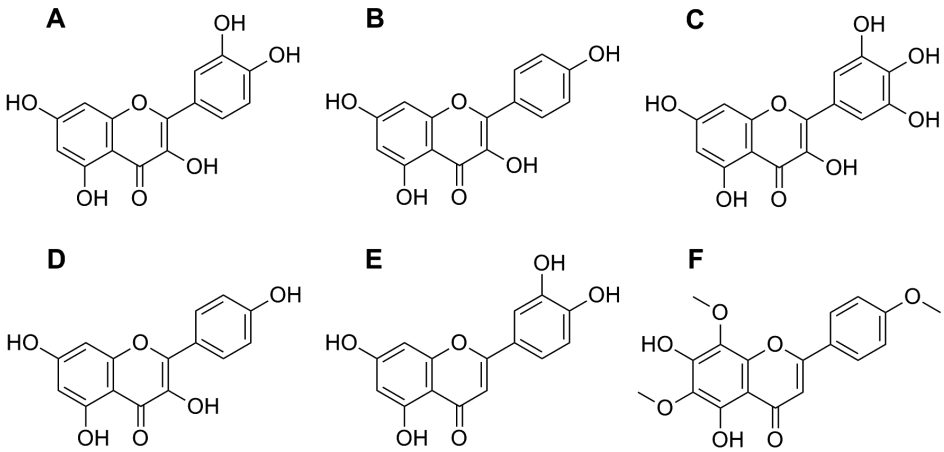


Figure 3. Structural formulas of (A) quercetin, (B) kaempferol, (C) myricetin, (D) apigenin, (E) luteolin, and (F) nevadensin, the major SULT inhibitors in alkenylbenzene-containing herbs and spices and/or throughout the diet.

Because the catalytic efficiency for the SULT-catalyzed conversion of 7HC into 7HCS and for 1′-hydroxyestragole into 1′-sulfooxyestragole is higher in the liver of rat compared with the liver of human (Wang *et al.*, 2006; Alhusainy *et al.*, 2010), in vitro incubations for inhibition of SULT activity by the selected flavonoids was performed using pooled male rat liver S9. Oxidation of 1′-hydroxyestragole to 1′-oxoestragole represents a minor metabolic route in the liver of male rat as compared with the liver of human suggesting that the inhibition of this pathway would hardly influence the overall bioactivation of estragole in the liver of male rat but may influence the overall metabolism in human. Therefore, in vitro incubations for inhibition of oxidation of 1′-hydroxyestragole by the selected flavonoids were performed in pooled human liver microsomes. In this way, the kinetics for inhibition of SULT activity and of oxidation of 1′-hydroxyestragole were determined, and the mode of inhibition and the respective *K*i values were defined.

Tables 2 and 3 display the Michaelis–Menten parameters (V_{max} and K_m) and inhibition constants (*K*i) for 7HCS and 1′-oxoestragole formation by pooled male rat liver S9 and pooled human liver microsomal fractions, respectively, in the presence or absence of the flavonoids quercetin, kaempferol, myricetin, and apigenin. The apparent V_{max} values for sulfonation were lowered significantly (minus 30–33%; $p < 0.001$) in the presence of each flavonoid, whereas the apparent K_m values were not statistically significantly different from the K_m value determined in the absence of each flavonoid. The apparent V_{max} values for oxidation of 1′-hydroxyestragole were lowered significantly (minus 52–77%; $p < 0.001$) in the presence of each flavonoid, whereas the apparent K_m values were not statistically significantly different from the K_m value determined in the absence of each flavonoid. These results point at a non-competitive type of inhibition of sulfonation, as well as oxidation by each individual flavonoid. Applying the Michaelis–Menten equation for non-competitive inhibition, the inhibition constant (*K*i) for SULT inhibition as calculated from the data amounted to, respectively, 1.5, 0.6, 11.9, and 0.7 μM for quercetin, kaempferol, myricetin, and apigenin.

The inhibition constants (*K*i) for inhibition of the conversion of 7HC to 7HCS by the different flavonoids are not expected to be significantly different when using 7HC instead of 1′-hydroxyestragole as a substrate. This is based on the type of inhibition that was shown to be non-competitive meaning that only the apparent V_{max} and not the apparent K_m of the enzyme was found to be affected, which corroborates the assumption that flavonoids do not interfere with the substrate binding site and exert their inhibiting effect by interaction with another site on the enzyme than the site involved in substrate binding (Simmons, 1996). In our previous work (Alhusainy *et al.*, 2010), the type of SULT inhibition (non-competitive) and the *K*i (4 nM) for this inhibition by the flavonoid nevadensin was similar in pooled male rat and pooled human liver S9 fractions. Based on this result, it was assumed that the *K*i and the type of SULT inhibition by the flavonoids quercetin, kaempferol, myricetin, and apigenin determined in pooled male rat liver S9 will also be similar in human liver S9, given that the type of inhibition by each of them was also non-competitive.

Table 2. Kinetic parameters of SULT-dependent formation of 7HCS by pooled male rat Liver S9 in the absence or presence of different flavonoids.

Inhibitor	Apparent V_{max} (nmol/min/mg protein)	Apparent K_m (μM)	*Ki* [a] (μM)
Quercetin (0.75 μM)	1.8±0.1*	4.3±0.6	1.5
Kaempferol (0.3 μM)	1.8±0.2*	4.0±1.1	0.6
Myricetin (5 μM)	1.9±0.01*	3.8±0.3	11.9
Apigenin (0.35 μM)	1.8±0.1*	3.5±0.6	0.7

[a]Calculated using the Michaelis–Menten equation for non-competitive inhibition ($V^{app}_{max}=V_{max}/(1+([I]/Ki))$, where [I] is the flavonoid concentration (μM).
An asterisk (*) indicates a significant inhibition compared to the incubation without inhibitor ($P<0.001$).
Note1: The control (no inhibitor) V_{max}=2.7±0.2 nmol/min/mg protein, and K_m=4.0±1.0 μM.

Table 3. Kinetic parameters of 1′-oxoestragole formation by pooled human liver microsomes in the absence or presence of different flavonoids.

Inhibitor	Apparent V_{max} (nmol/min/mg protein)	Apparent K_m (μM)	*Ki* [a] (μM)
Quercetin (5 μM)	4.8±1.0*	1046±258	4.5
Kaempferol (5 μM)	2.3±0.3*	605±69	1.5
Myricetin (10 μM)	3.5±0.9*	727±292	5.4
Apigenin (10 μM)	4.0±0.4*	613±66	6.7

[a]Calculated using the Michaelis–Menten equation for non-competitive inhibition ($V^{app}_{max}=V_{max}/(1+([I]/Ki))$, where [I] is the flavonoid concentration (μM).
An asterisk (*) indicates a significant inhibition compared to the incubation without inhibitor ($P<0.001$).
Note 1:The control (no inhibitor) V_{max}=10±1.4 nmol/min/mg protein, and K_m=688±119 μM.

The inhibition constant (*Ki*) for inhibition of oxidation of 1′-hydroxyestragole to 1′-oxoestragole by quercetin, kaempferol, myricetin, and apigenin amounted to 4.5, 1.5, 5.4, and 6.7 μM, respectively (Table 3).

Effect of selected flavonoid mixtures on SULT-catalyzed conversion of 7HC into 7HCS and on oxidation of 1′-hydroxyestragole into 1′-oxoestragole

Given that the diet may contain a variety of SULT inhibitors, experiments were performed to assess the effect of combined flavonoid exposure on SULT activity, as well as on oxidation of 1′-hydroxyestragole to 1′-oxoestragole. To this end, a test mixture was defined that mimics a realistic dietary flavonoid mixture and included four flavonoids which were found to be abundant in alkenylbenzene-containing herbs and spices and able to inhibit SULT activity, namely, quercetin, kaempferol, apigenin, and

nevadensin, the latter being previously identified as a potent SULT inhibitor present in basil (Alhusainy *et al.*, 2010).

The simultaneous addition of quercetin, kaempferol, apigenin, and nevadensin at concentrations equal to 0.2*K*i, 0.5*K*i, 1*K*i, 2*K*i, 5*K*i, or 10*K*i of each flavonoid together with 25 μM of the substrate 7HC lowered the SULT activity in incubations with pooled male rat liver S9 (Figure 4). The reduction in the formation of 7HCS was similar to the reduction predicted by the Michaelis–Menten equation defined for a series of independent non-competitive inhibitors (Figure 4), which verifies our assumption for a non-competitive type of inhibition by each individual flavonoid on SULT enzyme activity, as well as an additive interaction between the flavonoids in the mixture (Figure 4).

With the exception of nevadensin that had no effect on 1′-hydroxyestragole oxidation in pooled human liver microsomal fractions (Alhusainy *et al.*, 2010), the other SULT inhibitors identified in alkenylbenzene-containing herbs and spices, namely, quercetin, kaempferol, myricetin, and apigenin, had an inhibiting effect on the oxidation of 1′-hydroxyestragole. Therefore, incubations for testing the inhibition of conversion of 1′-hydroxyestragole to 1′-oxoestragole by a mixture of quercetin, kaempferol, myricetin, and apigenin were performed. Figure 5 shows that the simultaneous addition of quercetin, kaempferol, myricetin, and apigenin at concentrations equal to 0.1*K*i, 0.2*K*i, 0.5*K*i, 1*K*i, or 2*K*i, for each flavonoid together with 400 μM of the substrate 1′-hydroxyestragole reduced the formation of 1′-oxoestragole. The observed inhibition in the formation of 1′-oxoestragole in the presence of the flavonoid mixture at concentrations higher than their *K*i values matched the predicted values of the derived Michaelis–Menten formula defined for a series of independent non-competitive inhibitors, whereas at concentrations lower than their *K*i values, the predicted values were somewhat higher than the values actually observed, which may be due to different types of enzymes playing a role in the oxidation

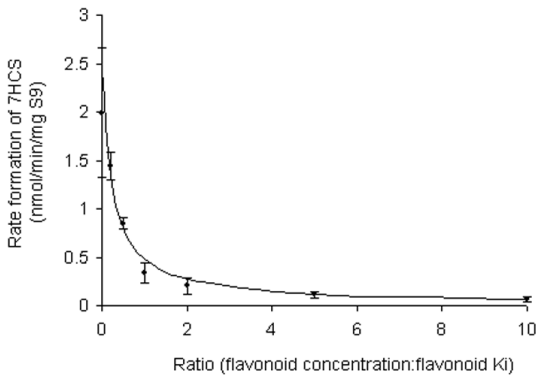


Figure 4. SULT-dependent formation of 7HCS as measured in incubations with pooled male rat liver S9 in the presence of increasing concentrations of flavonoid mixtures containing quercetin, kaempferol, apigenin, and nevadensin at 0*K*i, 0.2*K*i, 0.5*K*i, 1*K*i, 2*K*i, 5*K*i, or 10*K*i of each flavonoid (●),or as predicted by our derived Michaelis–Menten equation assuming an additive effect for a series of independent non-competitive inhibitors (solid line).

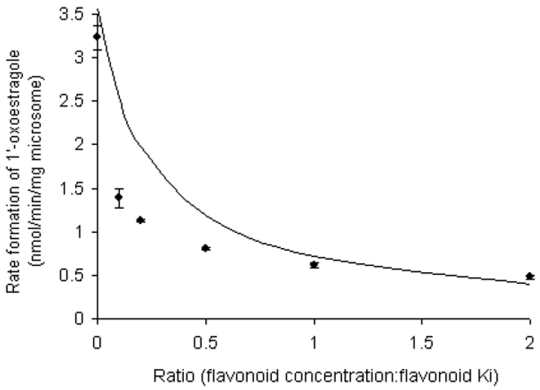


Figure 5. Formation of 1′-oxoestragole as measured in incubations with pooled human liver microsomes in the presence of increasing concentrations of flavonoid mixtures containing quercetin, kaempferol, myricetin, and apigenin at 0*K*i, 0.1*K*i, 0.2*K*i, 0.5*K*i, 1*K*i, or 2*K*i of each flavonoid (●),or as predicted by our derived Michaelis Menten equation assuming an additive effect for a series of independent non-competitive inhibitors (solid line).

of 1′-hydroxyestragole. Thus, other modes of interactions than an additive interaction at concentrations lower than *K*i values cannot be excluded. Nonetheless, at all concentrations of the flavonoid mixture, the difference between predicted values and observed ones was less than twofold. Moreover, a fit to the experimental data assuming the inhibition to be competitive instead of non-competitive matched the experimental data worse, showing deviations even at the high concentrations and larger variation (up to 2.2-fold) at the lower concentration range (data not shown). Therefore, it was concluded that the predictions made by the derived formula gives a reasonable first approximation.

Effect of flavonoid mixtures on conversion and DNA binding of 1′-hydroxyestragole in HepG2 cells

To investigate whether the inhibition of bioactivation and detoxification pathways of 1′-hydroxyestragole would also be observed in an intact cellular system, cells from the human HepG2 hepatoma cell line were exposed to 1′-hydroxyestragole in the absence or presence of different flavonoids and their combinations, followed by detection of the level of 1′-hydroxestragole oxidation, glucuronidation, and sulfonation, the latter reflected by the formation of the major estragole DNA adduct (E-3′-N^2-dGuo). Table 4 summarizes the different flavonoid mixtures and/or individual flavonoids which were investigated in the HepG2 model system. The outcomes of these studies will reveal whether selected flavonoids and/or their mixtures are able to inhibit SULT-mediated bioactivation of 1′-hydroxyestragole in this cellular system, resulting in an ultimate shift of metabolism in favour of detoxification at the cost of bioactivation.

The compounds were not cytotoxic to HepG2 cells under the conditions used in these experiments as observed by MTT activity measurements (data not shown). Figure 6A reveals that a significant reduction in the formation of E-3′-N^2-dGuo compared with

control (no flavonoid) is observed in the human HepG2 cells following co-administration of 50 μM of the substrate 1′-hydroxyestragole and 13, 5.4, 2.3, 1.5, and 0.34 μM of a flavonoid mixture containing quercetin, kaempferol, myricetin, apigenin, and luteolin respectively (each at a concentration corresponding to its relative contribution in the diet, Table 4). E-3′-N^2 dGuo formation in the HepG2 cells is also inhibited significantly by 100 μM of the same flavonoids in a mixture (each at 20 μM), by 50 μM kaempferol, or by 100 μM quercetin (Figure 6A).

The data indicate that the flavonoids are able to pass the cell membrane and exert an intracellular effect on SULT-mediated bioactivation of 1′-hydroxyestragole. HPLC-UV analysis of the media revealed a significant reduction in the formation of 1′-oxoestragole (measured as GS-1′-oxoestragole, Figure 6B) and a significant increase in the formation of 1′-hydroxyestragole glucuronide (Figure 6C). Altogether, the data indicate a shift metabolism from sulfonation and oxidation to glucuronidation, which is a detoxification pathway for 1′-hydroxyestragole.

PBBK model based predictions for estragole metabolism in the presence of selected SULT-inhibiting flavonoids

Figure 7A illustrates the PBBK model based predictions for the formation of 1′-sulfooxyestragole in the liver of rat and human after co-administration of 0.01 mg/kg bw/day of estragole, representing the estragole dose considered relevant for human dietary exposure from herbs and spices mainly (Smith *et al.*, 2002), and a mixture of the flavonoids quercetin, kaempferol, myricetin, and apigenin at a total EDI

Table 4. Flavonoid treatments tested in human HepG2 cells exposed to 50 μM of 1-hydroxyestragole for 22 h at 37°C.

Sample	Flavonoids treatments	Concentrations in 2.5 mL medium	Rationale
1	Quercetin, Kaempferol, Myricetin, Apigenin, Luteolin	13 μM 5.4 μM 2.3 μM 1.5 μM 0.34 μM	Based on dietary levels of 12, 4.6, 2.2, 1.2, and 0.3 mg/day (Lugasi *et al.*, 2003; Mullie *et al.*, 2008) assuming 3 liter plasma and 100% bioavailability
2	Quercetin, Kaempferol, Myricetin, Apigenin, Luteolin	20 μM 20 μM 20 μM 20 μM 20 μM	High dose mixture, up to a total level of 100 μM, shown to be without cytotoxicity.
3	Quercetin	100 μM	Based on highest dose without cytotoxicity given that the level in dietary quercetin supplements of 500 mg/day, assuming 3 liter plasma and 100% bioavailability would amount to 552 μM.
4	Kaempferol	50 μM	Kaempferol was the most potent flavonoid in inhibiting SULT activity and oxidation of 1′-hydroxyestragole. The concentration selected is equal to the concentration of the substrate, 1′-hydroxyestragole.

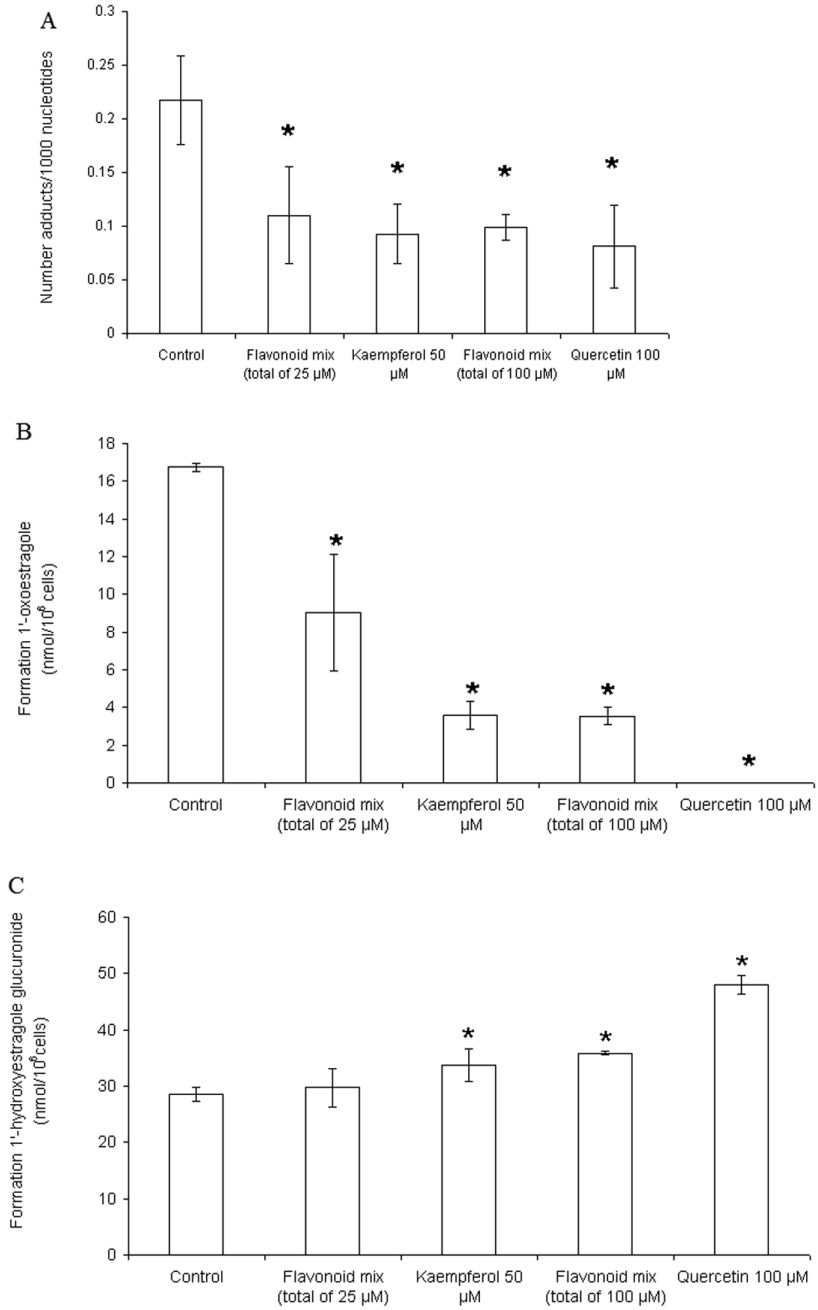


Figure 6. Formation of (A) E-3′-N^2-dGuo, (B) 1′-oxoestragole (measured as GSH adducts), and (C) 1′-hydroxyestragole glucuronide in HepG2 cells exposed for 22 h to 50 µM 1′-hydroxyestragole in the absence or presence of a mixture of the following flavonoids: quercetin, kaempferol, myricetin, apigenin, and luteolin (13, 5.4, 2.3, 1.5, and 0.34 µM respectively), 100 µM of the same flavonoids in a mixture (each at 20 µM), 50 µM kaempferol, or 100 µM quercetin.

level of 20.0 ± 6.3 mg/day (Lugasi *et al.*, 2003; Mullie *et al.*, 2008), which corresponds to 0.33 mg flavonoids/kg bw/day for a person of 60 kg. The results presented in Figure 6A reveal that the formation of 1′-sulfooxyestragole in the liver of rat after 24 h is predicted to decrease by 0.18%, 1.7%, 8%, and 14.4% compared with control when assuming, respectively, 1%, 10%, 50%, and 100% uptake of the flavonoid mixture, whereas in the liver of human, the formation of 1′-sulfooxyestragole was predicted to decrease by, respectively, 0.5%, 5%, 18%, and 27%.

In addition, the possible consequences of consumption of estragole together with a flavonoid food supplement containing 500 mg of quercetin, representing food supplements actually available on the market, was also studied. The PBBK model predictions indicated that the intake of 500 mg quercetin, which corresponds to 8.3 mg querectin/kg bw/day for a person of 60 kg, together with 0.01 mg/kg bw of estragole may decrease the formation of 1′-sulfooxyestragole in the liver of rat by 3%, 23%, 55%, and 69% when assuming 1%, 10%, 50%, and 100% uptake of quercetin, and in the liver of human, formation of 1′-sulfooxyestragole was predicted to decrease by, respectively, 8.6%, 36%, 56%, and 63% at these levels of quercetin uptake (Figure 7B).

In a third PBBK modeling scenario, the protective effect of nevadensin against SULT-mediated bioactivation of estragole in consumers of basil was evaluated. Assuming that basil contains 0.5% of essential oil (Smith *et al.*, 2002) and that the essential oil of basil contains 22.7% of estragole (Sanda *et al.*, 1998), an EDI of 0.01 mg/kg bw/day of estragole would result from an intake of 529 mg basil/day. Based on our previous work (Alhusainy *et al.*, 2010), the amount of nevadensin in 529 mg of basil is expected to be 0.34 mg, and this would result in an EDI for nevadensin of 0.0057 mg/kg bw/day for a person of 60 kg. At an estragole dose of 0.01 mg/kg bw/day and a nevadensin intake of 0.0057 mg/kg bw/day, the formation of 1′-sulfooxyestragole in the liver of rat is predicted to decrease by 0.77%, 7%, 26%, and 40% compared with control, whereas in the liver of human, the formation of 1′-sulfooxyestragole was predicted to decrease by 3.5%, 25%, 56%, and 68% when assuming, respectively, 1%, 10%, 50%, and 100% uptake of nevadensin (Figure 7C).

Finally, it is worth noting that even when the concentration of estragole was increased 1000-fold keeping the concentrations of the SULT-inhibiting flavonoids at the values defined in Table 5, the percentage inhibition of 1′-sulfooxyestragole formation remains the same as obtained at the 1000-fold lower dose of estragole. This is a characteristic of non-competitive inhibition, for which the level of inhibition depends only on the dose of the inhibitors (Simmons, 1996).

DISCUSSION

The alkenylbenzene estragole is an important natural constituent of herbs and spices. However, several studies have shown both genotoxic and carcinogenic properties for this compound in rodents (Drinkwater *et al.*, 1976; Miller *et al.*, 1983). These experiments were performed using the pure compound dosed by gavage in high doses, whereas in herbs and spices, estragole is present in relatively lower amounts together with other constituents in a food matrix, which can influence its biochemical

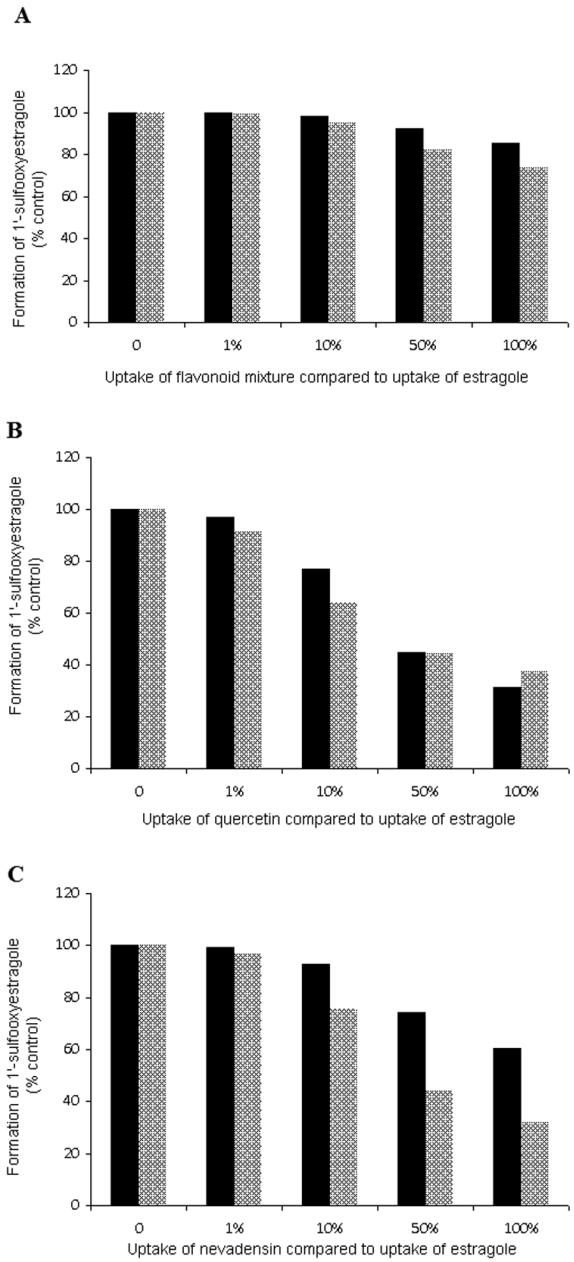


Figure 7. PBBK model based predictions for the formation of 1'-sulfooxyestragole in the liver of rat (black bars) and human (grey bars) after co-administration of 0.01 mg/kg bw/day of estragole and (A) a mixture of flavonoids at their EDI levels including 12 mg/day of quercetin, 4.6 mg/day of kaempferol, 2.2 mg/day of myricetin, and 1.2 mg/day of apigenin; (B) quercetin at a dose level present in food supplements amounting to 500 mg/day; or (C) nevadensin at 0.34 mg/day, representing the dose level resulting from intake of 529 mg of basil, which is the amount of basil resulting in 0.01 mg/kg bw/day of estragole (Sanda *et al.*, 1998; Smith *et al.*, 2002; Alhusainy *et al.*, 2010)

Table 5. Input data for PBBK models representing three possible intake scenarios of flavonoids and estragole at 0.01 mg/kg bw/day

Scenario	Constant (*L*)[a] (Flavonoid:estragole)	Input PBBK [flavonoid]]=CL_E×L×f
EDI of flavonoids[b]:		
Querectin (4×10^{-5} mol/day)	9.8	[quercetin]=$CL_E\times 9.8\times$f
Kaempferol (1.6×10^{-5} mol/day)	4.0	[kaempferol]=$CL_E\times 4.0\times$f
Myricetin (7×10^{-6} mol/day)	1.7	[myricetin]=$CL_E\times 1.7\times$ f
Apigenin (4.4×10^{-6} mol/day)	1.1	[apigenin]=$CL_E\times 1.1\times$f
Quercetin tablets (0.002 mol/day)	408	[quercetin]=$CL_E\times 408\times$f
3) Nevadensin (1.1×10^{-6} mol/day)[c]	0.25	[nevadensin]=$CL_E\times$0.25×f

Note. CL_E is the concentration of estragole in the liver (μmol/L), L is a constant that is determined based on the corresponding intake scenario and f is the molar ratio between estragole and each flavonoid that varies from 0 (no SULT inhibitors uptake into liver) to 1 (representing 100% uptake of a flavonoid into the liver).

[a]Constant (L) is calculated as the ratio between the EDI of a flavonoid and the EDI of estragole of 0.01 mg/kg bw/day (Smith *et al.*, 2002), which is equivalent to 4.1×10^{-6} mol/day given a molecular weight for estragole of 148 g/mol and assuming a body weight of 60 kg.

[b]Based on Lugasi *et al*. (2003); Mullie *et al*. (2008).

[c]Assuming that basil contains 0.5% of essential oil (Smith *et al.*, 2002) and that the essential oil of basil contains 22.7% of estragole (Sanda *et al.*, 1998), an EDI of 0.01 mg/kg bw/day of estragole would result from a 529 mg basil/day. The amount of nevadensin in 529 mg of basil is expected to result in a daily intake of 0.34 mg/day or 1.1×10^{-6} mol/day for a 60 kg person (Alhusainy *et al.*, 2010).

fate. The present study reports that methanolic extracts from different alkenylbenzene-containing herbs and spices were able to inhibit SULT activity involved in the formation of the proximate hepatocarcinogen 1′-sulfooxyestragole.

Flavonoids including nevadensin, quercetin, kaempferol, myricetin, and apigenin were the major constituents responsible for this inhibition of SULT activity with *K*i values in the nano- to micromolar range. Apart from SULT inhibitors present in herbs and spices and identified in the present study, the regular human diet is rich in other SULT inhibitors ranging from a number of natural dietary chemicals, such as polyphenols (Eaton *et al.*, 1996), to a number of food additives such as (±)-catechin, (+)-catechin, 4-chlorobenzoic acid, aspartame, benzoic acid, erythrosine, gallic acid, octyl gallate, *p*-hydroxybenzoic acid, propyl gallate, protocatechuic acid, saccharin, tannic acid, tartrazine, and vanillin, which have all been shown to be capable to inhibit the sulfonation of a number of xenobiotics and endobiotics in human liver cytosol to varying extents (Bamforth *et al.*, 1993).

A great volume of literature data supports the plausibility of non-competitive inhibition of sulfonation of various substrates by different flavonoids such as quercetin, genistein, (+)-catechin, kaempferol, apigenin, and diadzein (Walle *et al.*, 1995; Ghazali and Waring, 1999; Mesía -Vela and Kauffman, 2003).

The various flavonoids tested in this study were also shown to be able to significantly inhibit estragole DNA-adduct formation in human HepG2 cells exposed to the proximate carcinogen 1′-hydroxyestragole (50% reduction compared with control) even

at concentrations as low as 25 μM of the flavonoid mixture. The results of the present work also show that at levels of flavonoids sufficient to inhibit SULT activity, they can also inhibit the oxidation of 1′-hydroxyestragole, which is the major phase-II pathway of 1′-hydroxyestragole in the liver of human. Additional results of the present study revealed that in the human HepG2 cells exposed to 1′-hydroxyestragole in the presence of flavonoids, reduction in the activity of sulfonation and oxidation results in a metabolic shift toward glucuronidation, which serves as a detoxification pathway for 1′-hydroxyestragole.

In a next step, the kinetics for SULT inhibition and for the inhibition of 1′-oxoestragole were incorporated in the PBBK models for estragole in rat and human to predict the effect of co-exposure to estragole and (mixtures of) the different flavonoids on the bioactivation in vivo. The PBBK model based predictions indicate that the reduction of estragole bioactivation in rat and human by co-administration of the flavonoids is dependent on whether the intracellular liver concentrations of the flavonoids can reach their *K*i values determined in the present study. For example, high intake levels of SULT inhibitors (e.g. quercetin) at levels present in food supplements currently available on the market might result in significant inhibition of the formation of 1′-sulfooxyestragole even at 10% uptake of the flavonoid. Interestingly, for human liver, a dose of nevadensin of only 0.34 mg/day was predicted to result in comparable inhibition of 1′-sulfooxyestragole formation as a dose of quercetin of 500 mg/day (Figure 6B and C). This clearly illustrates that although nevadensin is not a major flavonoid constituent in the diet, its contribution to the SULT-mediated modulation of estragole is already achieved at relatively lower dose levels due to its *K*i value of 4 nM (Alhusainy *et al.*, 2010), which is 2–3 orders of magnitude lower than that for other flavonoids. Furthermore, the inhibitory effect of nevadensin in vivo may also be more easily achieved than that of the other flavonoids because nevadensin is a methylated flavonoid, and methylated flavones such as nevadensin have been demonstrated to be more metabolically stable and have a higher intestinal absorption than their unmethylated analogues increasing the oral bioavailability of methylated flavones compared with their unmethylated analogues (Wen and Walle, 2006).

In this context, it is also of interest to take into account the reported intracellular and circulating concentrations of the different flavonoid-type SULT inhibitors. Mean plasma levels of quercetin, for example, reached 7.6 μM in human volunteers after consuming a supplement of 160 g stewed and homogenized onions, which provided 331 μmol of quercetin glucosides (Graefe *et al.*, 2001), a plasma level which is sufficient to inhibit SULT activity giving a *K*i for quercetin-mediated SULT inhibition of 1.5 μM as determined in the present study. Moreover, after a single oral dose of 200 mg/kg bw of *Chrysanthemum morifolium* extract to rats apigenin reached maximum plasma levels of 16 μM (Chen *et al.*, 2007).

Clearly, plasma levels of quercetin and apigenin can reach levels higher than their *K*i. Data on human plasma levels of other flavonoids tested in the present study are lacking. However, plasma levels for other flavonoids, namely, the isoflavones genistein and daidzein, may exceed 0.5 μM in women who eat a traditional Japanese diet

(Uehara *et al.*, 2000) and may even reach 1 µM in individuals consuming some dietary supplements (Gooderham *et al.*, 1996), both levels are sufficient to inhibit SULT1A1 and SULT1EI significantly given the IC_{50} for inhibition of SULT1A1 and SULT1EI by genistein of 0.5 and 1 µM, respectively (Harris *et al.*, 2004). Moreover, plasma levels of other flavonoids, such as hesperetin, may reach 1–3 µM after ingestion of large amounts of fruit and vegetables (Erlund *et al.*, 2002), which are concentrations sufficient to inhibit both SULT1A1 and SULT1E1 (Harris *et al.*, 2004). It is important to note, however, that in vivo flavonoids undergo extensive conjugation to their corresponding glucuronic acid and sulfonate conjugates, and that in most in vivo studies, flavonoids are measured after deconjugation. Therefore, the reported plasma concentrations may reflect total flavonoid concentration in the conjugated and unconjugated forms instead of the concentrations of the aglycon.

This raises the question if in these conjugated forms the flavonoids are still able to interact with SULTs. In this regard, it is worth to note that conjugated metabolites of flavonoids can retain the biological activity of the parent compound (Harris *et al.*, 2004). This is highly dependent on the molecular site of conjugation. Moreover, studies in which cells in vitro were exposed to glucuronidated and sulfonated conjugates of quercetin demonstrated that within the cells, the flavonoid was no longer conjugated due to efficient extra- and/or intracellular deconjugation (Lee-Hilz *et al.*, 2008). Another issue of interest is if these flavonoids reported in literature can inhibit the same SULT isoforms which are involved in the sulfonation of the substrate 1′-hydroxyestragole, which have so far not been identified.

Several PBPK models have been developed and investigated chemical interactions at the level of metabolic competition (El-Masri *et al.*, 1996a,b; Dobrev *et al.*, 2002; Krishnan *et al.*, 2002). These models have identified thresholds for exposure, below which competitive inhibition is not expected. In this regard, we have shown in our paper that plasma levels of the different flavonoids can reach levels sufficiently high to inhibit SULT activity and in a non-competitive way.

Finally, it is important to note that the PBBK model analysis of the effect of the inhibitors on estragole bioactivation in vivo as presented is a fist approximation. More refined models could take into account i) sub-models for the ADME characteristics of the individual flavonoids and/or ii) non additive modes of interactions for the flavonoids in mixture on the conversion of 1′-hydroxyestragole to 1′-oxoestragole which better fit the experimental in vitro data for this inhibition. However, development of such refined models was beyond the scope of the present study.

In conclusion, dietary flavonoids may modulate the bioactivation and thus the ultimate cancer risk posed by estragole provided that the physiological liver concentrations of the inhibitors reach their *K*i values. Whether in vivo levels of flavonoids can reach their *K*i values and whether conjugated forms of the flavonoids can still inhibit SULT activity are important issues that need further investigation.

Overall, an in vivo modulation for estragole bioactivation is highly plausible given the facts that i) SULT inhibitors are abundant in the diet, ii) their inhibition constants

(*Ki*) for SULT activity are in the nano- to micromolar range, and iii) their combined effect on SULT enzyme activity was shown to be additive. If the PBBK based predicted data can be validated in vivo, which is an important issue for future research, this would imply that the likelihood of bioactivation and subsequent adverse effects might be lower when alkenylbenzenes are consumed in a matrix of food items containing SULT inhibitors than what would be expected on the basis of experiments using alkenylbenzenes as pure compounds. The safety of alkenylbenzene-containing botanicals could consequently be judged and regulated on the basis of the EDIs of the different alkenylbenzenes compared with the EDIs of the different respective SULT inhibitors which are present in the diet. Further research investigations are directed at investigating efficiency of SULT inhibitors in reducing-DNA adduct formation and the subsequent carcinogenic risk in vivo.

ACKNOWLEDGMENT

The authors would like to acknowledge Dr. Yiannis Fiamegos and Florian Viton for the synthesis of 1′-hydroxyestragole. This work was supported in part by a grant from the International Organization of the Flavour Industry (IOFI) and by the Nestlé Research Centre (Switzerland).

REFERENCES

1. Alhusainy, W., Paini, A., Punt, A., Louisse, J., Spenkelink, A., Vervoort, J., Delatour, T., Scholz, G., Schilter, B., Adams, T., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). Identification of nevadensin as an important herb-based constituent inhibiting estragole bioactivation and physiology-based biokinetic modeling of its possible in vivo effect. *Toxicology and Applied Pharmacology* **245**, 179-190.
2. Anthony, A., Caldwell, J., Gutt, A. J., and Smith, R. L. (1987). Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1′-hydroxyestragole. *Food and Chemical Toxicology* **25**, 799-806.
3. Bamforth, K. J., Jones, A. L., Roberts, R. C., and Coughtrie, M. W. H. (1993). Common food additives are potent inhibitors of human liver 17α-ethinyloestradiol and dopamine sulphotransferases. *Biochemical Pharmacology* **46**, 1713-1720.
4. Barter, Z. E., Bayliss, M. K., Beaune, P. H., Boobis, A. R., Carlile, D. J., Edwards, R. J., Houston, J. B., Lake, B. G., Lipscomb, J. C., Pelkonen, O. R., Tucker, G. T., and Rostami-Hodjegan, A. (2007). Scaling factors for the extrapolation of in vivo metabolic drug clearance from in vitro data: Reaching a consensus on values of human microsomal protein and hepatocellularity per gram of liver. *Current Drug Metabolism* **8**, 33-45.
5. Boberg, E. W., Miller, E. C., and Miller, J. A. (1983). Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1′-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1′-hydroxysafrole in mouse liver. *Cancer Research* **43**, 5163-5173.
6. Borchert, P., Wislocki, P. G., Miller, J. A., and Miller, E. C. (1973). The metabolism of the naturally occurring hepatocarcinogen safrole to 1′-hydroxysafrole and the electrophilic reactivity of 1′-acetoxysafrole. *Cancer Research* **33**, 575-589.
7. Brown, R. P., Delp, M. D., Lindstedt, S. L., Rhomberg, L. R., and Beliles, R. P. (1997). Physiological parameter values for physiologically based pharmacokinetic models. *Toxicology and Industrial Health* **13**, 407-484.
8. Chen, T., Li, L. P., Lu, X. Y., Jiang, H. D., and Su, Z. (2007). Absorption and excretion

of luteolin and apigenin in rats after oral administration of Chrysanthemum morifolium extract. *Journal of Agricultural and Food Chemistry* **55**, 273-277.

9. Dearlove, R. P., Greenspan, P., Hartle, D. K., Swanson, R. B., and Hargrove, J. L. (2008). Inhibition of protein glycation by extracts of culinary herbs and spices. *Journal of Medicinal Food* **11**, 275-281.
10. DeJongh, J., Verhaar, H. J. M., and Hermens, J. L. M. (1997). A quantitative property-property relationship (QPPR) approach to estimate in vitro tissue blood partition coefficients of organic chemicals in rats and humans. *Archives of Toxicology* **72**, 17-25.
11. Dobrev, I. D., Andersen, M. E., and Yang, R. S. H. (2002). In silico toxicology: Simulating interaction thresholds for human exposure to mixtures of trichloroethylene, tetrachloroethylene, and 1,1,1-trichloroethane. *Environmental Health Perspectives* **110**, 1031-1039.
12. Drinkwater, N. R., Miller, E. C., Miller, J. A., and Pitot, H. C. (1976). Hepatocarcinogenicity of estragole (1-Allyl-4-methoxybenzene) and 1'-Hydroxyestragole in the mouse and mutagenicity of 1'- acetoxyestragole in bacteria. *Journal of the National Cancer Institute* **57**, 1323-1331.
13. Eaton, E. A., Walle, U. K., Lewis, A. J., Hudson, T., Wilson, A. A., and Walle, T. (1996). Flavonoids, potent inhibitors of the human P-form phenolsulfotransferase: Potential role in drug metabolism and chemoprevention. *Drug Metabolism and Disposition* **24**, 232-237.
14. El-Masri, H. A., Constan, A. A., Ramsdell, H. S., and Yang, R. S. H. (1996a). Physiologically based pharmacodynamic modeling of an interaction threshold between trichloroethylene and 1,1-dichloroethylene in Fischer 344 rats. *Toxicology and Applied Pharmacology* **141**, 124-132.
15. El-Masri, H. A., Tessari, J. D., and Yang, R. S. H. (1996b). Exploration of an interaction threshold for the joint toxicity of trichloroethylene and 1,1 dichloroethylene: Utilization of a PBPK model. *Archives of Toxicology* **70**, 527-539.
16. Erlund, I., Silaste, M. L., Alfthan, G., Rantala, M., Kesäniemi, Y. A., and Aro, A. (2002). Plasma concentrations of the flavonoids hesperetin, naringenin and quercetin in human subjects following their habitual diets, and diets high or low in fruit and vegetables. *European Journal of Clinical Nutrition* **56**, 891-898.
17. European Commission (EC) (2008). Regulation (EC) No 1334/2008 of the Europian Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC.
18. Ghazali, R. A., and Waring, R. H. (1999). The effects of flavonoids on human phenolsulphotransferases: Potential in drug metabolism and chemoprevention. *Life Sciences* **65**, 1625-1632.
19. Gooderham, M. J., Adlercreutz, H., Ojala, S. T., Wähälä, K., and Holub, B. J. (1996). A soy protein isolate rich in genistein and daidzein and its effects on plasma isoflavone concentrations, platelet aggregation, blood lipids and fatty acid composition of plasma phospholipid in normal men. *Journal of Nutrition* **126**, 2000-2006.
20. Graefe, E. U., Wittig, J., Mueller, S., Riethling, A. K., Uehleke, B., Drewelow, B., Pforte, H., Jacobasch, G., Derendorf, H., and Veit, M. (2001). Pharmacokinetics and bioavailability of quercetin glycosides in humans. *Journal of Clinical Pharmacology* **41**, 492-499.
21. Grayer, R. J., Vieira, R. F., Price, A. M., Kite, G. C., Simon, J. E., and Paton, A. J. (2004). Characterization of cultivars within species of Ocimum by exudate flavonoid profiles. *Biochemical Systematics and Ecology* **32**, 901-913.
22. Harris, R. M., Wood, D. M., Bottomley, L., Blagg, S., Owen, K., Hughes, P. J., Waring, R. H., and Kirk, C. J. (2004). Phytoestrogens are potent inhibitors of estrogen sulfation: Implications for breast cancer risk and treatment. *Journal of Clinical Endocrinology and Metabolism* **89**, 1779-1787.
23. Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B., and Kromhout, D. (1993a). Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* **342**, 1007-1011.
24. Hertog, M. G. L., Hollman, P. C. H., Katan, M. B., and Kromhout, D. (1993b). Intake

of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutrition and Cancer* **20**, 21-29.

25. Hussain, R. F., Nouri, A. M. E., and Oliver, R. T. D. (1993). A new approach for measurement of cytotoxicity using colorimetric assay. *Journal of Immunological Methods* **160**, 89-96.
26. Jeurissen, S. M. F., Punt, A., Delatour, T., and Rietjens, I. M. C. M. (2008). Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1 '-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. *Food and Chemical Toxicology* **46**, 2296-2302.
27. Justesen, U., and Knuthsen, P. (2001). Composition of flavonoids in fresh herbs and calculation of flavonoid intake by use of herbs in traditional Danish dishes. *Food Chemistry* **73**, 245-250.
28. Kikuzaki, H., Miyajima, Y., and Nakatani, N. (2008). Phenolic glycosides from berries of Pimenta dioica. *Journal of Natural Products* **71**, 861-865.
29. Krishnan, K., Haddad, S., Béliveau, M., and Tardif, R. (2002). Physiological modeling and extrapolation of pharmacokinetic interactions from binary to more complex chemical mixtures. *Environmental Health Perspectives* **110**, 989-994.
30. Lee-Hilz, Y. Y., Stolaki, M., van Berkel, W. J. H., Aarts, J. M. M. J. G., and Rietjens, I. M. C. M. (2008). Activation of EpRE-mediated gene transcription by quercetin glucuronides depends on their deconjugation. *Food and Chemical Toxicology* **46**, 2128-2134.
31. Lugasi, A., Hóvári, J., Sági, K. V., and Bíró, L. (2003). The role of antioxidant phytonutrients in the prevention of diseases. *Acta Biologica Szegediensis* **47**, 119-125.
32. Medinsky, M. A., Leavens, T. L., Csanády, G. A., Gargas, M. L., and Bond, J. A. (1994). In vivo metabolism of butadiene by mice and rats: A comparison of physiological model predictions and experimental data. *Carcinogenesis* **15**, 1329-1340.
33. Mesía -Vela, S., and Kauffman, F. C. (2003). Inhibition of rat liver sulfotransferases SULT1A1 and SULT2A1 and glucuronosyltransferase by dietary flavonoids. *Xenobiotica* **33**, 1211-1220.
34. Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Research* **43**, 1124-1134.
35. Morimitsu, Y., Sugihara, N., and Furuno, K. (2004). Inhibitory effect of flavonoids on sulfo- and glucurono-conjugation of acetaminophen in rat cultured hepatocytes and liver subcellular preparations. *Biological and Pharmaceutical Bulletin* **27**, 714-717.
36. Mullie, P., Clarys, P., Deriemaeker, P., and Hebbelinck, M. (2008). Estimation of daily human intake of food flavonoids. *International Journal of Food Sciences and Nutrition* **59**, 291-298.
37. Paini, A., Punt, A., Viton, F., Scholz, G., Delatour, T., Marin-Kuan, M., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). A physiologically based biodynamic (PBBD) model for estragole DNA binding in rat liver based on in vitro kinetic data and estragole DNA adduct formation in primary hepatocytes. *Toxicology and Applied Pharmacology* **245**, 57-66.
38. Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. *Cancer Research* **41**, 176-186.
39. Punt, A., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2007). Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1'-hydroxyestragole. *Chemical Research in Toxicology* **20**, 991-998.
40. Punt, A., Freidig, A. P., Delatour, T., Scholz, G., Boersma, M. G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2008). A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat. *Toxicology and Applied Pharmacology* **231**, 248-259.
41. Punt, A., Paini, A., Boersma, M. G., Freidig, A. P., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2009). Use of physiologically based

biokinetic (PBBK) modeling to study estragole bioactivation and detoxification in humans as compared with male rats. *Toxicological Sciences* **110**, 255-269.

42. Ramsey, J. C., and Andersen, M. E. (1984). A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicology and Applied Pharmacology* **73**, 159-175.
43. Sanda, K., Koba, K., Nambo, P., and Gaset, A. (1998). Chemical investigation of Ocimum species growing in Togo. *Flavour and Fragrance Journal* **13**, 226-232.
44. Sangster, S. A., Caldwell, J., and Hutt, A. J. (1987). The metabolic disposition of [methoxy-^{14}C]-labelled *trans*-anethole, estragole and *p*-propylanisole in human volunteers. *Xenobiotica* **17**, 1223-1232.
45. SCF (2001). Opinion of the Scientific Committee on Food on Estragole (1-allyl-4-methoxybenzene). European Commission, Health and Consumer Protection Directorate. General, Report Series 10, Directorate C, Scientific Opinions, Brussels, Belgium. Obtained January 10, 2008, at http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.
46. Shan, B., Cai, Y. Z., Sun, M., and Corke, H. (2005). Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *Journal of Agricultural and Food Chemistry* **53**, 7749-7759.
47. Simmons, J. E. (1996). Application of physiologically based pharmacokinetic modelling to combination toxicology. *Food and Chemical Toxicology* **34**, 1067-1073.
48. Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - Methyl eugenol and estragole. *Food and Chemical Toxicology* **40**, 851-870.
49. Suhaj, M. (2006). Spice antioxidants isolation and their antiradical activity: a review. *Journal of Food Composition and Analysis* **19**, 531-537.
50. Uehara, M., Arai, Y., Watanabe, S., and Adlercreutz, H. (2000). Comparison of plasma and urinary phytoestrogens in Japanese and Finnish women by time-resolved fluoroimmunoassay. *BioFactors* **12**, 217-225.
51. Walle, T., Eaton, E. A., and Walle, U. K. (1995). Quercetin, a potent and specific inhibitor of the human P-form phenolsulfotransferase. *Biochemical Pharmacology* **50**, 731-734.
52. Wang, Q., Ye, C., Jia, R., Owen, A. J., Hidalgo, I. J., and Li, J. (2006). Inter-species comparison of 7-hydroxycoumarin glucuronidation and sulfation in liver S9 fractions. *In Vitro Cellular and Developmental Biology - Animal* **42**, 8-12.
53. Wen, X., and Walle, T. (2006). Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug Metabolism and Disposition* **34**, 1786-1792.

4

In vivo validation and physiologically based biokinetic modeling of the inhibition of SULT-mediated estragole DNA adduct formation in the liver of male Sprague–Dawley rats by the basil flavonoid nevadensin

Based on:
Alhusainy W, Paini A, van den Berg JHJ, Punt A, Scholz G, Schilter B, van Bladeren PJ, Taylor S, Adams TB, and Rietjens IMCM

Molecular Nutrition and Food Research 57 (2013), 1969-1978

Supplementary Material can be found at http://onlinelibrary.wiley.com/doi/10.1002/mnfr.201300144/suppinfo

ABSTRACT

The present work investigates whether the previous observation that the basil flavonoid nevadensin is able to inhibit sulfotransferase (SULT)-mediated estragole DNA adduct formation in primary rat hepatocytes could be validated in vivo. Estragole and nevadensin were co-administered orally to Sprague–Dawley rats, at a ratio reflecting their presence in basil. Moreover, previously developed physiologically based biokinetic (PBBK) models to study this inhibition in rat and in human liver were refined by including a sub-model describing nevadensin kinetics. Nevadensin resulted in a significant 36% reduction in the levels of estragole DNA adducts formed in the liver of rats. The refined PBBK model predicts the formation of estragole DNA adducts in the liver of rat with less than twofold difference compared to in vivo data and suggests more potent inhibition in the liver of human compared to rat due to less efficient metabolism of nevadensin in human liver and intestine. Given the role of the SULT-mediated DNA adduct formation in the hepatocarcinogenicity of estragole, the results of the present study suggest that the likelihood of bioactivation and subsequent adverse effects in rodent bioassays may be lower when estragole is dosed with nevadensin compared to dosing of pure estragole.

INTRODUCTION

The alkenylbenzene estragole (1-allyl-4-methoxybenzene) (Figure 1) is a constituent of many herbs and spices and their essential oils (Smith *et al.*, 2002). Estragole has been demonstrated to be hepatocarcinogenic in rodents at high doses due to its bioactivation by cytochromes P450 leading to the formation of the proximate carcinogen 1′-hydroxyestragole (Figure 1). Further bioactivation of 1′-hydroxyestragole requires the involvement of sulfotransferases (SULTs) that convert 1′-hydroxyestragole to the ultimate carcinogen 1′-sulfooxyestragole (Figure 1). The 1′-sulfooxy metabolite is unstable and, via a putative reactive carbocation intermediate, binds covalently to different endogenous nucleophiles including DNA leading to DNA adduct formation (Phillips *et al.*, 1984; Randerath *et al.*, 1984).

Therefore, 1′-sulfooxyestragole is considered to be the ultimate hepatotoxic and hepatocarcinogenic agent in rodents (Boberg *et al.*, 1983). This conclusion is supported by observations from experiments with mice revealing that co-administration with the SULT inhibitor pentachlorophenol (PCP) results in a reduction in hepatic DNA and RNA adduct formation and tumour formation upon long-term dietary administration of the closely related alkenylbenzene safrole or its 1′-hydroxy metabolite (Boberg *et al.*, 1983).

Given its genotoxicity and carcinogenicity, the addition of estragole as a pure substance in food stuffs has been prohibited since September 2008 within the European Union (European Commission, 2008). Intake of estragole at present occurs primarily from consumption of foods containing sweet basil, fennel, and anise or their essential oils (Smith *et al.*, 2002). A significant difficulty in evaluating the metabolic, biochemical, and toxicological data for the alkenylbenzene estragole and other alkenylbenzenes is that human exposure results from a complex mixture of food, spice, and spice oil constituents that may significantly impact the biochemical fate and toxicological risk of estragole.

In this respect, we have previously demonstrated that the natural basil flavone nevadensin (5,7-dihydroxy-6,8,4′- trimethoxyflavone) (Figure 2) was able to inhibit estragole DNA adduct (N^2-(*trans*-isoestragol-3′-y1)-2′-deoxyguanosine (E-3′-N^2-dGuo) formation in primary rat hepatocytes exposed to the proximate carcinogen, 1′-hydroxyestragole (Jeurissen *et al.*, 2008; Alhusainy *et al.*, 2010). This inhibition by nevadensin was shown to occur at the level of SULT-mediated bioactivation of 1′-hydroxyestragole into 1′-sulfooxyestragole (Jeurissen *et al.*, 2008; Alhusainy *et al.*, 2010).

However, whether a potent SULT inhibiting effect by nevadensin and subsequent reduction of estragole E-3′-N^2-dGuo DNA adduct formation occurs in vivo remained to be established. The aim of the present study therefore was to investigate whether the inhibition of SULT-mediated E-3′-N^2-dGuo formation by nevadensin observed in vitro could be validated in vivo. Furthermore, the physiologically based biokinetic (PBBK) models previously developed to study this inhibition in rat and human liver (Punt *et al.*, 2008, 2009; Alhusainy *et al.*, 2010, 2012) were refined by including a sub-model describing nevadensin kinetics and evaluated using the newly available experimental in vivo data in rat.

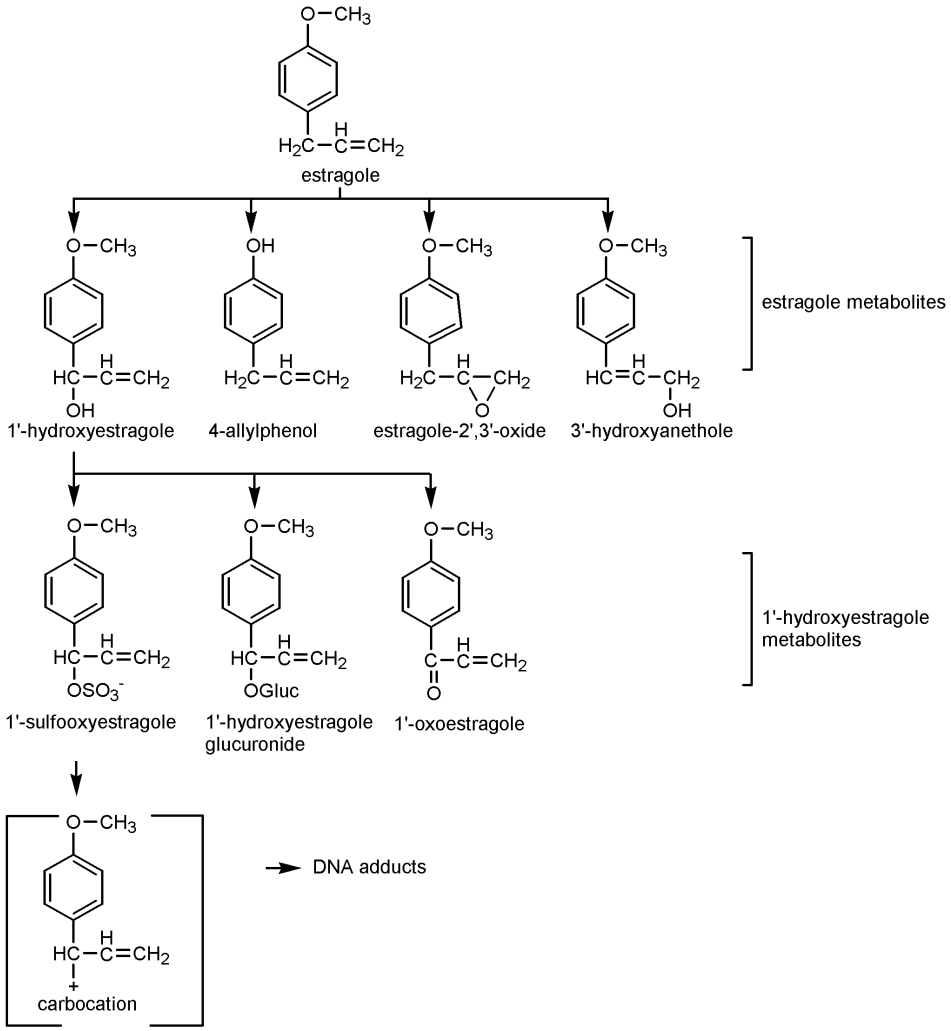


Figure 1. Bioactivation and detoxification pathways of estragole.

MATERIALS AND METHODS

Materials

Estragole, alamethicin, uridine 5′-diphosphoglucuronic acid (UDPGA), pentachlorophenol (PCP), 3′-phosphoadenosine-5′-phosphosulfate (PAPS), and (S-(5′-adenosyl)-L-methionine iodide) were purchased from Sigma-Aldrich (Steinheim, Germany). Tris and PBS were obtained from Gibco BRL Life Technologies (Paisley, UK). TFA was obtained from Mallinckrodt Baker B.V. (The Netherlands). Methanol (Ultra Liquid Chromatography/Mass Spectrometry, ULC/MS) and ACN (ULC/MS) were obtained from Biosolve (Valkenswaard, The Netherlands). Rats and their Teklad 2014C feed were purchased

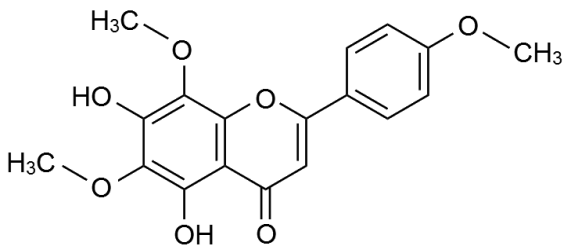


Figure 2. Molecular structure of the natural SULT inhibitor nevadensin.

from Harlan Laboratories (The Netherlands). Pooled mixed gender human and pooled male Sprague–Dawley rat intestinal S9 were purchased from Tebu-bio (Heerhugowaard, The Netherlands). Pooled male Sprague–Dawley rat and pooled human liver S9 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nevadensin was purchased from Sinova, Inc. (Bethesda, MD, USA) under the name lysionotin ($C_{18}H_{16}O_7$). Get pure DNA Kit-Cell was obtained from Dojindo Molecular Technology, Inc. (Kumamoto, Japan).

Animal experiment

The animal studies were in compliance with the Dutch Act on animal experimentation (Stb, 1977, 67; Stb 1996, 565), revised February 5, 1997. The study was approved by the ethical committee on animal experimentation of Wageningen University. All procedures were considered to avoid and minimize animal discomfort. Six-week-old white male outbred Sprague–Dawley rats were housed three rats per cage. Enrichment was provided for each cage. A 12-h light/dark cycle for the rats was applied during the animal acclimatization and treatment periods. Temperature (±20°C) and humidity (±55%) were monitored daily. Feed and tap water were provided *ad libitum*. The total number of animals was 21 rats (136.6±5.1 g).

After a 5-day acclimatization period, animals were put into metabolic cages 24 h prior to gavage and were kept in the metabolic cages up to time of sacrifice (48 h after gavage). Rats were assigned to three groups, each containing seven rats. All compounds were dissolved in corn oil, placed in a water bath at 30°C and vortexed before each gavage and the volume of gavages was 2 mg/kg bw.

The first group, representing the control group, was dosed once with 150 mg/kg bw estragole only. In a study by Paini et al. (Paini *et al.*, 2012a), the dose of 150 mg/kg bw was the lowest dose of estragole able to induce significant levels of estragole DNA adducts (E-3′-N^2-dGuo) in the liver, lungs, and kidneys of male outbred Sprague–Dawley rats 48 h after gavage compared to the solvent control (vehicle) group.

The second group was dosed once with 150 mg/kg bw estragole and 87.2 mg/kg bw of nevadensin. The dose of nevadensin of 87.2 mg/kg bw was selected to mimic the ratio of estragole to nevadensin (1:0.25) present in basil (Alhusainy *et al.*, 2012) and the two compounds were dosed simultaneously to also mimic the real-life situation where the two compounds are ingested together.

The third group, representing a positive control group for SULT inhibition, was dosed once with 150 mg/kg bw estragole and the SULT inhibitor PCP at 11 mg/kg bw, dosed simultaneously. The dose of PCP was based on literature data where a dose of PCP of 11 mg/kg bw/day was reported to reduce the incidence of hepatomas resulting from a single intraperitoneal dose of 111 mg/kg bw estragole after 10 months (Wiseman *et al.*, 1987).

After anesthesia of the animals with a mixture of isoflurane and oxygen, blood was removed before removing livers. Livers were then weighted, cut into pieces, snap frozen in liquid nitrogen, and stored at -80°C until further processing.

Tissue DNA extraction and digestion

Prior to DNA extraction, liver samples were homogenized as follows: Two samples from the left lateral lobe of the liver were taken randomly and each sample was around 1 g. Then, 4 mL cold PBS was added to each 1 g liver sample and the sample was homogenized on ice at 1000 rpm using a mechanical cell homogenizer (B. Braun,Melsungen, Germany). Thereafter, homogenates were placed in 15-mL tubes and centrifuged at 3000×*g* for 5 min (Sigma centrifuge, type 4K10, Germany) to obtain pellets. DNA extraction from the pellets obtained was performed using a Get pure DNA Kit-Cell protocol for tissue following the manufacturer's instructions. The final DNA pellet was dissolved in 200 µL MilliQ water. Characterization of the yield and purity of the extracted DNA, digestion of DNA, and quantification of E-3'-N^2-dGuo DNA adducts by LC–ESI–MS/MS were all performed as described previously (Paini *et al.*, 2010).

Glucuronidation, sulfonation, and methylation of nevadensin by liver and intestinal S9 fractions

To construct a nevadensin PBBK model describing nevadensin kinetics, the following studies were performed to obtain the kinetic parameters (V_{max} and K_m) for the main pathways involved in the conversion of nevadensin.

To examine glucuronidation of nevadensin, 0.01 mg/mL pooled male rat liver or intestinal S9 and 0.05 mg/mL pooled human liver or intestinal S9 were incubated with (final concentrations) 5 mM UDGPA in 0.2 M Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$ in a total volume of 200 µL. Incubations were pre-treated with 0.025 mg/mL of the pore-forming peptide alamethicin added from a 200 times concentrated stock in methanol on ice for 15 min to overcome enzyme latency and obtain maximal glucuronidation activity (Fisher *et al.*, 2000). Incubation mixtures were preincubated at 37°C for 1 min, after which the reactions were started by the addition of the substrate nevadensin from 200 times concentrated stock solutions in DMSO to obtain final concentrations ranging between 0.5 and 50 µM. The reactions were carried out for 10 min at 37°C.

To examine sulfonation of nevadensin, 1 mg/mL pooled male rat or pooled human liver or intestinal S9 were incubated with (final concentrations) 0.5 mM PAPS in 0.1 M Tris-HCl (pH 7.4) in a total volume of 100 µL. Incubation mixtures were pre-incubated at 37°C for 1 min, after which the reactions were started by the addition of the substrate nevadensin

from 100 times concentrated stock solutions in DMSO to obtain final concentrations ranging between 1 and 500 μM. The reactions were carried out at 37°C for 30 min.

To examine methylation of nevadensin, 1 mg/mL pooled male rat or pooled human liver or intestinal S9 were incubated with (final concentrations) 2 mM S-adenosylmethionine in 0.1 M potassium phosphate (pH 7.4) in a total volume of 100 μL. Incubation mixtures were pre-incubated at 37°C for 1 min, after which the reactions were started by the addition of the substrate nevadensin from 100 times concentrated stock solutions in DMSO to obtain final concentration of 5 or 50 μM. The reactions were carried out at 37°C for either 10 min or 1 h. Cold ACN was added to terminate all reactions (50 μL for glucuronidation and 25 μL for sulfonation and methylation) and incubations were centrifuged for 5 min at 5°C and 16000×*g* to precipitate the proteins and the supernatants were stored at −20°C until UPLC analysis.

UPLC–UV detection and quantification of nevadensin metabolites

Samples were analyzed by UPLC–UV on a waters Acquity™ ultra performance LC system coupled to an Acquity UPLC® BEH C18 1.7 μm 2.1x 50 mm column (Waters, Ireland). To detect the conjugates of nevadensin, the gradient was made with ultra-pure water containing 0.1% v/v TFA and ACN and the flow rate was set to 0.6 mL/min. The gradient was started with 20% ACN. ACN was kept at 20% for 2 min, increased to 25% in 4 min, to 80% in 0.3 min, and to 100% in 0.7 min, and kept at 100% for 0.3 min. Initial conditions were reset in 0.2 min and then the system was equilibrated at these conditions for 0.5 min. Detection was carried out using an Acquity™ ultra performance LC photodiode array detector (Waters, Milford, MA, USA). Given that the nevadensin conjugates have a similar UV spectrum as that of nevadensin, they were quantified by comparison of their peak area at a wavelength of 280 nm to the calibration curve of nevadensin.

Data analysis

To test whether the change in formation of E-3′-N^2-dGuo levels in the control group (treated with estragole only) compared to the groups treated with estragole plus nevadensin or PCP was significant, a two-sample *t*-test (one sided) was performed after determining equality of variances by the Levene's version of the F tested with SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL, USA). The kinetic constants (V_{max} and K_m) for sulfonation and glucuronidation of nevadensin were derived from plots by fitting the data to the standard Michaelis–Menten equation using the Life Science Workbench (LSW) data analysis toolbox (version 1.1.1, MDL Information Systems, Inc.).

PBBK model based predictions for formation of E-3′-N^2-dGuo in the liver of rat and human in the presence or absence of nevadensin

In our previous work (Alhusainy *et al.*, 2010, 2012), the estragole PBBK models in rat (Punt *et al.*, 2008) and human (Punt *et al.*, 2009) were used to obtain insight into the quantitative dose- and species-dependent effects of SULT inhibition by nevadensin on the formation of the ultimate carcinogenic metabolite, 1′-sulfooxyestragole, in the liver.

Nevadensin was assumed to follow a similar time-dependent concentration curve in the liver of rat and human as estragole and different scenarios of uptake and bioavailability ranging between 1 and 100% were also simulated (Alhusainy *et al.*, 2010, 2012).

In the present study, we aimed at further refinement of these former PBBK models and evaluation of the models obtained against the newly generated in vivo data. This was performed by combining the previously defined estragole PBBK models with the PBBK models describing the dose-dependent kinetics of nevadensin in male rat and human. A schematic diagram for the rat and human estragole PBBK model is shown in Figure 3A and that for the rat and human nevadensin PBBK model in Figure 3B. The original PBBK models for estragole were described in detail previously (Punt *et al.*, 2008, 2009) and therefore in this section only the newly developed nevadensin PBBK models in rat and human are described. Similar to the PBBK models for estragole, the model for nevadensin included separate compartments for liver, fat, and rapidly and slowly perfused tissues. Unlike the PBBK models of estragole, the nevadensin PBBK models contained a compartment for intestine that was found to be involved in the metabolism of nevadensin.

Absorption rate constant of nevadensin (Ka_N) was set to 0.75/h, representing a median value for absorption constants between the unmethylated flavonoid quercetin (0.5/h) (Li *et al.*, 2009) and the fully methylated flavonoid nobiletin (1/h) (Singh *et al.*, 2011). All other PBBK model parameters and kinetic constants are provided in the Supplementary Material (Tables S1–S6). The apparent V_{max} values for the glucuronidation and sulfonation of nevadensin in the liver and intestine, expressed as (nmol/min/mg S9 protein), were determined in vitro in the present study, and scaled to the liver or intestine using an S9 protein yield of 143 mg/g liver (Medinsky *et al.*, 1994) or 11 mg/g intestine (van de Kerkhof *et al.*, 2007). All apparent K_m values were determined in vitro and were assumed to correspond to the apparent in vivo K_m values.

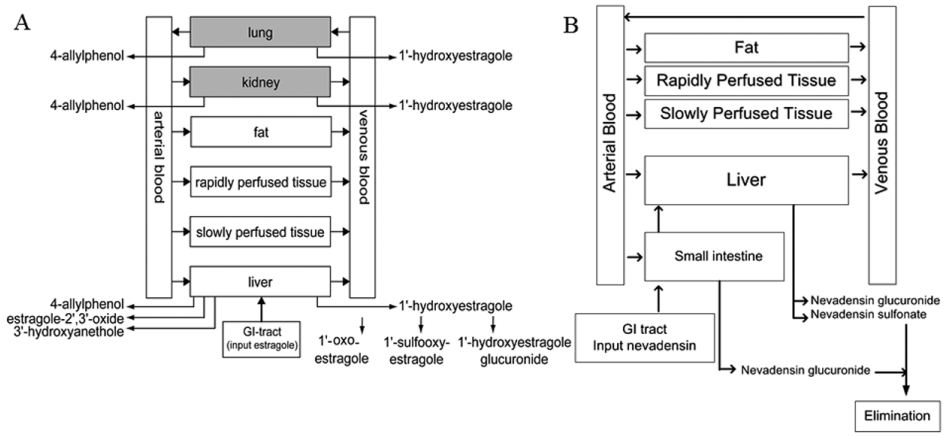


Figure 3. Schematic diagram of the PBBK model for (A) estragole and (B) nevadensin, in rat and human with the grey colour indicating organ compartments included in the PBBK model for rat but not in that for human.

Mass balance equations for nevadensin in metabolizing tissues are as follows:

Liver : dAL_N/dt

$= QL_N \times CA_N + QI_N \times CVI_N - (QL_N + QI_N) \times CVL_N - V_{maxLNG} \times (CL_N/PL_N)/(K_{mLNG} + (CL_N/PL_N)) - V_{maxLNS} \times (CL_N/PL_N)/(K_{mLNS} + (CL_N/PL_N))$

$CL_N = AL_N/VL_N$

$CVL_N = CL_N/PL_N$

Intestine : dAI_N/dt

$= dUptake_N/dt + QI_N \times (CA_N - CVI_N) - V_{maxING} \times (CI_N/PI_N)/(K_{mING} + (CI_N/PI_N))$

$dUptake_N/dt = dAGI_N/dt = Ka_N \times AGI_N$;

$AGI_N(0)$= oral dose

$CI_N = AI_N/VI_N$

$CVI_N = CI_N/PI_N$

where $dUptake_N/dt$ is the amount of nevadensin taken up from the gastrointestinal lumen (μmol), AGI_N (μmol) is the amount of nevadensin remaining in the gastrointestinal lumen, AL_N and AI_N are the amount of nevadensin in liver and intestine, respectively. Correspondingly, CL_N and CI_N are the nevadensin concentration in liver and intestine, respectively (μmol/L), CA_N is the nevadensin concentration in the arterial blood (μmol/L). QL_N and QI_N are the blood flow rates to liver (L) and intestine (I) (L/h), QC is the cardiac output (L/h), VL and VI are the volumes of liver and intestine, PL_N is the liver/blood partition coefficient of nevadensin and PI_N is the intestine/blood partition coefficient of nevadensin and V_{maxLNG}, V_{maxLNS}, and V_{maxING} are the maximum rates (μmol/h) for the formation of nevadensin glucuronide (NG) and nevadensin sulfonate (NS) in the liver (L) or intestine (I), and K_{mLNG}, K_{mLNS}, and K_{mING} are the Michaelis–Menten constants (μM) for the formation for the same metabolites of nevadensin.

The estragole and nevadensin PBBK models were connected to form a binary PBBK model taking into account the type of interaction between estragole and nevadensin defined in our previous work to be a non-competitive type of inhibition (Alhusainy *et al.*, 2010). Thus, the Michaelis–Menten equation representing the sulfonation pathway was modified accordingly (Alhusainy *et al.*, 2010). Moreover, nevadensin in the unconjugated form (CVL_N) is the presumed active form of nevadensin able to inhibit SULT activity.

To predict E-3′-N^2-dGuo DNA adduct levels in the liver in the absence or presence of nevadensin, the following equation describing the relationship between the PBBK predicted levels of 1′-sulfooxyestragole and the E-3′-N^2-dGuo DNA adduct formation levels in the liver of Sprague–Dawley rats, as described previously (Paini *et al.*, 2012b), was integrated into the model:

$DNAdGuo = 0.006 \times CL_{HES}$

where DNAdGuo is the amount of E-3′-N^2-dGuo DNA adducts (#adducts/1000 nt) formed in the liver and CL_{HES} is the amount of 1′-sulfooxyestragole (nmol/g liver) predicted by the PBBK model to be formed in the liver (Paini *et al.*, 2012b). The full code for the binary estragole-nevadensin PBBK model in rat and human are provided in the Supplementary Material (Model Code S1 and Model Code S2, respectively). Model equations were coded and numerically integrated in Berkeley Madonna 8.0.1 (Macey and Oster, UC Berkeley, CA,USA) using the Rosenbrock's algorithm for stiff systems.

Sensitivity analysis

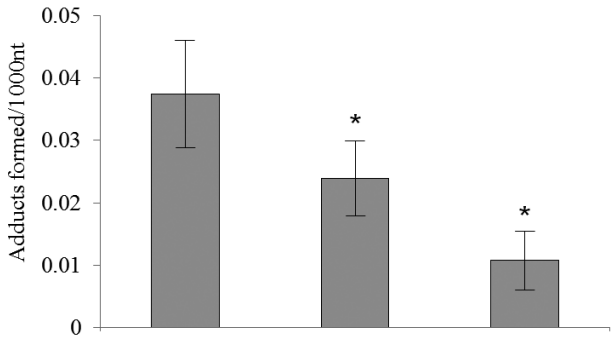
A sensitivity analysis was performed to identify key parameters that influence estragole E-3′-N^2-dGuo formation in the binary estragole-nevadensin model. A 1% increase in each parameter value without changing the other model parameters was chosen for this analysis. Normalized sensitivity coefficients for each parameter of the model were determined according to the following equation: SC = (C′−C)/(P′−P) × (P/C)

where C is the initial value of model output, C′ is the modified value of the model output resulting from an increase in parameter value, P is the initial parameter value, and P′ is the modified parameter value (Evans and Andersen, 2000).

RESULTS

Inhibition of E-3′-N^2-dGuo formation by nevadensin

As depicted in Figure 4, co-administration of 150 mg/kg bw estragole with 87.2 mg/kg bw nevadensin, representing their molar ratio in basil, or with 11 mg/kg bw PCP resulted in a significant 36 and 71% reduction in E-3′-N^2-dGuo formation in the liver of the rats, respectively.



Estragole (mg/kg bw)	150	150	150
Nevadensin (mg/kg bw)	0	87.2	0
PCP (mg/kg bw)	0	0	11

Figure 4. Formation E-3′-N^2-dGuo estragole DNA adducts in the liver of male Sprague–Dawley rats exposed to 150 mg/kg bw of estragole in the absence or presence of 87.2 mg/kg bw nevadensin or 11 mg/kg bw pentachlorophenol (PCP), the latter included as a positive control for SULT inhibition

Identification and kinetics of nevadensin metabolite formation by rat and human liver and intestinal S9

Figure 5 presents the HPLC–UV chromatograms from incubations of a pooled male rat liver S9 with 50 μM nevadensin in the presence of (A) UDGPA or (B) PAPS. The peak eluting at 4.1 min (Figure 5A) represents a nevadensin glucuronide since blank incubations without the cofactor UDGPA or the S9 fraction lacked this peak. The peak at 5.0 min (Figure 5B) represents a nevadensin sulfonate since blank incubations, without the cofactor PAPS or the S9 fraction, lacked this peak. The peak eluting at 6.5 min in the two chromatograms was identified as nevadensin since it shows the same retention time and UV spectrum as a commercially available nevadensin standard.

Moreover, LC–MS analysis using the same conditions used for the detection of E-3′-N^2-dGuo DNA adduct using negative ion mode instead of a positive ion mode and a collision energy of −18 eV revealed the presence of the following ions; *m/z* 519→343 (RT 3.16 min) corresponding to a nevadensin glucuronide and *m/z* 423→343 344 (RT 4.44 min) corresponding to a nevadensin sulfonate. Formation of methyl conjugates was not observed under the experimental conditions applied.

Glucuronidation seems to be the most efficient conjugation pathway for nevadensin in the liver and intestine of rat and human (Table 1). Overall, the catalytic efficiency for glucuronidation is 3.0- and 3.3-fold higher in the liver and intestine of rats compared to that in human, respectively, whereas the catalytic efficiency for sulfonation is 3.3-fold higher in the liver of rat compared to the liver of human (Table 1).

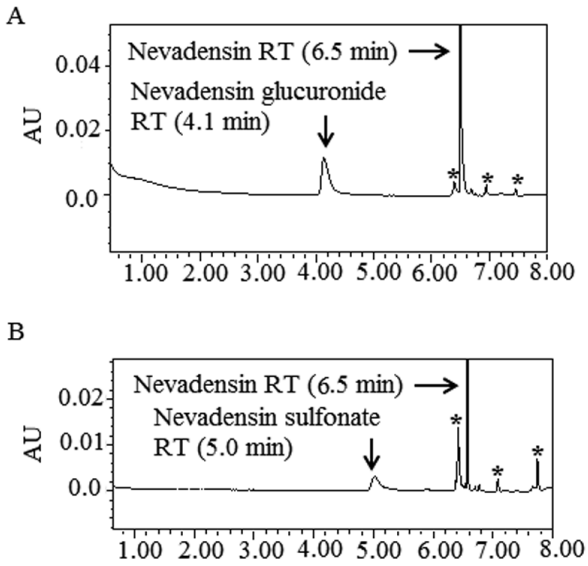


Figure 5. UPLC–UV chromatograms of incubations of nevadensin with rat S9 and (A) UDPGA for 30 min or (B) PAPS for 10 min. Peaks marked with an asterisk were also present in blank incubations without the respective cofactors.

Table 1. Kinetic parameters for glucuronidation and sulfonation of nevadensin in incubations with pooled male rat and human liver and intestinal S9 fractions

Protein	V_{max} (nmol/min/mg S9)	K_m (μM)	Catalytic efficiency (V_{max}/K_m)[a]
Glucuronidation			
Rat liver S9	0.62±0.10	0.78±0.16	0.79
Rat intestinal S9	0.58±0.07	0.86±0.39	0.66
Human liver S9	0.22±0.06	0.86±0.37	0.26
Human intestinal S9	0.55±0.03	2.80±0.07	0.20
Sulfonation			
Rat liver S9	0.05±0.005	26.0±3.2	0.002
Human liver S9	0.003±0.0006	5.1±1.0	0.0006

[a] Catalytic efficiency (V_{max}/K_m) is in (mL/min/mg S9 protein).

PBBK model based predictions for formation of E-3′-N^2-dGuo in the liver of rat and human in the absence or presence of nevadensin

Two different PBBK model results are presented in Figure 6, one being the prediction obtained using the previously described PBBK model assuming the nevadensin uptake to be 1% of that of estragole in the liver during the modeling time (48 h) (Alhusainy *et al.*, 2010, 2012) and the other prediction being obtained using the newly defined PBBK model including a sub-model describing the kinetics of nevadensin. As shown in Figure 6A, in the absence of nevadensin, the two models give the same result and predict the E-3′-N^2-dGuo level in the liver of rat after 48-h exposure to 150 mg/kg estragole to amount to 0.058 adducts/1000 nt. At the same dose level of estragole, the experimentally observed level of E-3′-N^2-dGuo in rat liver was 0.037±0.0086 adducts/1000 nt, which is only 1.6-fold lower than the predicted value.

For co-administration of 150 mg estragole/kg bw and 87.2 mg nevadensin/kg bw, the PBBK model predicts the E-3′-N^2-dGuo level to decrease to 0.002 adducts/1000 nt or to 0.012 adducts/1000 nt using either the previous or the new modeling approach, respectively. At the dose levels of estragole and nevadensin mentioned above, the experimentally observed level of E-3′-N^2-dGuo in rats was 0.024±0.006 adducts/1000 nt, which is, respectively, 12-fold or twofold higher than the predicted values.

Clearly, the new modeling approach that takes the kinetics for nevadensin into account better matches the experimental data compared to our previous modeling approach. According to Figure 6B, upon dosing 150 mg/kg bw of estragole in the presence of nevadensin, the formation of E-3′-N^2-dGuo in the liver of human after 48 h is predicted to decrease from 0.21 adducts/1000 nt to 0.009 adducts/1000 nt or to 0.02 adducts/1000 nt using either the previous or the new PBBK modeling approach, respectively. Thus, reduction in E-3′-N^2-dGuo formation is predicted to be larger in the liver of human compared to rat.

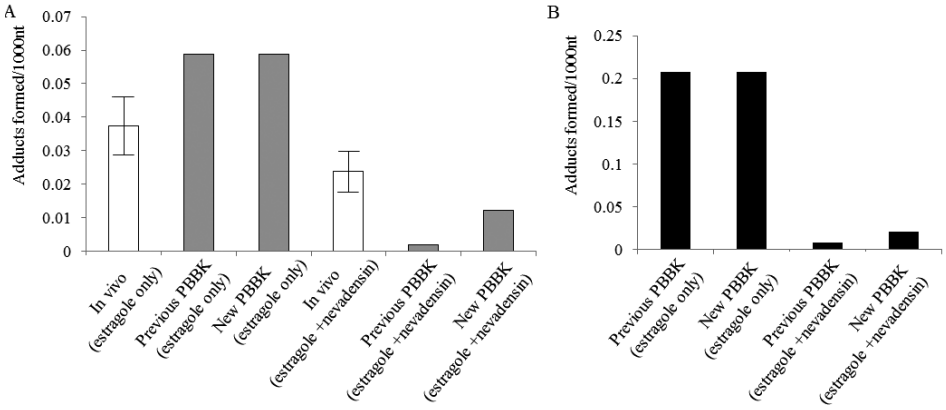


Figure 6. PBBK model based predictions for formation of E-3′-N^2-dGuo in the liver after a single oral dose of 150 mg/kg bw of estragole in the absence or presence of 87.2 mg/kg bw nevadensin in (A) rat and (B) human according to two PBBK modeling approaches; first, our previous PBBK models (Alhusainy *et al.*, 2010, 2012) assuming a nevadensin uptake in the liver that equals 1% of that of estragole concentration during the modeling time (48 h) and second, the newly defined PBBK models including a PBBK sub-model describing nevadensin kinetics.

Sensitivity analysis

The common parameters with an impact on the model predictions for formation of E-3′-N^2-dGuo in the liver of rat and human in the presence of 150 mg/kg bw estragole and 87.2 mg/kg bw nevadensin are the kinetic parameters (V_{max} and K_m) for formation of nevadensin glucuronide (NG) in the liver and the inhibition constant (*K*i) for the formation of ultimate estragole carcinogen, 1′-sulfooxyestragole (HES) by nevadensin (Figure S1A and S1B in the Supplementrary Material). The previous parameters have higher impact in the human compared to the rat model and higher impact in both species than the kinetic parameters (V_{max} and K_m) for the formation of the proximate carcinogen, 1′-hydroxyestragole (HE). Also of importance are the kinetic parameters (V_{max} and K_m) for the most important detoxification routes in each species, namely glucuronidation of 1′-hydroxyestragole resulting in the formation of 1′-hydroxyestragole glucuronide (HEG) in the liver of rat and oxidation of 1′-hydroxyestragole resulting in the formation of 1′-oxoestragole (HEO) in the liver of human.

DISCUSSION

The present work is the first report presenting data showing that a natural food-borne SULT inhibitor is able to inhibit SULT enzyme activity in vivo. This was demonstrated by a significant inhibition of SULT-mediated formation of E-3′-N^2-dGuo estragole DNA adduct in the liver of Sprague–Dawley rats exposed to the alkenylbenzene estragole and nevadensin simultaneously at a molar ratio of the two compounds reflecting their natural occurrence in basil. This observation is of importance given that the only phenolics for which in vivo SULT inhibitory activity has been reported so far are PCP and 2,6-dichloro-4-nitrophenol

(DCNP) (Koster *et al.*, 1979). Many SULT inhibitors including a number of natural dietary chemicals, such as polyphenols and a number of food additives, were also shown to have a SULT-inhibiting activity in vitro (Bamforth *et al.*, 1993; Morimitsu *et al.*, 2004; Alhusainy *et al.*, 2012). Different reports have also anticipated a SULT-inhibiting activity for some flavonoids in vivo because reported plasma concentration can reach levels higher than their *K*i (Gooderham *et al.*, 1996; Alhusainy *et al.*, 2012). It is important to note, however, that the reported plasma concentrations often reflect total flavonoid concentrations including both conjugated and unconjugated forms instead of the concentrations of the aglycon, whereas the active form of the flavonoid exerting SULT inhibition in most in vitro experiments is the aglycone. Nevadensin may be an effective in vivo SULT inhibitor because it possesses the structural requirements for effective SULT inhibitory activity including the presence of C5 and C7 hydroxyl substituents on the A-ring and a C2–C3 double bond in conjunction with the C4 carbonyl group on the C-ring (Morimitsu *et al.*, 2004). Furthermore, methylated flavones such as nevadensin have been demonstrated to be much more metabolically stable and have a higher intestinal absorption than their unmethylated analogs (Wen and Walle, 2006; Walle, 2007).

The estragole DNA adduct measured in the present study is E-3′-N^2-dGuo, which was shown to be the major DNA adduct formed in several in vitro and rodent studies with estragole (Phillips *et al.*, 1981; Wiseman *et al.*, 1985; Punt *et al.*, 2007) and a similar DNA adduct was also the major adduct formed with the related alkenylbenzene methyleugenol in in vitro studies and even in a human study (Herrmann *et al.*, 2012, 2013). Some other studies reported E-3′-N^6-dA to be the major adduct formed in rodents (Ishii *et al.*, 2011; Suzuki *et al.*, 2012). In the present study, as a first approximation, we assumed that nevadensin-mediated inhibition of formation of E-3′-N^2-dGuo would reflect the reduction in all estragole DNA adducts. This is based on the fact that all estragole DNA adducts will result from a chemical reaction with 1′-sulfooxyestragole, which is the ultimate carcinogenic metabolite, and that it is the formation of 1′-sulfooxyestragole that is inhibited by nevadensin. If all adducts would be reduced to a similar extent, one can subsequently assume that the resulting mutagenicity and carcinogencity would be reduced to a similar extent as well, even when different adducts would display different mutagenicity.

The newly refined PBBK modeling approach that takes the kinetics for conversion of nevadensin into account was shown to adequately match the experimental data with only twofold difference in predicted estragole E-3′-N^2-dGuo level compared to 12-fold difference using the previous approach (Alhusainy *et al.*, 2010, 2012). Also, PBBK model predictions reveal a more potent inhibition of E-3′-N^2-dGuo formation in the liver of human compared to rat due to lower catalytic efficiencies for glucuronidation and sulfonation of nevadensin in the liver and intestine of human compared to rat resulting in higher liver concentrations of nevadensin in its unconjugated form, which is the form required for SULT inhibition.

The question that might be raised now is how to integrate the current results on nevadensin-mediated modulation of estragole bioactivation in the risk assessment of

estragole. The approach suggested by the European Food Safety Authority for the risk assessment of compounds that are both genotoxic and carcinogenic is the so-called margin of exposure (MOE) approach, which can be used to set priorities in risk management (EFSA, 2005). The reported lower confidence bound of the benchmark dose that gives 10% extra cancer incidence ($BMDL_{10}$) value of estragole-based tumour data in mice (Miller *et al.*, 1983) varies between 3.3 and 6.5 mg/kg bw/day (van den Berg *et al.*, 2011). Using these reported $BMDL_{10}$ values and the estimated daily intake for estragole of 0.01 mg/kg bw/day (Smith *et al.*, 2002), the MOE value varies between 350 and 650. Matrix modulation of estragole toxicity by nevadensin cannot easily be reflected in the numerical MOE but one may suggest to translate the reduction in estragole E-3′-N^2-dGuo DNA adduct levels into a proportional reduction in the incidence of hepatomas and then to calculate a refined $BMDL_{10}$ value that can be used to estimate a refined MOE. The newly developed PBBK models were used to calculate the possible reduction in E-3′-N^2-dGuo at the estragole dose levels used in the study of Miller et al. (Miller *et al.*, 1983) when nevadensin would be co administered with estragole at a molar ratio of estragole to nevadensin of 1:0.25 reflecting their natural occurrence in basil as shown in the Supplementary Material (Table S7). Thereafter, the percentage reduction in E-3′-N^2-dGuo formation was used to calculate a possible percentage reduction in hepatoma incidences at the dose levels of estragole used in the study of Miller et al. (Miller *et al.*, 1983) as shown in Table S7 in the Supplementary Material. Subsequently, a benchmark dose (BMD) analysis of the newly generated dose response curves (Figure S2 in the Supplementary Material) was performed using BMDS software version 2.1.2. providing newly estimated refined $BMDL_{10}$ values. Using these refined $BMDL_{10}$ values as shown in Table S8 in the Supplementary Material and the EDI for estragole of 0.01 mg/kg bw/day (Smith *et al.*, 2002), the refined MOE for estragole amounted to 4,300 and 7,350 pointing at a lower priority for risk management, than when the MOE calculation would be based on the tumour data obtained with pure estragole. Although this linear relation between E-3′-N^2-dGuo formation and hepatoma formation remains to be established, the assumption was used as a first approximation to estimate the possible consequences of the nevadensin-mediated inhibition of estragole bioactivation to its DNA reactive carcinogenic metabolite.

Overall, given the role of the SULT-mediated formation of estragole DNA adduct formation in the hepatocarcinogenicity of estragole in rodents, the results of the present study indicate that the likelihood of bioactivation and subsequent adverse effects in rodent bioassays may be lower when estragole is dosed in a matrix containing SULT inhibitors such as nevadensin compared to experiments using estragole as a single compound.

ACKNOWLEDGEMENT

This work was supported, in part, by a grant from the International Organization of the Flavour Industry (IOFI) and from Nestlé Research Centre (Switzerland).

REFERENCES

1. Alhusainy, W., Paini, A., Punt, A., Louisse, J., Spenkelink, A., Vervoort, J., Delatour, T., Scholz, G., Schilter, B., Adams, T., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). Identification of nevadensin as an important herb-based constituent inhibiting estragole bioactivation and physiology-based biokinetic modeling of its possible in vivo effect. *Toxicology and Applied Pharmacology* **245**, 179-190.
2. Alhusainy, W., van den Berg, S. J. P. L., Paini, A., Campana, A., Asselman, M., Spenkelink, A., Punt, A., Scholz, G., Schilter, B., Adams, T. B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2012). Matrix modulation of the bioactivation of estragole by constituents of different alkenylbenzene-containing herbs and spices and physiologically based biokinetic modeling of possible in vivo Effects. *Toxicological Sciences* **129**, 174-187.
3. Bamforth, K. J., Jones, A. L., Roberts, R. C., and Coughtrie, M. W. H. (1993). Common food additives are potent inhibitors of human liver 17α-ethinyloestradiol and dopamine sulphotransferases. *Biochemical Pharmacology* **46**, 1713-1720.
4. Boberg, E. W., Miller, E. C., and Miller, J. A. (1983). Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1'-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1'-hydroxysafrole in mouse liver. *Cancer Research* **43**, 5163-5173.
5. EFSA (2005). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. *EFSA Journal* **282**, 1-31.
6. European Commission (EC) (2008). Regulation (EC) No 1334/2008 of the Europian Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC.
7. Evans, M. V., and Andersen, M. E. (2000). Sensitivity analysis of a physiological model for 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD): Assessing the impact of specific model parameters on sequestration in liver and fat in the rat. *Toxicological Sciences* **54**, 71-80.
8. Fisher, M. B., Campanale, K., Ackermann, B. L., Vandenbranden, M., and Wrighton, S. A. (2000). In vitro glucuronidation using human liver microsomes and the pore- forming peptide alamethicin. *Drug Metabolism and Disposition* **28**, 560-566.
9. Gooderham, M. J., Adlercreutz, H., Ojala, S. T., Wähälä, K., and Holub, B. J. (1996). A soy protein isolate rich in genistein and daidzein and its effects on plasma isoflavone concentrations, platelet aggregation, blood lipids and fatty acid composition of plasma phospholipid in normal men. *Journal of Nutrition* **126**, 2000-2006.
10. Herrmann, K., Engst, W., Appel, K. E., Monien, B. H., and Glatt, H. (2012). Identification of human and murine sulfotransferases able to activate hydroxylated metabolites of methyleugenol to mutagens in Salmonella typhimurium and detection of associated DNA adducts using UPLC-MS/MS methods. *Mutagenesis* **27**, 453-462.
11. Herrmann, K., Schumacher, F., Engst, W., Appel, K. E., Klein, K., Zanger, U. M., and Glatt, H. (2013). Abundance of DNA adducts of methyleugenol, a rodent hepatocarcinogen, in human: Liver samples. *Carcinogenesis* **34**, 1025-1030.
12. Ishii, Y., Suzuki, Y., Hibi, D., Jin, M., Fukuhara, K., Umemura, T., and Nishikawa, A. (2011). Detection and quantification of specific DNA adducts by liquid chromatography-tandem mass spectrometry in the livers of rats given estragole at the carcinogenic dose. *Chemical Research in Toxicology* **24**, 532-541.
13. Jeurissen, S. M. F., Punt, A., Delatour, T., and Rietjens, I. M. C. M. (2008). Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1 '-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. *Food and Chemical Toxicology* **46**, 2296-2302.
14. Koster, H., Scholtens, E., and Mulder, G. J. (1979). Inhibition of sulfation of phenols in vivo by 2,6-dichloro-4-nitrophenol: Selectivity of its action in relation to other conjugations in the rat in vivo. *Medical Biology* **57**, 340-344.

15. Li, H., Zhao, X., Ma, Y., Zhai, G., Li, L., and Lou, H. (2009). Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. *Journal of Controlled Release* **133**, 238-244.
16. Medinsky, M. A., Leavens, T. L., Csanády, G. A., Gargas, M. L., and Bond, J. A. (1994). In vivo metabolism of butadiene by mice and rats: A comparison of physiological model predictions and experimental data. *Carcinogenesis* **15**, 1329-1340.
17. Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Research* **43**, 1124-1134.
18. Morimitsu, Y., Sugihara, N., and Furuno, K. (2004). Inhibitory effect of flavonoids on sulfo- and glucurono-conjugation of acetaminophen in rat cultured hepatocytes and liver subcellular preparations. *Biological and Pharmaceutical Bulletin* **27**, 714-717.
19. Paini, A., Punt, A., Scholz, G., Gremaud, E., Spenkelink, B., Alink, G., Schilter, B., Van Bladeren, P. J., and Rietjens, I. M. C. M. (2012a). In vivo validation of DNA adduct formation by estragole in rats predicted by physiologically based biodynamic modelling. *Mutagenesis* **27**, 653-663.
20. Paini, A., Punt, A., Viton, F., Scholz, G., Delatour, T., Marin-Kuan, M., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). A physiologically based biodynamic (PBBD) model for estragole DNA binding in rat liver based on in vitro kinetic data and estragole DNA adduct formation in primary hepatocytes. *Toxicology and Applied Pharmacology* **245**, 57-66.
21. Paini, A., Scholz, G., Boersma, M. G., Spenkelink, A., Schilter, B., van Bladeren, P. J., Rietjens, I. M. C. M., and Punt, A. (2012b). Evaluation of interindividual human variation in bioactivation and DNA binding of estragole in liver predicted by physiologically based biodynamic (PBBD) and Monte Carlo modeling. In *Generation of in vitro data to model dose dependent in vivo DNA binding of genotoxic carcinogens and its consequences: the case of estragole.*, pp. 70-85.
22. Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. *Cancer Research* **41**, 176-186.
23. Phillips, D. H., Reddy, M. V., and Randerath, K. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. *Carcinogenesis* **5**, 1623-1628.
24. Punt, A., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2007). Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1'-hydroxyestragole. *Chemical Research in Toxicology* **20**, 991-998.
25. Punt, A., Freidig, A. P., Delatour, T., Scholz, G., Boersma, M. G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2008). A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat. *Toxicology and Applied Pharmacology* **231**, 248-259.
26. Punt, A., Paini, A., Boersma, M. G., Freidig, A. P., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2009). Use of physiologically based biokinetic (PBBK) modeling to study estragole bioactivation and detoxification in humans as compared with male rats. *Toxicological Sciences* **110**, 255-269.
27. Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis* **5**, 1613-1622.
28. Singh, S. P., Wahajuddin, Tewari, D., Patel, K., and Jain, G. K. (2011). Permeability determination and pharmacokinetic study of nobiletin in rat plasma and brain by validated high-performance liquid chromatography method. *Fitoterapia* **82**, 1206-1214.
29. Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J.,

and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - Methyl eugenol and estragole. *Food and Chemical Toxicology* **40**, 851-870.

30. Suzuki, Y., Umemura, T., Ishii, Y., Hibi, D., Inoue, T., Jin, M., Sakai, H., Kodama, Y., Nohmi, T., Yanai, T., Nishikawa, A., and Ogawa, K. (2012). Possible involvement of sulfotransferase 1A1 in estragole-induced DNA modification and carcinogenesis in the livers of female mice. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis* **749**, 23-28.
31. van de Kerkhof, E. G., de Graaf, I. A. M., and Groothuis, G. M. M. (2007). In vitro methods to study intestinal drug metabolism. *Current Drug Metabolism* **8**, 658-675.
32. van den Berg, S. J. P. L., Restani, P., Boersma, M. G., Delmulle, L., and Rietjens, I. M. C. M. (2011). Levels of Genotoxic and Carcinogenic Compounds in Plant Food Supplements and Associated Risk Assessment. *Food and Nutrition Sciences* **2**, 989-1010.
33. Walle, T. (2007). Methylation of dietary flavones greatly improves their hepatic metabolic stability and intestinal absorption. *Molecular Pharmaceutics* **4**, 826-832.
34. Wen, X., and Walle, T. (2006). Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug Metabolism and Disposition* **34**, 1786-1792.
35. Wiseman, R. W., Fennell, T. R., Miller, J. A., and Miller, E. C. (1985). Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1′-hydroxysafrole and 1′-hydroxyestragole in vitro and in mouse liver in vivo, including new adducts at C-8 and N-7 of guanine residues. *Cancer Research* **45**, 3096-3105.
36. Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F1 mice. *Cancer Research* **47**, 2275-2283.

5 Inhibition of methyleugenol bioactivation by the herb based constituent nevadensin and prediction of possible in vivo consequences using physiologically based biokinetic modeling

Based on:
Al-Subeihi A, Alhusainy W, Paini A, Punt A, Vervoort J, van Bladeren PJ, and Rietjens IMCM

Food and Chemical Toxicology 59 (2013), 564–571

Supplementary Material can be found at http://www.sciencedirect.com/science/article/pii/S0278691513004225#

ABSTRACT

Methyleugenol occurs naturally in a variety of spices, herbs, including basil, and their essential oils. Methyleugenol induces hepatomas in rodent bioassays following its conversion to a DNA reactive metabolite. In the present study, the basil constituent nevadensin was shown to be able to inhibit SULT-mediated DNA adduct formation in HepG2 cells exposed to the proximate carcinogen 1′-hydroxymethyleugenol in the presence of nevadensin. To investigate possible in vivo implications of SULT inhibition by nevadensin on methyleugenol bioactivation, the rat physiologically based biokinetic (PBBK) model developed in our previous work to describe the dose-dependent bioactivation and detoxification of methyleugenol in male rat was combined with the recently developed PBBK model describing the dose-dependent kinetics of nevadensin in male rat. The resulting binary methyleugenol–nevadensin PBBK model was used to predict the possible nevadensin- mediated reduction in methyleugenol DNA adduct formation and resulting carcinogenicity at the doses of methyleugenol used by the NTP carcinogenicity study. Using these data an updated risk assessment using the margin of exposure (MOE) approach was performed. The results obtained point at a potential reduction of the cancer risk when rodents are orally exposed to methyleugenol within a relevant food matrix containing SULT inhibitors compared to exposure to pure methyleugenol.

INTRODUCTION

Methyleugenol is a compound naturally occurring in a variety of spices and herbs, including clove, allspice, cinnamon leaves, walnuts, basil, nutmeg, anise, pimento, citronella, laurel fruits leaves and others, as well as in their essential oils (Smith *et al.*, 2002). The general population is primarily exposed to methyleugenol via ingestion of food stuffs flavoured with methyleugenol containing essential oils (Smith *et al.*, 2010), including for instance, candy, cookies such as gingersnaps, ice cream, tomato ketchup and relish (Burdock, 1995; Leung, 1998). Realistic daily intake levels of methyleugenol have been estimated to range from 0.014 mg/kg bw/day (Smith *et al.*, 2002) to 0.217 mg/kg bw/day (SCF, 2001). Given that at present addition of methyleugenol as a pure compound to individual food categories is no longer allowed within the European Union (European Commission, 2008), the value of 0.014 mg/kg bw/day, resulting mainly from herbs and spices and their essential oils (Smith *et al.*, 2002), is taken in the present study as the value for current levels of dietary human intake.

In 2000, the carcinogenicity of methyleugenol was investigated in mice and rats (NTP, 2000). The NTP reported that methyleugenol is carcinogenic in both rats and mice when dosed at high dose levels as a pure compound suspended in 0.5 % methylcellulose (NTP, 2000). Methyleugenol is unreactive by itself but undergoes metabolic activation to produce an electrophilic metabolite that acts as the DNA reactive intermediate (Miller *et al.*, 1983; Randerath *et al.*, 1984; Gardner *et al.*, 1997; Smith *et al.*, 2002). Figure 1 displays the bioactivation pathway of methyleugenol which starts with hydroxylation of the 1′-position of methyleugenol to produce the proximate carcinogenic metabolite 1′-hydroxymethyleugenol (Miller *et al.*, 1983; Gardner *et al.*, 1997; Smith *et al.*, 2002). In a next step, 1′-hydroxymethyleugenol can be sulfonated by SULT to form 1′-sulfoxymethyleugenol that can form DNA adducts (Smith *et al.*, 2002). Recently, studies using *Salmonella typhimurium* TA100 strains expressing different human SULTs revealed that human SULT1A1 and SULT1C2 are specifically able to activate 1′-hydroxymethyleugenol to DNA reactive metabolites (Herrmann *et al.*, 2012).

It is important to note that the carcinogenicity observed in studies in which animals are exposed to high dose levels of the pure compound by gavage may not be representative for the situation where humans are exposed to methyleugenol at low levels via dietary intake with a food matrix being present. For the related alkenylbenzene estragole it was actually demonstrated that other ingredients present

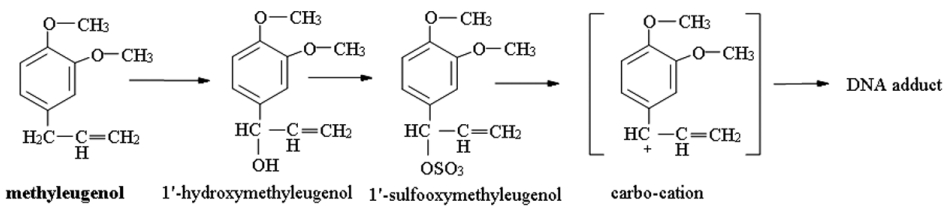


Figure 1. Structural representation of the pathway for bioactivation of methyleugenol.

in alkenylbenzene containing herbs or food items may inhibit the SULT-mediated bioactivation of estragole to its ultimate DNA reactive 1′-sulfooxy metabolite (Jeurissen *et al.*, 2008; Alhusainy *et al.*, 2010). For instance a methanolic basil extract was shown to cause a dose dependent inhibition of DNA adduct formation in 1′-hydroxyestragole exposed human HepG2 cells (Jeurissen *et al.*, 2008). In a subsequent study, the flavonoid nevadensin (Figure 2) was identified as the potent SULT inhibitor present in these methanolic basil extracts (Alhusainy *et al.*, 2010). So far the existence of such matrix dependent interactions with the bioactivation of alkenylbenzenes was only demonstrated for estragole (Alhusainy *et al.*, 2010, 2012).

Therefore the objective of the present study was to study the potential of nevadensin to inhibit the SULT-mediated bioactivation and subsequent DNA adduct formation of methyleugenol using human HepG2 cells as an in vitro model. To obtain some insight in the in vivo relevance of these observations our physiologically based biokinetic (PBBK) model for bioactivation and detoxification of methyleugenol in male rat (Al-Subeihi *et al.*, 2011) was combined with the recently developed PBBK model for nevadensin in male rat (Alhusainy *et al.*, 2013). This combined PBBK model was previously validated using in vivo data on liver adduct formation in male Sprague–Dawley rats orally dosed with the structurally related alkenylbenzene estragole and nevadensin (Alhusainy *et al.*, 2013). Using this binary PBBK model the effects of combined in vivo exposure to methyleugenol and nevadensin could be quantified as well given that the SULT inhibition by nevadensin is non-competitive in nature and therefore by definition independent of the nature of the SULT substrate.

MATERIALS AND METHODS

Materials

2′-Deoxyguanosine was purchased from Sigma (Basel, Switzerland), and 1,2,3,7,9-$^{15}N_5$-2′-deoxyguanosine was obtained from Cambridge Isotope Laboratories (Cambridge, MA). Fetal bovine serum (FBS), DMEM/F12 (L-glutamine, 15 mM HEPES) medium and phosphate buffered saline (PBS) (pH 7.4) were purchased from Gibco (UK). Dimethylsulfoxide (DMSO), methanol, zinc sulphate (heptahydrate), phosphodiesterase I from *Crotalus adamanteus* (venom phosphodiesterase), phosphodiesterase II from

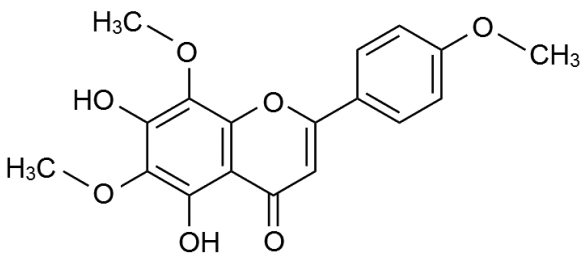


Figure 2. Structural formula of nevadensin.

bovine spleen (spleen phosphodiesterase), nuclease P1 and alkaline phosphatase were purchased from Sigma (Schnelldorf, Germany). Acetonitrile was purchased from Biosolve BV (Valkenswaard, The Netherlands). Formic acid and ethanol were obtained from VWR Merck (Darmstadt, Germany). Nevadensin was purchased from Apin Chemicals (Milton, UK). Pentachlorophenol (PCP; 98%) was obtained from Sigma-Riedel de Haen (Seelze, Germany). 1′-Hydroxymethyleugenol and 1′-acetoxymethyleugenol were synthesized as described previously (Al-Subeihi *et al.*, 2011). Human HepG2 cells were purchased from the American type culture collection (Manassas, Virginia).

Synthesis of N^2-(*trans*-isomethyleugenol-3′-yl)-2′-deoxyguanosine (ME-3′-N^2-dGuo) and $^{15}N_5$-labeled N^2-(*trans*-isomethyleugenol-3′-yl)-2′-deoxyguanosine ($^{15}N_5$) ME-3′-N^2-dGuo)

ME-3′-N^2-dGuo was synthesized via a reaction between 1′-acetoxymethyleugenol and 2′-deoxyguanosine following the protocol of Punt *et al.* (2007). In brief, 250 µL of a 0.01 g/mL solution of 1′-acetoxymethyleugenol in DMSO was added to 2250 µL of 2.5 mM 2′-deoxyguanosine in 2.5 mM ammonium carbonate (pH 7.4). The same reaction was performed for the synthesis of ($^{15}N_5$) ME-3′-N^2-dGuo in which 1′-acetoxymethyleugenol was allowed to react with $^{15}N_5$-labeled 2′-deoxyguanosine. The incubations were stirred for 48 h at 37°C. Both ME-3′-N^2-dGuo and ($^{15}N_5$) ME-3′-N^2-dGuo were purified by HPLC–UV on a M600 liquid chromatography system (Waters, Milford, MA) equipped with an Alltima C18 5 µm column, 150 × 4.6 mm (Alltech, Breda, The Netherlands). The gradient was made with ultrapure water and acetonitrile. The flow rate was 1 mL/min. A linear gradient was applied from 10% to 15% acetonitrile over 30 min after which the percentage of acetonitrile was increased to 100% over 2 min, kept at 100% for 1 min, lowered to 10% in 2 min, and kept at these initial conditions for 10 min for equilibration of the system. Detection was carried out using a Waters 966 photodiode array detector (Waters, Milford, MA) at 260 nm. Both ME-3′-N^2-dGuo and ($^{15}N_5$) ME-3′-N^2-dGuo were collected at a retention time of 11.4 min. The purity of both ME-3′-N^2-dGuo and ($^{15}N_5$) ME-3′-N^2-dGuo was more than 98%, according to LC–MS/MS and HPLC analyses.

Cytotoxicity test

The cytotoxicity of nevadensin, 1′-hydroxymethyleugenol, pentachlorophenol, and DMSO was evaluated using the MTT test (Mosmann, 1983; Hussain *et al.*, 1993). HepG2 cells were plated in a 96-well plate at a density of 2 $\times 10^4$ cells per well 24 h prior to exposure. Just before exposure the medium was removed and cells were washed with 100 µL HBSS. Cells were exposed to 0.5% DMSO (control) or the test compounds in exposure medium (DMEM/F12 + glutamax + 50 µg/mL gentamicin) at concentrations of nevadensin, 1′-hydroxymethyleugenol, and/or pentachlorophenol as indicated and added from respectively 5000, 1000, and 400 times concentrated stock solutions in DMSO for 4 h in a humidified atmosphere at 37°C. Then, 5 µL of a 5 mg/mL MTT solution in PBS was added and the cells were incubated for another hour.

Thereafter, the medium was removed and 100 µL of DMSO was added to all wells to dissolve the formazan crystals. The absorbance was measured at 562 nm and cell viability was defined as the ratio between the absorbance measured for nevadensin, 1′-hydroxymethyleugenol, pentachlorophenol or DMSO treated cell samples and the absorption measured for untreated cell samples.

Incubation of HepG2 cells with 1′-hydroxymethyleugenol

Human HepG2 cells were cultured in a 75 cm^2 flask (Corning, NY, USA) with DMEM/F12 medium containing glutamax, 10% FBS and incubated in a humidified incubator under, 5% CO_2 and 95% air at 37°C. Once reaching 80-90% confluence the cells were incubated overnight (~14 h) in the presence of 1′-hydroxymethyleugenol at a final concentration of 100 µM (added from a 1000 times concentrated stock solution in DMSO) and nevadensin at a final concentration of 0, 0.002, 0.02, 0.2, or 2 µM (added from 5000 times concentrated stock solutions in DMSO) or pentachlorophenol at a final concentration of 12.5 µM (added from a 400 times concentrated stock solution in DMSO). After overnight incubation cells were washed twice with phosphate buffered saline (PBS), scraped, collected into an Ependorf tube, and centrifuged at 1500 rpm for 5 min (Eppendorf Centrifuge 5424). DNA was extracted from the pellet and thereafter digested as reported in the next section.

DNA extraction and digestion

DNA was extracted from human HepG2 cells as previously described (Jeurissen *et al.*, 2008; Paini *et al.*, 2010). Briefly, DNA was extracted using the Get pure DNA Kit-Cell protocol (Dojindo Molecular Technology Inc., Kumamoto, Japan) with 3-10×10^6 cells per sample (following the manufacturer's instructions). The yields and purity of the extracted DNA were determined using the Nanodrop technique measuring the absorbance ratio A260 nm/A280 nm. DNA samples with an absorbance ratio of 1.8-2 were considered sufficiently pure. The quantity of DNA per sample was calculated from the Nanodrop output in ng/mL using a molar extinction coefficient for double stranded DNA of 50 (L/mol × cm) at a wavelength of 260 nm. Digestion of isolated DNA was done as reported previously (Paini *et al.*, 2010). In short, to 50 µg DNA in 80 µL water, 20 µL buffer P1 (300 mM sodium acetate, 1 mM $ZnSO_4$, pH 5.3), 11 µL SPDE (spleen phosphodiesterase) solution (0.0004 U/µL in water), and 10 µL nuclease P1 (0.5 µg/µL in water) were added and the resulting solution was incubated for 4 h at 37 °C. Following incubation, 20 µL buffer (500 mM Tris-HCl, 1 mM EDTA, pH 8.0), 12 µL VPDE (venom phosphodiesterase) solution (0.00026 U/µL in water), and 6 µL alkaline phosphatase (0.764 U/µL in water) were added and the mixture was incubated for 3 h at 37°C (Paini *et al.*, 2010; Al-Subeihi *et al.*, 2011). After these incubations samples were filtered using eppendorf tubes with a cut off membrane of 5.000 nominal molecular weight limit (NMWL) (Millipore). The hydrolyzed filtered samples were evaporated to dryness and reconstituted in 50 µL water and stored at -20°C until LC–ESI–MS/MS analysis.

LC–ESI–MS/MS method for detection and quantification of ME-3′-N^2-dGuo

LC–ESI–MS/MS analysis was performed on a Perkin Elmer 200 Series HPLC System (Perkin Elmer, Waltham, Massachusetts) coupled to an API 3000 system (Applied Biosystem, Foster City, California). Samples were injected on an Agilent Zorbax Extend-C18 column, 2.1 × 50 mm, 3.5 Micron 80 Å (Basel, Switzerland), with a Zorbax guard column. The gradient was made with ultrapure water containing 0.1% (v/v) formic acid and 100% acetonitrile. The flow rate was set at 0.3 mL/min. A linear gradient was applied from 10% to 50% acetonitrile over 3 min, after which the percentage of acetonitrile was brought to 100% in 1 min, and kept at 100% acetonitrile over 2 min. The amount of acetonitrile was lowered to 10% over 1 min, and the column was equilibrated at these initial conditions for 8 min. ME-3′-N^2-dGuo eluted at 2.58 min. The mass spectrometric analysis was performed with the following settings: nebulizer gas (air) was set at 15 psi, curtain gas (nitrogen, which is used to keep the analyzer region clean) was set at 10 psi, the ion spray voltage at 4700 V, the collision gas (CAD) was 5 eV, the ion source temperature was set at 300 °C, the declustering potential was set at 37 V, the focusing potential was set at 200 V, the entrance potential at 9 V, and the collision cell exit was set at 15 V. Nitrogen was used as sheath gas turbo, ion spray, with a pressure of 7000 L/h. The dwell time per transition was 0.05 s. A divert valve was used in order to discard the gradient after elution of the peak. Quantification of the DNA adduct was carried out using selected-ion detection in the multiple reaction-monitoring mode (MRM), the characteristic transitions for the ME-3′-N^2-dGuo and for the ($^{15}N_5$) ME-3′-N^2-dGuo are displayed in Table 1.

Statistical analysis

To test whether the effect of nevadensin on formation levels of ME-3′-N^2-dGuo adducts was significant, a two sample *t*-test (one-sided, equal variances) was performed, after an F test for equal variances was done, using Excel (Microsoft Office 2000).

PBBK model based predictions

To investigate possible in vivo implications of SULT inhibition by nevadensin for methyleugenol bioactivation, the PBBK model developed in our previous work to describe the dose-dependent bioactivation and detoxification of methyleugenol in male rat (Al-Subeihi *et al.*, 2011) was combined with the recently developed PBBK

Table 1. Transition reaction (m/z) and the collision energy used to obtain the daughter fragments by LC–ESI–MS/MS.

	Transition (m/z) (used for quantification)	Collision energy (eV)	Transition (m/z) (used for confirmation)	Collision energy (eV)
ME-3′-N^2-dGuo	444→328	18	444→177 444→164	37 40
($^{15}N_5$)ME-3′-N^2-dGuo	449→333	18	449→169	40

model describing the dose-dependent kinetics of nevadensin in male rat (Alhusainy *et al.*, 2013). A schematic diagram for the male rat methyleugenol PBBK model is shown in Figure 3A and that for the male rat nevadensin PBBK model in Figure 3B. The original PBBK model for methyleugenol was described in detail previously (Al-Subeihi *et al.*, 2011). The male rat nevadensin PBBK model was also described previously (Alhusainy *et al.*, 2013). Similar to the male rat PBBK model of methyleugenol, the model for nevadensin included separate compartments for liver, fat, rapidly and slowly perfused tissues. Unlike the PBBK model of methyleugenol, the nevadensin PBBK model contained a compartment for intestine which was found to be involved in the metabolism of nevadensin (Alhusainy *et al.*, 2013).

The physiological, physico-chemical parameters and kinetic constants for the metabolism of methyleugenol and nevadensin used in the male rat PBBK models are described in detail in our previous work (Al-Subeihi *et al.*, 2011; Alhusainy *et al.*, 2013) and they are summarized in the Supplementary Material (Table S1-S4)

The methyleugenol and nevadensin PBBK models were connected to form a binary PBBK model taking into account the non-competitive type of inhibition of SULT by nevadensin in line with our previous work with estragole and nevadensin (Alhusainy *et al.*, 2010, 2012, 2013). The non-competitive nature of the SULT inhibition by nevadensin implies that the inhibition and its *K*i are independent of the nature of the substrate and therefore the *K*i for the inhibition of SULT enzyme activity by nevadensin identified previously using the standard substrate 7-hydroxycoumarin (Alhusainy *et al.*, 2010) can also be used in the combined methyleugenol nevadensin PBBK modeling.

The Michaelis–Menten equation in the model that represents the sulfonation pathway was modified to reflect the non-competitive type of inhibition by introducing a modulation factor $(1+([CVL_N]/K_i)$ for the apparent V_{max} value. Nevadensin in the unconjugated form (CVL_N) is assumed to be the active form of nevadensin able to inhibit SULT activity. The resulting equation for sulfonation then is as follows:

$$dAM_{1'HMES}/dt = V_{max,L\_1'HMES}/(1+([CVL_N]/K_i)) \times CL_{1'HME}/PL_{1'HME}/(K_{m,L-1'HMES}+CL_{1'HME}/PL_{1'HME})$$

where $AM_{1'HMES}$ is the amount of 1′-sulfooxymethyleugenol (μmol), $V_{max,L\_1'HMES}$ is the maximum rate of formation of 1′-sulfooxymethyleugenol, $[CVL_N]$ is the free concentration of nevadensin in the liver (μmol/L), K_i is the inhibition constant for inhibition of the sulfonation by nevadensin (μmol/L), $CL_{1'HME}$ is the concentration of 1′-hydroxymethyleugenol in the liver (μmol/L), $PL_{1'HME}$ is the liver/blood partition coefficient of 1′-hydroxymethyleugenol, and $K_{m,L-1'HMES}$ is the Michaelis–Menten constant for the formation of 1′-sulfooxymethyleugenol (μmol/L) in the liver. Using our binary methyleugenol–nevadensin PBBK model for methyleugenol bioactivation and detoxification, the time dependent concentration of 1′-sulfooxymethyleugenol ($CL_{1'HMES}$) in the liver of male rat following an exposure to different doses of methyleugenol in the presence of varying amounts of nevadensin can be predicted.

In line with our previous work (Alhusainy *et al.*, 2013), the equation describing the relationship between the PBBK predicted levels of the 1′-sulfooxy metabolite and the levels of DNA adduct formation in the liver (Paini *et al.*, 2012b) was as follows;

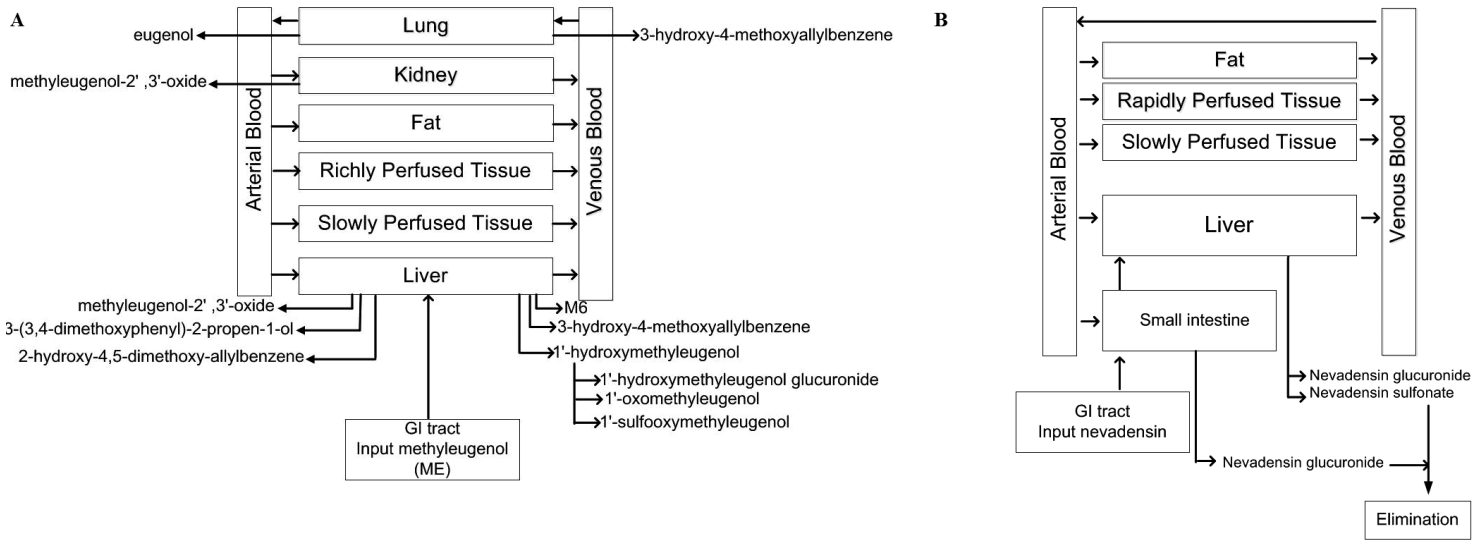


Figure 3. Representation of the conceptual model for the physiologically based biokinetic model for methyleugenol (A) and nevadensin (B) in male rat.

$DNA_{dGuo}=0.006\times CL_{1'HMES}$

Where DNA_{dGuo} is the amount of ME-3′-N^2-dGuo DNA adducts (#adducts/1000nt) formed in the liver and $CL_{1'HMES}$ is the amount of the 1′-sulfooxy metabolite (nmol/g liver) predicted by the PBBK model to be formed in the liver.

Model equations were coded and numerically integrated in Berkeley Madonna 8.0.1 (Macey and Oster, UC Berkeley, CA, USA) using the Rsenbrock's algorithm for stiff systems.

$BMDL_{10}$ and MOE analysis

In order to estimate how reduction in methyleugenol bioactivation in the presence of nevadensin may in theory affect the risk assessment of methyleugenol, we investigated the possible consequences arising from the co-administration of methyleugenol and nevadensin on the so-called margin of exposure (MOE). The MOE was suggested by the European Food Safety Authority (EFSA) to be used to set priorities in risk management with respect to compounds that are both genotoxic and carcinogenic and is defined as the ratio between the lower confidence limit of the benchmark dose that gives 10% extra cancer incidence ($BMDL_{10}$) and the estimated daily intake (EDI) (EFSA, 2005). 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 $BMDL_{10}$ values of methyleugenol for female and male rats were obtained from the literature (van den Berg *et al*., 2011) where the values were calculated using BMDS software version 2.1.2 based on the available incidence of hepatocellular carcinomas in female and male F344/N rats administered methyleugenol by gavage 5 days per week for up to 105 weeks (NTP, 2000). The binary methyleugenol–nevadensin PBBK model in male rat was used to calculate the possible reduction in ME-3′-N^2-dGuo at the methyleugenol dose levels used in the NTP study for female and male rats when methyleugenol would be co-administered with nevadensin at a molar ratio of methyleugenol to nevadensin of 1:0.20 reflecting their natural occurrence in basil (Smith *et al.*, 2002; Alhusainy *et al.*, 2012). Thereafter, the percentage reduction in ME-3′-N^2-dGuo formation was used to calculate the percentage reduction in incidence of hepatocellular carcinomas at the respective dose levels of methyleugenol and nevadensin assuming a corresponding reduction in ME-3′-N^2-dGuo DNA adduct and incidences of hepatocellular carcinomas. Subsequently a BMD analysis of the newly generated dose response curves was performed to estimate refined $BMDL_{10}$ values. Using these refined $BMDL_{10}$ values and the EDI for methyleugenol of 0.014 mg/kg bw/day (Smith *et al.*, 2002), the refined MOE for methyleugenol was estimated as shown in the results section.

RESULTS

Synthesis and characterization of ME-3′-N^2-dGuo and ($^{15}N_5$) ME-3′-N^2-dGuo

Incubation of 1′-acetoxymethyleugenol with 2′-deoxyguanosine resulted in the formation of several adducts with 2′-deoxyguanosine as detected by HPLC–UV (Figure 4). Our chromatographic profile was comparable to that reported in the literature for the same reaction between the structurally related 1′-acetoxyestragole and 2′-deoxyguanosine (Punt *et al.*, 2007). Based on analogy to outcomes of the synthesis of N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine (E-3′-N^2-dGuo) from 1′-acetoxyestragole by the same procedure it was expected that the most abundant peak at 11.4 min (Figure 4) corresponds to the adduct of interest ME-3′-N^2-dGuo. Figure 5 presents the structures of the various adducts detected based on this analogy to previous adducts identified for E-3′-N^2-dGuo.

This nature of the major adduct is also in line with results reported previously (Phillips *et al.*, 1981; Wiseman *et al.*, 1985; Punt *et al.*, 2007) for the nature of the major DNA adduct of estragole; E-3′-N^2-dGuo. ME-3′-N^2-dGuo thus obtained was collected at 11.4 min and subsequently used as a standard to confirm the formation of the adduct of interest and to develop the quantitative LC–ESI–MS/MS method for detecting and quantifying DNA nucleotide adducts of methyleugenol.

A similar chromatographic profile was obtained when the reaction was performed using ($^{15}N_5$) ME-3′-N^2-dGuo, and the isolation of the labeled adduct was undertaken in the same way as described for the non-labeled analog. The characterizations of ME-3′-N^2-dGuo and ($^{15}N_5$) ME-3′-N^2-dGuo were carried out on the basis of the LC–ESI–MS/MS results depicted in Figure 6. In Figure 6A, the transitions used for the MS/MS spectrum of ME-3′-N^2-dGuo provided a molecular ion at *m/z* 444 corresponding to the protonated molecule [M+H]+. The 5 Da up mass shift observed in the MS/MS spectrum of ($^{15}N_5$) ME-3′-N^2-dGuo (*m/z* 449) (Figure 5B) was consistent with the *penta*-^{15}N labelling of the guanine moiety. When fragmented by collision, the ion of ME-3′-N^2-dGuo at *m/z* 444 gave rise to three major fragment ions at *m/z* 328, 177, and 164 (insert Figure 6A). The

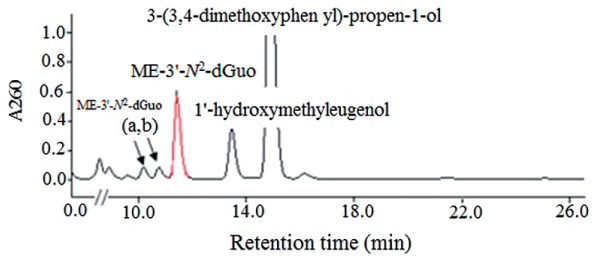
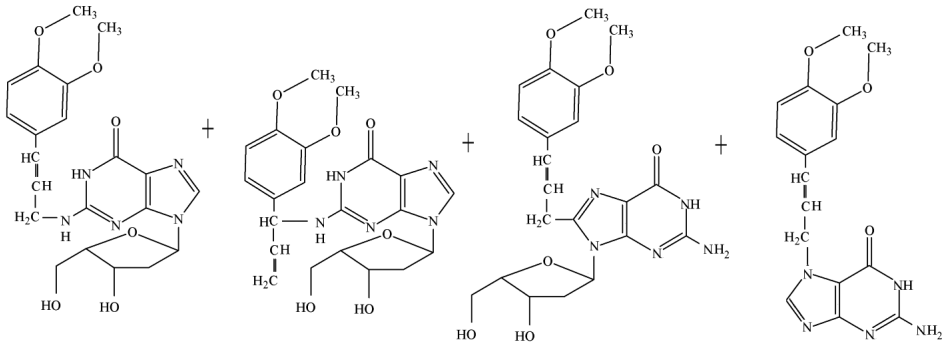


Figure 4. HPLC–UV chromatogram of methyleugenol adducts with 2′dGuo formed in a reaction between 1′ACME and 2′dGuo. On the basis of previous identification by Phillips et al. (1981), Wiseman et al. (1985), and Punt et al. (2007) for the 2′dGuo adduct of estragole, peaks were characterized as two diastereomers of ME-1′-N^2-dG and ME-3′-N^2-dGuo.



major adduct ME-3′-N^2-dGuo | minor adduct ME-1′-N^2-dGuo | minor adduct ME-3′-8-dGuo | minor adduct ME-3′-7-dGuo

Figure 5. Expected structure of adducts of ME with 2′dGuo based on the analogy of the reaction of estragole with 2′dGuo as identified by Phillips et al. (1981) and Wiseman et al. (1985). dGuo = 2′-deoxyguanosine, ME-3′-N^2-dGuo = N^2-(*trans*-isomethyleugenol-3′-yl)-2′-deoxyguanosine, ME-3′-C8-dGuo = 8-(*trans*-isomethyleugenol-3′yl)-2′-deoxyguanosine, ME-1′-N^2-dGuo = N^2-(methyleugenol-1′-yl)-2′-deoxyguanosine, and ME ME-3′-N7-dGuo= 7-(*trans*-methyleugenol-3V-yl)-2′-guanine.

fragment ions at *m/z* 328, m/z 177, and *m/z* 164 were rationalized in terms of a loss of the deoxyribosyl group, loss of the 2′-deoxyguanosine group, and loss of deoxyribosyl and phenylethyl groups, respectively. The same MS/MS spectrum of ME-3′-N^2-dGuo was obtained in the study of Herrmann *et al*. (2012). Identification of ME-3′-N^2-dGuo was further supported by the observation of the mass loss of the deoxyribosyl group and the phenylethyl group in the transition *m/z* 449 → 333 and *m/z* 449 → 169, respectively obtained in the case of ($^{15}N_5$) ME-3′-N^2-dGuo (insert Figure 6B).

Inhibition of ME-3′-N^2-dGuo formation in the human hepatoma cell line HepG2

Figure 7A shows the LC–ESI–MS/MS chromatogram for the 444 → 328 transition of hydrolyzed DNA isolated from human HepG2 incubated with 100 μM 1′-hydroxymethyleugenol. The peak of ME-3′-N^2-dGuo appears at 2.7 min in the chromatogram. Figure 7B presents the ME-3′-N^2-dGuo formation detected in HepG2 cells exposed to 100 μM 1′-HME for ~14 h in the absence and presence of increasing concentrations of nevadensin or 12.5 μM pentachlorophenol added as a positive control for SULT inhibition. At the concentrations tested the compounds were not toxic to the HepG2 cells according to the results of the MTT test (data not shown). The results of Figure 7B reveal that ME-3′-N^2-dGuo formation in HepG2 cells exposed to 1′-hydroxymethyleugenol was almost completely blocked by co-exposure to the model SULT inhibitor pentachlorophenol. For nevadensin, a dose dependent inhibition was observed with 0.2 and 2 μM inhibiting ME-3′-N^2-dGuo formation in HepG2 cells exposed to 1′-hydroxymethyleugenol by 80 and 88 % respectively.

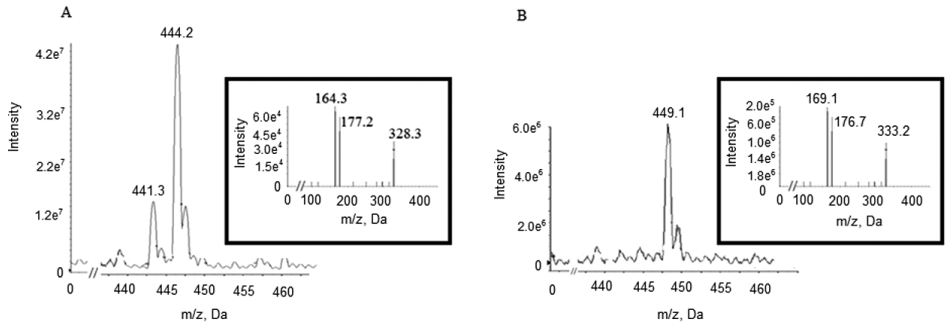
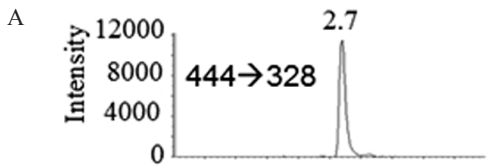
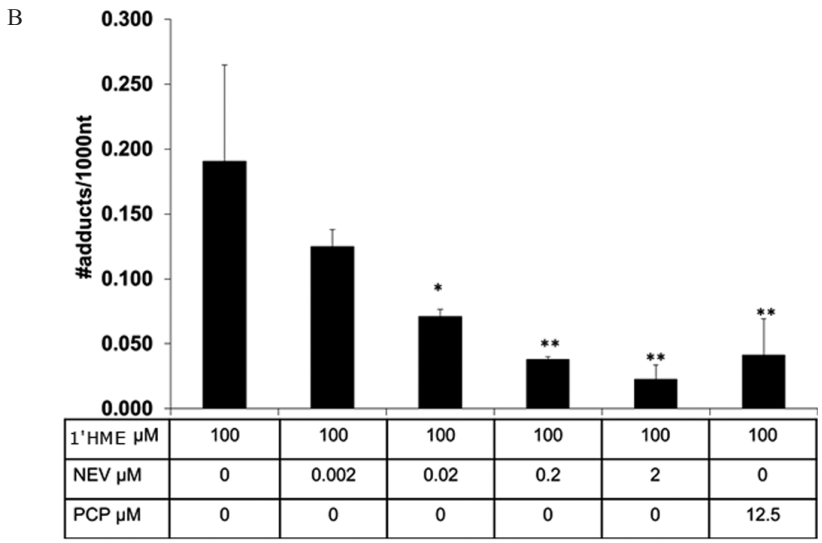


Figure 6. API 3000 Q1 spectrum of the parent ion representing: (A) the non-labeled synthesised ME-3′-N^2-dGuo (444 m/z, Da). The insert represents the daughter ions (328, 177, 164 m/z, Da) formed from the parent ion (444 m/z, Da) and (B) the labeled synthesised ($^{15}N_5$) ME 3′-N^2-dGuo (449 m/z, Da). In the small figure representation of the daughter ions (338, 169 m/z, Da) formed from the parent ion (449 m/z, Da).





1'HME µM	100	100	100	100	100	100
NEV µM	0	0.002	0.02	0.2	2	0
PCP µM	0	0	0	0	0	12.5

Figure 7. LC–ESI–MS/MS chromatogram of the adduct (444→328) formed in human HepG2 cells exposed to 100 µM methyleugenol (A) and DNA adduct levels detected in human HepG2 cells treated with 100 µM 1′HME and increasing concentration of nevadensin or 12.5 µM PCP as positive control (B). Data points represent mean (±SD) of triplicate measurements obtained in independent experiments. An asterisk (*) indicates a significant inhibition compared to the incubation without inhibitor,*$p < 0.05$; **$p \leq 0.01$.

PBBK model based predictions for formation of ME-3'-N^2-dGuo in the liver of rat in the absence or presence of nevadensin

To investigate possible in vivo implications for SULT inhibition by nevadensin on methyleugenol bioactivation, the rat PBBK model developed in our previous work to describe the dose-dependent bioactivation and detoxification of methyleugenol in male rat (Al-Subeihi *et al.*, 2011) was combined with the recently developed PBBK model describing the dose-dependent kinetics of nevadensin in male rat (Alhusainy *et al.*, 2013). Subsequently, the binary methyleugenol–nevadensin PBBK model was used to predict the percentage reduction in ME-3'-N^2-dGuo formation at the doses of methyleugenol used in the NTP study (NTP, 2000) of 0, 37, 75 and 150 mg/kg bw/day when nevadensin would be co-administered with methyleugenol at respectively 0, 14, 29 and 85 mg/kg bw/day representing a molar ratio of methyleugenol to nevadensin of 1:0.20 reflecting their natural occurrence in basil (Smith *et al.*, 2002; Alhusainy *et al.*, 2010). As presented in Tables S5 and Table S6 in the Supplementary Material, our binary methyleugenol–nevadensin PBBK model predicts a 77%, 82% and 83% inhibition in ME-3'-N^2-dGuo formation at the respective doses of methyleugenol and nevadensin.

Refined $BMDL_{10}$ and MOE for methyleugenol in the presence of nevadensin

The $BMDL_{10}$ values for methyleugenol derived from the data from the NTP study (NTP, 2000) were previously reported to vary between 49 to 74 mg/kg bw/day for female rats and from 15 to 34 mg/kg bw/day for male rats (van den Berg *et al.*, 2011).

Based on an EDI of 0.014 mg/kg bw/day (Smith *et al.*, 2002), these $BMDL_{10}$ values result in MOE values that vary between about 3,500 to 5,300 for female rats and from about 1,100 and 2,400 for male rats. Table S5 and S6 in the Supplementary Material present an overview of the incidence of hepatocellular carcinomas in female and male F344/N rats respectively with increasing doses of methyleugenol as reported by the NTP study (NTP, 2000) and also the refined incidence of hepatocellular carcinomas calculated in the present study assuming nevadensin would be co-administered to rats together with methyleugenol at a molar ratio of methyleugenol to nevadensin of 1:0.20. These refined incidences of hepatocellular carcinomas at the respective dose levels of methyleugenol and nevadensin were calculated using the binary male rat methyleugenol–nevadensin PBBK model based predictions for the nevadensin-mediated reduction in ME-3'-N^2-dGuo formation assuming a corresponding reduction in hepatocellular carcinoma formation. Although this linear relation between ME-3'-N^2-dGuo formation and hepatocellular carcinoma formation remains to be established, the assumption was used as a first approximation to estimate the possible consequences of the nevadensin-mediated inhibition of methyleugenol bioactivation to its DNA reactive carcinogenic metabolite. The BMDS analysis of the newly generated refined data resulted in a $BMDL_{10}$ value that varies from about 159 to 254 mg/kg bw/day for female rats (Table S7 in the Supplementary Material) and from about 129 to 142 mg/kg bw/day for male rats (Table S8 in the Supplementary Material). Using these newly estimated

refined $BMDL_{10}$ values and the EDI for methyleugenol of 0.014 mg/kg bw/day (Smith *et al.*, 2002), the MOE for methyleugenol would amount to about 11,000 to 18,000 for female rats and from about 9,200 to 10,000 for male rats. Clearly, co-administration of nevadensin together with methyleugenol is thus predicted to increase the MOE levels substantially pointing at a lower priority for risk management, than when the MOE calculation would be based on the tumour data obtained with pure methyleugenol.

DISCUSSION

Methyleugenol is an alkenylbenzene that occurs in many herbs and spices. The compound is carcinogenic in both rats and mice when administered at high dose levels (NTP, 2000). The hepatocarcinogenicity of methyleugenol is attributed to its bioactivation by hydroxylation followed by sulfonation leading to DNA adduct formation and eventually tumour formation as demonstrated in animal models (Smith *et al.*, 2002).

In a previous study it was demonstrated that the flavonoid nevadensin, which is an important constituent of herbs like basil (Grayer *et al.*, 1996, 2000) is able to inhibit the bioactivation of the related alkenylbenzene estragole to its DNA reactive metabolite at the level of the SULT-mediated conversion of 1′-hydroxyestragole to 1′-sulfoxyestragole (Jeurissen *et al.*, 2008; Alhusainy *et al.*, 2010).

The objective of the present study was to examine whether the herb based constituent nevadensin would also be able to inhibit the SULT-mediated bioactivation of methyleugenol and its subsequent formation of DNA adducts, and to investigate the possible in vivo consequences of such an inhibition using PBBK modeling. The effect of nevadensin on the SULT-mediated DNA adduct formation by 1′-hydroxymethyleugenol was investigated in the human hepatoma cell line HepG2. HepG2 cells contain different enzymes involved in the metabolism of 1′-hydroxymethyleugenol such as SULT and UDP-glucuronosyltransferases (Knasmüller *et al.*, 1998; Brandon *et al.*, 2003, 2006). A method for the quantification of ME-3′-N^2-dGuo was developed based on a recently described LC–ESI–MS/MS method to quantify the corresponding adducts from estragole (Paini *et al.*, 2012a). Incubations with human HepG2 cells exposed to 1′-hydroxymethyleugenol in the presence of increasing concentrations of nevadensin revealed a dose-dependent inhibition of ME-3′-N^2-dGuo formation by nevadensin at concentrations ranging from 0.002 to 2 μM. At 2 μM ME-3′-N^2-dGuo formation was inhibited by 88%.

The DNA adduct measured in the present study (ME-3′-N^2-dGuo) was shown to be the major adduct formed with methyleugenol in in vitro studies and even in a human study (Herrmann *et al.*, 2012, 2013). It was also shown by the same studies that in Ames tests using the *S. typhimurium* TA100 strain expressing the SULT1A1 enzyme and exposed to varying doses of the hydroxylated metabolites of methyleugenol that the levels of the ME-3′-N^2-dGuo adduct increased continuously with the dose and that adduct levels correlated with the mutagenic effect induced. In the present study, as a first approximation, we assumed that nevadensin-mediated inhibition of formation of ME-3′-N^2-dGuo would reflect the reduction in all possible methyleugenol DNA adducts. This assumption can be made given that all methyleugenol DNA adducts will result from

a chemical reaction with 1′-sulfooxymethyleugenol which is the ultimate carcinogenic metabolite, and that it is the formation of 1′-sulfooxymethyleugenol that is inhibited by nevadensin. If all adducts would be reduced to a similar extent one can subsequently assume that the resulting mutagenicity and carcinogencity would be reduced to a similar extent as well, even when different adducts would display different mutagenicity.

To investigate possible in vivo implications of SULT inhibition by nevadensin on methyleugenol bioactivation, the rat PBBK model developed in our previous work to describe the dose-dependent bioactivation and detoxification of methyleugenol in male rat (Al-Subeihi *et al.*, 2011) was combined with the recently developed nevadensin PBBK model in male Sprague–Dawley. The nevadensin PBBK model in male rat was validated in an in vivo study in which male Sprague–Dawley rats were co-administered the structurally related alkenylbenzene estragole and nevadensin orally (Alhusainy *et al.*, 2013). Given the structural resemblance between estragole and methyleugenol and the similar effects of the two compounds in standard mutagenicity tests (Dorange *et al.*, 1977; Sekizawa and Shibamoto, 1982; To *et al.*, 1982; Mortelmans *et al.*, 1986; Zeiger *et al.*, 1987; Schiestl *et al.*, 1989; Zani *et al.*, 1991; Brennan *et al.*, 1996) and in rodent carcinogenicity studies (NTP, 2000, 2008) together with the fact that the type of inhibition exerted by nevadensin is non-competitive and thus by definition should be evident regardless of the substrate it is likely to expect an effect of nevadensin on methyleugenol bioactivation in vivo will occur as well.

In a next step, we investigated the possible consequences arising from the co-administration of methyleugenol and nevadensin on the reported MOE of methyleugenol. Our binary methyleugenol–nevadensin PBBK model in rat was used to predict the possible reduction in ME-3′-N^2-dGuo at the methyleugenol dose levels used in the NTP study when methyleugenol would be co-administered with nevadensin at a molar ratio of methyleugenol to nevadensin of 1:0.20. Thereafter, the reduction in the incidence of hepatocellular carcinomas was calculated assuming a proportional reduction in the formation E-3′-N^2-dGuo and the incidence of hepatocellular carcinomas. Data analysis showed that co-administration of nevadensin together with methyleugenol increased the MOE levels substantially pointing at a lower priority for risk management, than when the MOE calculations would be based on the tumour data obtained with pure methyleugenol.

In conclusion, the present study points at an important matrix effect of combined exposure to methyleugenol and nevadensin. SULT inhibition was shown to lead to the inhibition of the formation of the ultimate DNA reactive metabolite. This implies that dosing methyleugenol to rodents within a matrix of sweet basil or other food items containing SULT inhibitors would pose a risk that is lower than what would be estimated based on results from rodent bioassays in which methyleugenol is dosed as a pure compound by gavage without the relevant food matrix being present.

ACKNOWLEDGEMENT

The authors would like to thank Nestlé Research Centre for kindly providing the LC-MS/MS API 3000 instrument. This work was supported by a grant from sandwich PhD fellowship program/Wageningen University, the Netherlands.

REFERENCES

1. Al-Subeihi, A. A. A., Spenkelink, B., Rachmawati, N., Boersma, M. G., Punt, A., Vervoort, J., van Bladeren, P. J., and Rietjens, I. M. C. M. (2011). Physiologically based biokinetic model of bioactivation and detoxification of the alkenylbenzene methyleugenol in rat. *Toxicology in Vitro* **25**, 267-285.
2. Alhusainy, W., Paini, A., Punt, A., Louisse, J., Spenkelink, A., Vervoort, J., Delatour, T., Scholz, G., Schilter, B., Adams, T., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). Identification of nevadensin as an important herb-based constituent inhibiting estragole bioactivation and physiology-based biokinetic modeling of its possible in vivo effect. *Toxicology and Applied Pharmacology* **245**, 179-190.
3. Alhusainy, W., Paini, A., Van den Berg, J. H. J., Punt, A., Scholz, G., Schilter, B., van Bladeren, P. J., Taylor, S., Adams, T. B., and Rietjens, I. M. C. M. (2013). In vivo validation and physiologically based modeling of the inhibition of SULT-mediated estragole DNA adduct formation in the liver of male Sprague-Dawley rats by the basil flavonoid nevadensin. *Molecular Nutrition and Food Research* DOI 10.1002/mnfr.201300144, http://eproof.aptaracorp.com/powerproof2/adp.do?aid=1357984731012469844.
4. Alhusainy, W., van den Berg, S. J. P. L., Paini, A., Campana, A., Asselman, M., Spenkelink, A., Punt, A., Scholz, G., Schilter, B., Adams, T. B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2012). Matrix modulation of the bioactivation of estragole by constituents of different alkenylbenzene-containing herbs and spices and physiologically based biokinetic modeling of possible in vivo Effects. *Toxicological Sciences* **129**, 174-187.
5. Brandon, E. F. A., Bosch, T. M., Deenen, M. J., Levink, R., Van Der Wal, E., Van Meerveld, J. B. M., Bijl, M., Beijnen, J. H., Schellens, J. H. M., and Meijerman, I. (2006). Validation of in vitro cell models used in drug metabolism and transport studies; Genotyping of cytochrome P450, phase II enzymes and drug transporter polymorphisms in the human hepatoma (HepG2), ovarian carcinoma (IGROV-1) and colon carcinoma (CaCo-2, LS180) cell lines. *Toxicology and Applied Pharmacology* **211**, 1-10.
6. Brandon, E. F. A., Raap, C. D., Meijerman, I., Beijnen, J. H., and Schellens, J. H. M. (2003). An update on in vitro test methods in human hepatic drug biotransformation research: Pros and cons. *Toxicology and Applied Pharmacology* **189**, 233-246.
7. Brennan, R. J., Kandikonda, S., Khrimian, A. P., DeMilo, A. B., Liquido, N. J., and Schiestl, R. H. (1996). Saturated and monofluoro analogs of the oriental fruit fly attractant methyl eugenol show reduced genotoxic activities in yeast. *Mutation Research - Genetic Toxicology* **369**, 175-181.
8. Burdock, G. A., ed. (1995). Fenaroli's Handbook of Flavor Ingredients, Vol. II, 3rd Edition, CRC Press, Boca Raton, FL.
9. Dorange, J. L., Delaforge, M., Janiaud, P., and Padieu, P. (1977). Mutagenicity of the metabolites of the epoxide diol pathway of safrole and analogues. Study on Salmonella typhimurium. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales* **171**, 1041-1048.
10. EFSA (2005). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. *EFSA Journal* **282**, 1-31.
11. European Commission (EC) (2008). Regulation (EC) No 1334/2008 of the Europian Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC.
12. Gardner, I., Wakazono, H., Bergin, P., De Waziers, I., Beaune, P., Kenna, J. G., and Caldwell, J. (1997). Cytochrome P450 mediated bioactivation of methyleugenol to 1'-hydroxymethyleugenol in Fischer 344 rat and human liver microsomes. *Carcinogenesis* **18**, 1775-1783.
13. Grayer, R. J., Bryan, S. E., Veitch, N. C., Goldstone, F. J., Paton, A., and Wollenweber, E. (1996). External flavones in sweet basil, Ocimum basilicum, and related taxa. *Phytochemistry* **43**, 1041-1048.

14. Grayer, R. J., Kite, G. C., Abou-Zaid, M., and Archer, L. J. (2000). The application of atmospheric pressure chemical ionisation liquid chromatography-mass spectrometry in the chemotaxonomic study of flavonoids: Characterisation of flavonoids from Ocimum gratissimum var. gratissimum. *Phytochemical Analysis* **11**, 257-267.
15. Herrmann, K., Engst, W., Appel, K. E., Monien, B. H., and Glatt, H. (2012). Identification of human and murine sulfotransferases able to activate hydroxylated metabolites of methyleugenol to mutagens in Salmonella typhimurium and detection of associated DNA adducts using UPLC-MS/MS methods. *Mutagenesis* **27**, 453-462.
16. Herrmann, K., Schumacher, F., Engst, W., Appel, K. E., Klein, K., Zanger, U. M., and Glatt, H. (2013). Abundance of DNA adducts of methyleugenol, a rodent hepatocarcinogen, in human: Liver samples. *Carcinogenesis* **34**, 1025-1030.
17. Hussain, R. F., Nouri, A. M. E., and Oliver, R. T. D. (1993). A new approach for measurement of cytotoxicity using colorimetric assay. *Journal of Immunological Methods* **160**, 89-96.
18. Jeurissen, S. M. F., Punt, A., Delatour, T., and Rietjens, I. M. C. M. (2008). Basil extract inhibits the sulfotransferase mediated formation of DNA adducts of the procarcinogen 1 '-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. *Food and Chemical Toxicology* **46**, 2296-2302.
19. Knasmüller, S., Parzefall, W., Sanyal, R., Ecker, S., Schwab, C., Uhl, M., Mersch-Sundermann, V., Williamson, G., Hietsch, G., Langer, T., Darroudi, F., and Natarajan, A. T. (1998). Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis* **402**, 185-202.
20. Leung, A. Y., ed. (1998). Encyclopedia of Common Natural Ingredients. John Wiley and Sons, New York.
21. Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Research* **43**, 1124-1134.
22. Mortelmans, K., Haworth, S., Lawlor, T., Speck, W., Tainer, B., and Zeiger, E. (1986). Salmonella mutagenicity tests: II. Results from the testing of 270 chemicals. *Environmental Mutagenesis* **8**, 1-119.
23. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**, 55-63.
24. NTP (2000). National Toxicology Program on toxicology and carcinogenesis studies of Methyleugenol (CAS NO. 93-15-12) in F344/N rats and B6C3F1 mice (Gavage Studies). DRAFT NTP-TR-491; NIH Publication No. 98-3950., 1-412.
25. NTP (2008). National Toxicology Program on toxicology and carcinogenesis studies of estragole in F344/N rats and B6C3F1 mice. U.S. Dept Of health Human services. NIH Publication TOX-82.
26. Paini, A., Punt, A., Scholz, G., Gremaud, E., Spenkelink, B., Alink, G., Schilter, B., Van Bladeren, P. J., and Rietjens, I. M. C. M. (2012a). In vivo validation of DNA adduct formation by estragole in rats predicted by physiologically based biodynamic modelling. *Mutagenesis* **27**, 653-663.
27. Paini, A., Punt, A., Viton, F., Scholz, G., Delatour, T., Marin-Kuan, M., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). A physiologically based biodynamic (PBBD) model for estragole DNA binding in rat liver based on in vitro kinetic data and estragole DNA adduct formation in primary hepatocytes. *Toxicology and Applied Pharmacology* **245**, 57-66.
28. Paini, A., Scholz, G., Boersma, M. G., Spenkelink, A., Schilter, B., van Bladeren, P. J., Rietjens, I. M. C. M., and Punt, A. (2012b). Evaluation of interindividual human variation in bioactivation and DNA binding of estragole in liver predicted by physiologically based biodynamic (PBBD) and Monte Carlo modeling. In *Generation of in vitro data to model dose dependent in vivo DNA binding of genotoxic carcinogens and its consequences: the case of estragole.*, pp. 70-85.
29. Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the

DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1′-hydroxyestragole. *Cancer Research* **41**, 176-186.

30. Punt, A., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2007). Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1′-hydroxyestragole. *Chemical Research in Toxicology* **20**, 991-998.
31. Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis* **5**, 1613-1622.
32. SCF (2001). Opinion of the Scientific Committee on Food on methyleugenol (4-allyl-1,2-dimethoxybenzene.
33. Schiestl, R. H., Chan, W. S., Gietz, R. D., Mehta, R. D., and Hastings, P. J. (1989). Safrole, eugenol and methyleugenol induce intrachromosomal recombination in yeast. *Mutation Research* **224**, 427-436.
34. Sekizawa, J., and Shibamoto, T. (1982). Genotoxicity of safrole-related chemicals in microbial test systems. *Mutation Research* **101**, 127-140.
35. Smith, B., Cadby, P., Leblanc, J. C., and Setzer, R. W. (2010). Application of the margin of exposure (MOE) approach to substances in food that are genotoxic and carcinogenic. Example: Methyleugenol, CASRN: 93-15-2. *Food and Chemical Toxicology* **48**, S89-S97.
36. Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - Methyl eugenol and estragole. *Food and Chemical Toxicology* **40**, 851-870.
37. To, L. P., Hunt, T. P., and Andersen, M. E. (1982). Mutagenicity of *trans*-anethole, estragole, eugenol, and safrole in the Ames Salmonella typhimurium assay. *Bulletin of Environmental Contamination and Toxicology* **28**, 647-654.
38. van den Berg, S. J. P. L., Restani, P., Boersma, M. G., Delmulle, L., and Rietjens, I. M. C. M. (2011). Levels of Genotoxic and Carcinogenic Compounds in Plant Food Supplements and Associated Risk Assessment. *Food and Nutrition Sciences* **2**, 989-1010.
39. Wiseman, R. W., Fennell, T. R., Miller, J. A., and Miller, E. C. (1985). Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1′-hydroxysafrole and 1′-hydroxyestragole in vitro and in mouse liver in vivo, including new adducts at C-8 and N-7 of guanine residues. *Cancer Research* **45**, 3096-3105.
40. Zani, F., Massimo, G., Benvenuti, S., Bianchi, A., Albasini, A., Melegari, M., Vampa, G., Bellotti, A., and Mazza, P. (1991). Studies on the genotoxic properties of essential oils with Bacillus subtilis rec-assay and Salmonella/microsome reversion assay. *Planta Medica* **57**, 237-241.
41. Zeiger, E., Anderson, B., Haworth, S., Lawlor, T., Mortelmans, K., and Speck, W. (1987). Salmonella mutagenicity tests: III. Results from the testing of 255 chemicals. *Environmental Mutagenesis* **9**, 1-110.

6 The natural basil flavonoid nevadensin protects against methyleugenol-induced markers of hepatocarcinogenicity in male F344 rat

Based on:
Alhusainy Wasma, Williams GM, Jeffrey AM , Iatropoulos M J, Duan JD, Taylor S, AdamsTB, and Rietjens IMCM

(Submitted)

Supplementary Material can be found at the end of this chapter

ABSTRACT

The alkenylbenzene methyleugenol occurs naturally in a variety of spices and herbs, including basil, and their essential oils. At high dose levels methyleugenol induces hepatocellular cancer in rodents following bioactivation to 1'-sulfooxymethyleugenol which forms DNA adducts. The present study investigated whether the inhibitory effect of the basil flavonoid nevadensin on sulfotransferase (SULT)-mediated bioactivation of methyleugenol observed in vitro would also be reflected in a reduction of DNA adduct formation in the liver and a reduction in early markers for liver carcinogenesis in an 8-week in vivo rat study. Co-exposure to methyleugenol and nevadensin by intragastric instillation resulted in a significant inhibition of liver methyleugenol DNA adduct formation and also in a significant inhibition of induction of hepatocellular altered foci, representing indicators for initiation of neoplasia. These results suggest that tumour formation could be lower in rodent bioassays when methyleugenol would be dosed in a matrix containing SULT inhibitors such as nevadensin compared to experiments using the pure methyleugenol. An estimate of the possible consequences of this reduction for the risk assessment of methyleugenol indicates that it would result in increased $BMDL_{10}$ (lower confidence limit of the benchmark dose that gives 10% extra cancer incidence) and margin of exposure (MOE) values and thus a lower priority for risk management.

INTRODUCTION

The alkenylbenzene methyleugenol (3,4-dimethoxyallylbenzene) (Figure 1) is a natural constituent of many herbs, spices and their essential oils. Although methyleugenol is generally present in many foods at low levels, intake of methyleugenol occurs primarily from nutmeg, allspice, sweet basil, and fennel (Williams and Mattia, 2009). The National Toxicology Program reported that methyleugenol was hepatocarcinogenic in both rats and mice (NTP, 2000). The hepatocarcinogenicity of methyleugenol, like that of the chemically related safrole, is ascribed to its bioactivation by cytochrome P450 enzymes, leading in the case of methyleugenol to the formation of the proximate carcinogen 1′-hydroxymethyleugenol (Figure 1) (Miller *et al.*, 1983; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Smith *et al.*, 2002). Further bioactivation of 1′-hydroxymethyleugenol requires the involvement of sulfotransferases (SULTs) that convert 1′-hydroxymethyleugenol to the ultimate carcinogen 1′-sulfooxymethyleugenol (Figure 1) (Boberg *et al.*, 1983; Wiseman *et al.*, 1987). This sulfonated metabolite is unstable and, via a putative reactive carbonation intermediate, binds covalently to endogenous nucleophiles including DNA which results in DNA adduct formation in mice (Phillips *et al.*, 1981, 1984; Randerath *et al.*, 1984) and rats (Williams *et al.*, 2013).

Given its genotoxicity and carcinogenicity, the addition of methyleugenol as a pure substance to food has been prohibited since September 2008 within the European Union (EU) (European Commission, 2008), whereas in the USA the use of methyleugenol is limited to low annual volumes (Gavin et al., 2008). A main issue that remains to be investigated is whether the animal studies that use high levels of the pure compound without the normal food matrix being present, are an appropriate starting point for the risk assessment of methyleugenol. In this respect, we have recently shown that a methanolic extract from basil, which contained methyleugenol and the structurally related alkenylbenzene estragole, also contained the flavone nevadensin (5,7-dihydroxy-6,8,4′- trimethoxyflavone) (Figure 2). Nevadensin was able to reduce SULT-mediated estragole DNA adduct formation significantly in primary rat hepatocytes exposed in vitro to 1′-hydroxyestragole and nevadensin simultaneously (Alhusainy *et al.*, 2010). Nevadensin was also able to significantly ($p<0.01$) reduce the SULT-mediated methyleugenol DNA adduct formation in human HepG2 cells exposed

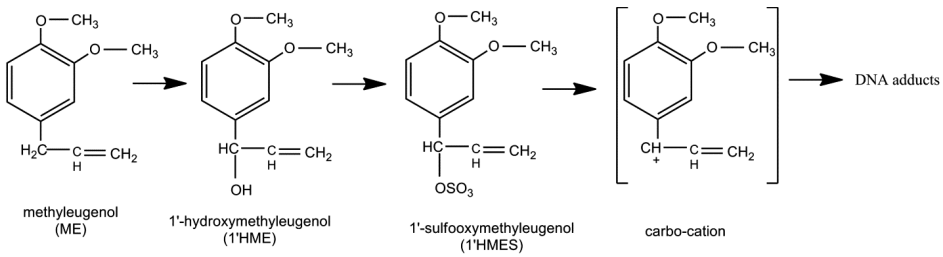


Figure 1. Structural representation of the pathway for bioactivation of methyleugenol which is also representative for other alkenylbenzenes.

to 1′-hydroxymethyleugenol and nevadensin simultaneously (Al-Subeihi *et al.*, 2013). Even more interestingly, nevadensin was able to reduce the formation of estragole DNA adduct formation significantly (by 36% $p<0.001$) in the liver of Sprague–Dawley rats when exposed to estragole and nevadensin simultaneously at a molar ratio of the two compounds reflecting their presence in basil (Alhusainy *et al.*, 2013). For methyleugenol this reduction in DNA adduct formation by nevadensin has only been demonstrated in an in vitro model system using HepG2 cells (Al-Subeihi *et al.*, 2013).

The objective of the present study was to investigate whether the inhibitory effect of nevadensin on SULT-mediated methyleugenol bioactivation and DNA adduct formation observed in the vitro model would also be observed in vivo. In addition, it would be appropriate to elucidate whether such a reduction would be accompanied by a reduction in early markers of liver carcinogenesis. To this end, an in vivo rat study was performed in which the animals were exposed for 8 weeks to either methyleugenol alone, methyleugenol in the presence of nevadensin or methyleugenol in the presence of pentachlorophenol (PCP), the latter included as a positive control for SULT inhibition and reduction in alkenylbenzene DNA adduct and tumour formation in vivo (Boberg *et al.*, 1983). The dose level of methyleugenol to be tested was selected based on a recent study by Williams *et al.* (2013) which quantified the methyleugenol dose-dependent effects on endpoints indicative of neoplastic initiation, including formation of hepatocellular altered foci (HAF), changes in hepatocellular proliferation and DNA adduct formation. This paradigm was previously used to demonstrate anticarcinogenic effects of butylated hydroxyanisole and butylated hydroxytoluene (Williams and Iatropoulos, 1996), hydroquinone (Williams *et al.*, 2007a) and acetaminophen (Williams *et al.*, 2007b).

MATERIALS AND METHODS

Materials

Methyleugenol (3,4-dimethoxyallylbenzene, CAS 87-86-5, lot number T1959A), (>98% pure) was obtained from Vigon International (East Stroudsburg, PA, USA). Pentachlorophenol (2,3,4,5,6-pentachlorophenol, PCP, CAS, lot number MKBG5282V) (>97% pure) was obtained from Sigma-Aldrich (St Louis, MO, USA). Nevadensin (5,7-dihydroxy-6,8,4′- trimethoxyflavone, CAS 10176-66-6, product number 34463n) (>98%

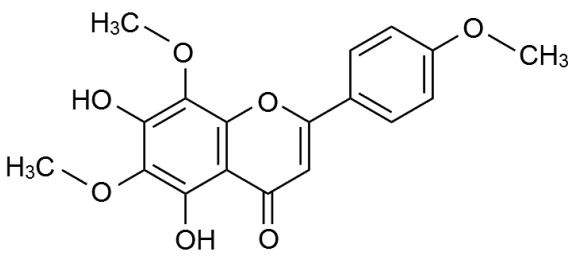


Figure 2. Molecular structure of the natural SULT inhibitor nevadensin.

pure) was obtained from Apin Chemicals (Milton, UK). Emulsions for gavage were prepared by dispersion with a Dounce homogenizer in 0.5% aqueous methylcellulose. Methylcellulose (CAS 9004-67-5, catalog number 1489B) was obtained from ICN Biochemicals Inc. (Aurora, OH, USA). 2′-Deoxyguanosine was purchased from Sigma (Basel, CH), zinc sulphate (heptahydrate), phosphodiesterase I from *Crotalus adamanteus* (venom phosphodiesterase), phosphodiesterase II from bovine spleen (spleen phosphodiesterase), nuclease P_1 and alkaline phosphatase were purchased from Sigma (Schnelldorf, Germany). Micrococcal nuclease, nuclease P_1 and spleen phosphodiesterase used for DNA adduct digestion for ^{32}P nucleotide postlabeling analysis was obtained from Sigma-Aldrich (St Louis, MO, USA). γ-^{32}P-Adenosine triphosphate (~6000 Ci/mmole) was obtained from Perkin-Elmer (Waltham, MA, USA). Acetonitrile (ACN) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Formic acid and ethanol were obtained from VWR Merck (Darmstadt, Germany). Synthesis and characterization of N^2-(*trans*-isomethyleugenol-3′-yl)-2′-deoxyguanosine (ME-3′-N^2-dGuo) was performed as previously reported (Al-Subeihi *et al.*, 2013) using a method adapted from a protocol for the synthesis of the estragole DNA adduct, N^2-(*trans*-isoestragol-3′-y1)-2′-deoxyguanosine (E-3′-N^2-dGuo) (Punt *et al.*, 2007).

Animals and experiment

In the present study male F344 rats were used because that strain was used by the NTP (2000) carcinogenicity study, and in the dose-response study of Williams *et al*. (2013). In the latter study, male F344 rats were used to elucidate the methyleugenol cancer initiating effects since liver preneoplasia can be readily quantified in this species (Williams, 1998, 1999). In addition, male F344 were more sensitive to hepatocarcinogenesis by methyleugenol than females (NTP, 2000). The study was approved by the Institutional Animal Care & Use Committee (IACUC) of New York Medical College. Male F344 rats, 5-6 week old at the start of the study, obtained from Taconic Farms, Inc. (Germantown, NY, USA), were housed in a conventional animal facility, accredited by the American Association for the Accreditation of Laboratory Animal Care. The care provided conforms to the Guide for the Care and Use of laboratory Animals (NIH-78-23). Rats were housed in solid bottom polycarbonated cages, with irradiated corn cob bedding and a microisolator filter on top (Ancare, Bellmore, NY, USA). The cages for groups dosed with methyleugenol were in a separate room from the groups not dosed with methyleugenol to isolate the later rats from exposure to any volatile methyleugenol.

Room temperature was maintained at 20 ± 2°C and relative humidity at 55 ± 20%. Fresh air was supplied to rooms with 18-22 exchanges/hr. Twelve hours of continuous low level fluorescent lighting (5 ft candles) was provided daily (7 AM to 7 PM) followed by 12 hours of dark cycle. Water was available *ad libitum* throughout the study and was supplied by an automatic watering system. Monitoring of the drinking water for microbiological and chemical contaminants was routinely conducted. Upon receipt, the rats were maintained under observation for two weeks on 5001 diet (WF Fisher & Son, Inc., Somerville, NJ, USA).

The rats were allocated to groups as shown in Table 1 of the result section. The total number of animals at the start of the experiment was 54 male F344 rats. Rats were assigned to 6 groups and each group consisted of 9 rats which were housed 3 rats per cage. The first group, representing the vehicle group, was doses with a 0.5% methylcellulose in aqueous solution, representing the vehicle for all compounds tested in the present study. All tested compounds including the vehicle solution were administered at 5 mL/kg bw by intragastric instillation (gavage) 3 days (Monday, Wednesday and Friday) per week for 8 weeks. The second group received 250 mg/kg bw/day methyleugenol from a stock solution containing 50 mg/mL methyleugenol in 0.5% methylcellulose, 3 days per week. The third group received 11 mg/kg bw/day PCP from a stock solution containing 2.2 mg/mL PCP in 0.5% methylcellulose together with sufficient 1M NaOH to bring pH to neutrality and enhance solubility. The fourth group received 120 mg/kg bw/day nevadensin from a stock solution containing 24 mg/mL nevadensin in 0.5% methylcellulose. The fifth group received 250 mg/kg bw/day methyleugenol together with 120 mg/kg bw/day nevadensin, both dosed simultaneously using a stock solution containing 50 mg/mL methyleugenol and 24 mg/mL nevadensin in 0.5% methylcellulose. The sixth group, representing a positive control group for SULT inhibition, was dosed with 250 mg/kg bw/day methyleugenol together with 11 mg/kg bw/day of the SULT inhibitor PCP, both dosed simultaneously using a stock solution containing 50 mg/mL methyleugenol and 2.2 mg/mL PCP in 0.5% methylcellulose.

Morbidity, mortality and clinical observations were conducted twice daily. BW was recorded the day prior to study initiation, thereafter weekly to determine gavage volumes and before necropsy. Overnight, prior to necropsy, all rats were fasted. Rats were exsanguinated under isoflurane anesthesia. Thereafter, blood was removed and livers were harvested and weighed. Slices of all liver lobes were placed on a metal block on ice for cutting into smaller pieces and either frozen in liquid nitrogen and stored at -80°C for DNA adduct analysis or two standard liver slices were taken and immersed in 10% neutral (phosphate) buffered formalin for routine hematoxylin and eosin (H&E) and immunohistochemistry for both proliferating cell nuclear antigen (PCNA) and placental-type (π) glutathione S-transferase (GST-P) microscopic evaluation.

Dose and time selection

The dose of methyleugenol of 250 mg/kg bw/day was given to rats by gavage three days per week for 8 weeks based on the study of Williams *et al*. (2013). In that study, the formation of methyleugenol DNA adducts, changes in PCNA and GST-P, the latter representing an indicator to initiation of hepatocellular cancer in rats (Williams, 1980; Bannasch, 1986; Williams, 1989; Enzmann *et al*., 1998; Williams, 1998; Williams, 1999) were significantly increased compared to the vehicle group (0.5% methylcellulose) after administration of 250 mg/kg bw/day of methyleugenol given orally to male F344 rats three days per week for a period of 8 weeks (Williams *et al*., 2013). A dose of 250 mg/kg bw/day of methyleugenol dosed 3 times per week yields a weekly cumulative dose of 750 mg/kg bw/week, which is identical to the weekly cumulative

dose achieved with a dose of 150 mg/kg bw/day methyleugenol, 5 days per week in the NTP carcinogenicity study (NTP, 2000).

A dose of nevadensin of 120 mg/kg bw/day (3 days per week) was selected (at a molar ratio of methyleugenol to nevadensin of 1:0.25) from a previous study where rats were gavaged with nevadensin together with the closely related alkenylbenzene estragole at the same molar ratio representing a molar ratio at which the two compounds are present in basil (Alhusainy *et al.*, 2013). In addition, the ratio was close to the molar ratio of methyleugenol and nevadensin in basil (1:0.20). These ratios were calculated based on a level of nevadensin of 0.64 mg nevadensin/g dried basil detected by us previously in a dried basil sample obtained from the Dutch market (Alhusainy *et al.*, 2012), and methyleugenol levels reported in the literature for the Genovese Gigante cultivar of basil, the most commonly used cultivar in pesto preparation in north-western Italy which contains at least 2 mg methyleugenol/g basil (Smith *et al.*, 2002).

The dose of PCP was based on literature where a dose of PCP of 11 mg/kg bw/day was reported to reduce the incidence of hepatomas resulting from a single intraperitoneal dose of 111 mg/kg bw estragole following 10 months of observation (Wiseman *et al.*, 1987). It was also based on a no-observed-adverse-effect level (NOAEL) for PCP of 10 mg/kg bw/day for both reproductive and general toxicity studies in Sprague–Dawley rats (Bernard *et al.*, 2002). The dose of PCP is also in accordance to our previous work where a dose of 11 mg/kg bw PCP resulted in a significant 73% reduction in estragole DNA adduct formation in the liver of Sprague–Dawley rats when measured 48 hr after they were exposed to a single oral dose of PCP and estragole simultaneously (Alhusainy *et al.*, 2013).

^{32}P nucleotide postlabeling (NPL) analysis for overall DNA adducts

DNA was isolated using QIAGEN (Valencia, CA, USA) G100 columns following the manufacturer's protocol (Qiagen, 2001). The yield and purity of the extracted DNA was determined using its 230/260/280 ratios measured on a Cary 1E UV-visible spectrophotometer and associated software (Cary WinUV Version 3.0 Bio Suite (Varian Inc, Walnut Creek, CA, USA) in Tris HCl buffer, pH 7.2. DNA was then processed for DNA adduct analysis by NPL performed as previously described (Williams, 1998; Jeffrey *et al.*, 2002). Owing to the high DNA adduct levels, 1 µg of isolated DNA was diluted with 9 µg of calf thymus DNA before digestion in order to obtain a more linear response with respect to DNA adduct levels in the different samples. Isolated DNA (10 µg) was enzymatically digested into 2'-deoxyribonucleoside 3'-monophosphates using micrococcal nuclease and spleen phosphodiesterase. The digest was then enriched using nuclease P_1 digestion (Reddy and Randerath, 1986). This enrichment procedure for modified DNA bases provides a better sensitivity for measurement of methyleugenol DNA adducts. The DNA modified bases were subsequently labeled using 100 µCi γ-^{32}P adenosine triphosphate (~6000 Ci/mmole, Perkin-Elmer, Waltham, MA, USA). The labeled modified bases were then resolved using a two-directional thin-layer chromatography (TLC) system. The first elution, bottom to top direction,

used 2.0 M sodium phosphate pH 5.5 with a wick for 16 hours followed by washing by 1.2 M lithium formate/2.8 M urea pH 3.5 in the same direction. Again the plates were washed and eluted this time left to right with 0.6 M lithium chloride, 0.25 M Tris HCl, 4 M urea, pH 8.0. Plates were again washed and dried and then exposed to phosphor screen for 40 min. The radioactivity on the TLC plates was detected using a Molecular Dynamics Storm 860 system (GE Health Care Life Sciences, Edison, NJ, USA).

Tissue DNA extraction and digestion for LC–ESI–MS/MS analysis of methyleugenol DNA adduct formation

DNA extraction and digestion for the LC–ESI–MS/MS analysis was processed differently from the procedure used for ^{32}P nucleotide postlabeling analysis and was in line with our previous work (Alhusainy *et al.*, 2013). Prior to DNA extraction, liver samples were homogenised as follows: one gram of a liver slice was taken randomly and dissolved in 4 mL cold phosphate buffered saline (PBS) and the mixture was then homogenized on ice at 1000 rpm using a mechanical cell homogenizer (B. Braun, Melsungen, Germany). Thereafter, homogenates were placed in 15 mL tubes and centrifuged at 3,000 g for 5 min at 4°C (Sigma centrifuge, type 4K10, Germany) to obtain pellets. DNA extraction from these pellets was performed using a Get pure DNA Kit-Cell protocol (Dojindo Molecular Technology Inc., Kumamoto, Japan) for tissue following the manufacturer's instructions. The final DNA pellet was dissolved in 100 µL MilliQ water. The yield and purity of the extracted DNA was determined using the Nanodrop ND-100 spectrophometer (Thermo Fisher Scientific, Inc.) by measuring the absorbance ratio A260 nm/A280 nm. DNA samples with an absorbance ratio of 1.8-2.0 were considered sufficiently pure. All DNA samples were diluted to achieve 50 µg DNA/100 µL water before digestion of the DNA. Digestion of DNA and quantification of the major methyleugenol DNA adduct (ME-3′-N^2-dGuo) by LC–ESI–MS/MS were performed as described previously (Paini *et al.*, 2010; Al-Subeihi *et al.*, 2013).

Quantification of hepatocellular proliferation

PCNA immunohistochemistry was applied to monitor the rate of hepatocellular proliferation of individual hepatocytes, as done in other studies of hepatocarcinogenesis (Iatropoulos and Williams, 1996; Williams *et al.*, 1998). Cell proliferation was assessed by scoring of PCNA positive cells (Iatropoulos and Williams, 1996). Neutral phosphate buffered formalin fixed liver sections (2 per liver and rat) were processed, cut and stained for PCNA at the Histology Core Facility, Department of Pathology, New York Medical College, (Valhalla, NY, USA) according to appropriate standard operating procedures. The PCNA evaluation reflected the percent replicating fraction (RF), which is derived from total PCNA positive nuclei counts over total hepatocellular nuclei counted from about 5 graticule areas (Graticules, Ltd, Tonbridge, UK). From each liver only areas with the highest (most intense) PCNA staining from every liver section across all groups were selected to be counted at a total magnification of 200x. The average range of hepatocellular nuclei counted across all groups was 725 – 1000 nuclei per rat liver.

Quantification of hepatocellular altered foci (HAF)

Preneoplastic liver lesions, designated as HAF, are useful precursor markers of neoplasia (Williams, 1980; Bannasch, 1986; Williams, 1989; Enzmann *et al.*, 1998; Williams, 1998; Williams, 1999). These lesions precede the development of neoplasms, and, although they greatly exceed the number of neoplasms, they are quantitatively related to them (Williams, 1980; Williams, 1982). Among many useful markers of HAF, placental-type (π) glutathione *S*-transferase (GST-P) has proven to be a very effective one (Sato *et al.*, 1984; Williams, 1999). Quantification of HAF was therefore used to assess initiation of hepatocarcinogenesis (Williams, 1980). From each rat, two standard liver sections were taken; the first liver section was taken from a liver lobe with visible macroscopic lesions and the second liver section was taken from the largest left lobe. Per liver (and rat) the average range of the liver section surface area was 2 to 4.6 cm^2. Only foci in clusters of 3 or more contiguous GST-P^+ HAF hepatocytes over the entire microscopic glass slide surface area were counted. In addition, the greatest diameter in µm of each GST-P^+ HAF was measured with a graticule (Graticules, Ltd, Tonbridge, UK). The total microscopic magnification was 100x. Subsequently, the average incidence of GST-P^+ HAF per cm^2 of liver tissue per exposure group was calculated.

Statistical analyses

Statistical analysis was undertaken using SigmaStat for Windows version 3.11.0 (Systat Software Inc., Chicago, IL, USA). Selected statistical analysis for the data sets was an one-way ANOVA against vehicles for body weight, absolute and relative liver weights, while the DNA adducts, PCNA and GST-P evaluations were against methyleugenol (Systat Software Manual, 2004).

RESULTS

Evaluation of experimental conditions

No deviations occurred in ambient temperature, relative humidity or any other condition in the animal room that would affect animal health or the integrity of the study. One death occurred in the second group dosed with 250 mg/kg bw/day methyleugenol in the first week of gavage due to a gavage error.

Change in body and liver weights

The summary of mean body weights (BW) in grams at week 0 and week 8 and the mean absolute (ALW) and relative liver weights (RLW) at week 8 are given in Table 1. At the start of the experiment (week 0), all groups had similar mean BWs. At 8 weeks, a significant decrease in mean BW was observed in rats dosed with methyleugenol only (7%), methyleugenol/nevadensin (6%), or methyleugenol/PCP (7%) compared to the vehicle control group. Also, at 8 weeks, there was a significant increase in RLW in groups dosed with methyleugenol (15%), methyleugenol/nevadensin (13%) and methyleugenol/PCP (16%) compared to the vehicle group.

Table 1. Body weights, absolute and relative liver weights.

Group (mg/kg bw/day)	BW (week 0)	BW (week 8)	ALW (week 8)	RLW (week 8)
1.Vehicle	254±7	317±9	14.22±0.82	4.49±0.20
2.Methyleugenol (250) [a]	252±7	295±12*	15.64±0.58 *	5.30±0.21*
3.PCP (11) [a]	253±8	311±7	14.77±0.54	4.74±0.23
4.Nevadensin (120) [a]	253±7	326±24	14.81±0.70	4.57±0.46
5.Methyleugenol /nevadensin (250/120) [a]	253±6	298±8*	15.38±0.68 *	5.17±0.28*
6.Methyleugenol /PCP (250/11) [a]	253±7	295±12*	15.75±0.75 *	5.34±0.27*

[a] Individual doses mg/kg delivered by gavage 3 days per week for 8 weeks.
* indicates values statistically significant at $p<0.05$ compared to vehicle group; there were 9 rats per group except for the methyleugenol group consisting of 8 rats due to a gavage error.

Effect of nevadensin on methyleugenol-induced DNA adduct formation

DNA isolation from the livers after 8 weeks exposure provided adequate yields of DNA and all samples had appropriate 260/280 nm absorbance ratio (1.85 $\pm$ 0.03 and 1.88 $\pm$ 0.0.01 using the QIAGEN and Dojindo kits respectively) indicating that purity was adequate. Figure 3 presents the spot pattern for the overall DNA adducts analyzed by the NPL, similar to that described previously (Williams et al, 2013; Zhou *et al.*, 2007) and shows the presence of one major DNA adduct and one minor DNA adduct in the liver of rats dosed with methyleugenol only and methyleugenol/ nevadensin compared to faint DNA adducts in the livers of rats from the vehicle group. This is in line with findings from two recent studies where the formation of ME-3′-N^2-dGuo as the major methyleugenol DNA adduct (Figure 4) and ME-3′-N^6-dA as a minor methyleugenol DNA adduct were detected in vitro, in mice and in a human study (Herrmann *et al.*, 2012, 2013).

The results of NPL analysis for overall DNA adducts and the LC–ESI–MS/MS analysis for the major methyleugenol DNA adduct (ME-3′-N^2-dGuo) are shown in Figure 5A and 5B respectively. The NPL analysis revealed a constant ratio of major to minor spots averaging 4.3 to 4.6, but significant ($p<0.05$) reductions in the formation of the total DNA adducts in the liver of rats dosed with methyleugenol/nevadensin (49%) and rats dosed with methyleugenol/PCP (44%) compared to the rats dosed with methyleugenol only. In line with the NPL analysis, the LC–ESI–MS/MS analysis for ME-3′-N^2-dGuo also showed a significant ($p<0.001$) reduction in the liver of rats dosed with methyleugenol/ nevadensin (88%) and rats dosed with methyleugenol/PCP (89%) compared to rats dosed with methyleugenol only (Figure 5B). The NPL detected the levels of total DNA adducts formed in the liver of rats whereas the LC–ESI–MS/MS analysis detected the levels of only the major DNA adduct formed (ME-3′-N^2-dGuo). The levels of ME-3′-N^2-dGuo DNA adduct detected by the LC–ESI–MS/MS analysis was higher than the total DNA adducts detected by the NPL analysis. This is because NPL analysis tends to underestimate the absolute adduct levels due to incomplete recovery (Beland *et al.*, 1999; Morden *et al.*, 2008), and the lack of an internal standard.

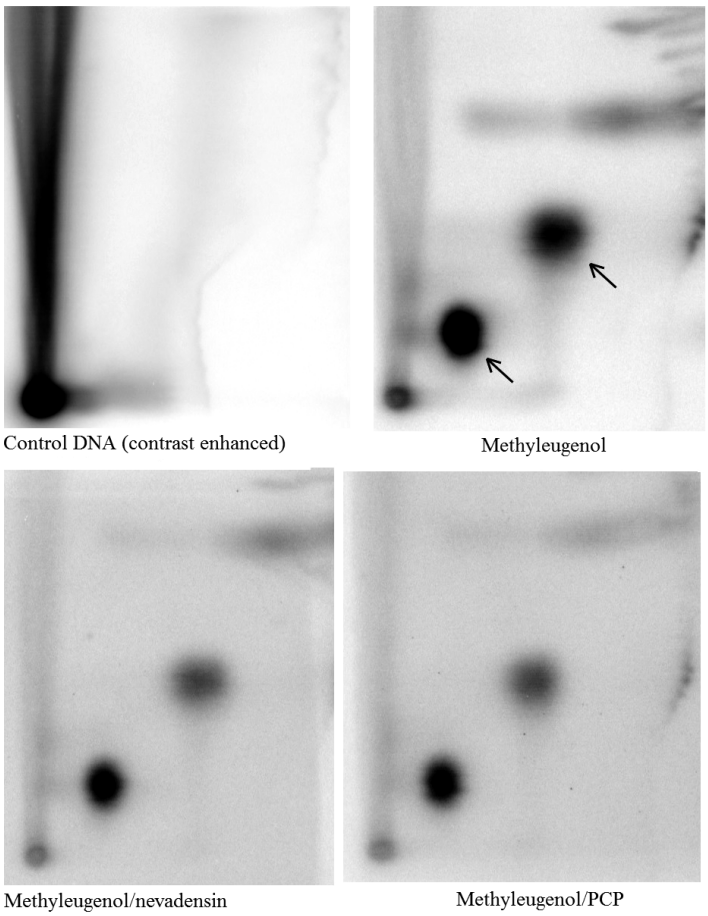


Figure 3. DNA spot pattern by NPL. The pattern was similar to that described by (Zhou *et al.*, 2007). Arrows in Figure indicate adducts used in quantitation.

Effect of nevadensin on proliferating cell nuclear antigen (PCNA)

The replicating fraction (RF) values, expressed in percent of PCNA-positive cells over total hepatocytes counted, are shown in Table 2. After 8 weeks, no statistically significant increase in the RF was present in any of the groups compared to the vehicle group. Nevertheless, there was a numerically greater RF in rats dosed with methyleugenol only compared to rats in the vehicle group (Table 2). The vehicle group value was the lowest of all groups (Tables 2). Table 1 of the Supplementary Material provides all individual PCNA data.

Effect of nevadensin on hepatocellular altered foci (HAF)

Table 3 presents summary results on incidence, multiplicity, and size (greatest diameter of GST-P$^+$ HAF) at 8 weeks, and Table 2 in the Spplementary Material provides all individual

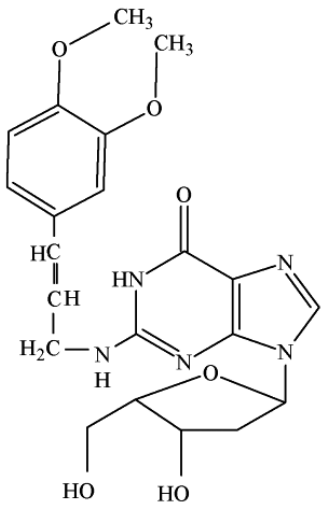


Figure 4. Molecular structure of the major methyleugenol DNA adduct; ME-3'-N^2-dGuo (N^2-(*trans*-isomethyleugenol-3'-yl)-2'-deoxyguanosine).

Table 2. Proliferating cell nuclear antigen (PCNA) marker of hepatocellular replicating fraction (RF) in percent.

Group	Replicating fraction (mean±SD)
1.Vehicle	3.23 ± 0.41
2.Methyleugenol	4.43 ± 1.09
3.PCP	3.33 ± 0.67
4.Nevadensin	4.04 ± .062
5.Methyleugenol/nevadensin	3.58 ± 0.37
6.Methyleugenol /PCP	3.74 ± 0.27

PCP, pentachlorophenol; none of the groups was significant compared to the vehicle control group.

GST-P data. Figure 6 presents microscopic images (at 200x magnification) of the GST-P$^+$ HAF at 8 weeks from the liver of one methyleugenol rat of group 2 which had 2 different GST-P$^+$ HAF, one with the smallest HAF being 102 µm (Figure 6A) and a second (Figure 6B) with the greatest diameter being 204 µm. The third GST-P$^+$ HAF is from the liver of a methyleugenol/nevadensin rat which has a reduced smallest diameter of 51 µm (Figure 6C). Comparing the two smallest diameters, it is evident that nevadensin reduces by half the methyleugenol-induced HAF (Figure 6). As shown in Table 3, no GST-P$^+$ HAF were detected in the livers of rats dosed with either the vehicle control, or nevadensin groups. A significant ($p<0.05$) increase in the incidence and mean multiplicity of the GST-P$^+$ HAF was observed in rats dosed with methyleugenol only. Thus, the incidence (80%), multiplicity (2.4 GSTP$^+$ HAF per rat liver, or 12-fold difference), and size (with 204 µm as the greatest diameter per rat liver) of group 2 (methyleugenol), were significantly ($p<0.05$) different from all other groups (Table 3).

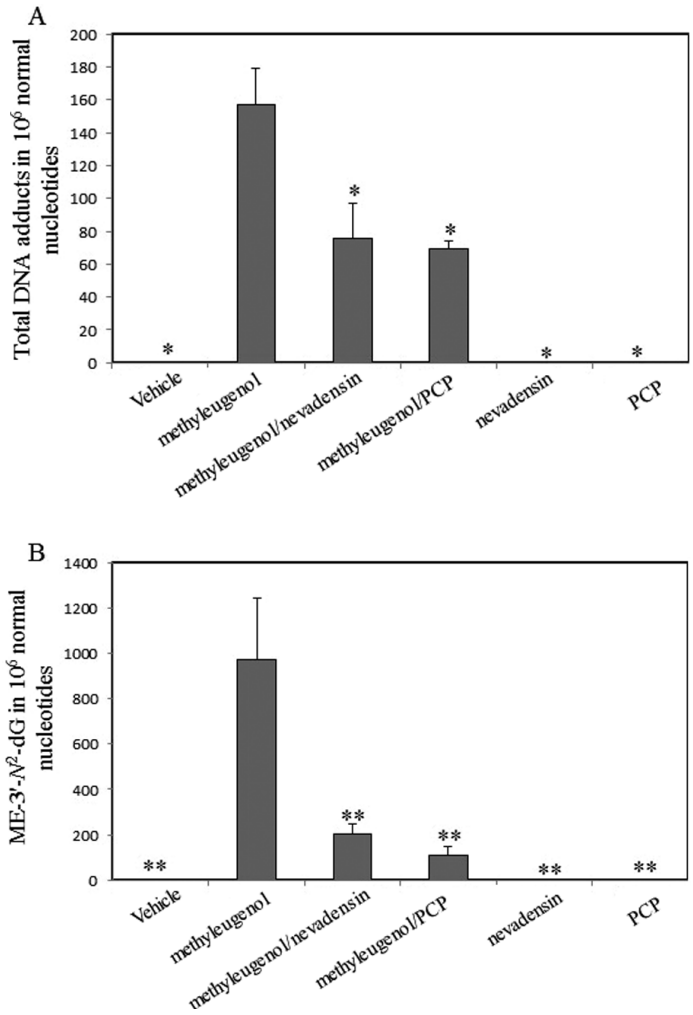


Figure 5. Formation of A) total methyleugenol DNA adducts in 106 normal nucleotides measured by NPL (N=3), and B) of ME-3'-N2-dGuo DNA adducts in 106 normal nucleotides measured by LC–ESI–MS/MS, in the liver of male F344 rats exposed orally to 250 mg/kg bw/day methyleugenol in the absence or presence of 120 mg/kg bw/day nevadensin or 11 mg/kg bw/day pentachlorophenol (PCP) versus rats dosed with vehicle, nevadensin only or PCP only for 8 weeks (3 days per week). Note: * and ** indicate values statistically significant at $p<0.05$ and $p<0.001$ respectively compared to methyleugenol.

DISCUSSION

The aim of the present study was to investigate whether the inhibitory effect of the basil flavonoid nevadensin on SULT-mediated methyleugenol bioactivation and DNA adduct formation observed in vitro (Al-Subeihi *et al.*, 2013) would also be observed in vivo, and to elucidate whether such a reduction would be accompanied by a

Table 3. Incidence, multiplicity and size of microscopic preneoplastic hepatocellular GST-P$^+$ HAF.

Group	Incidence (%)[a]	Multiplicity[b]	Size in µm[c]
1.Vehicle	0/5 (0) *	0.0 ± 0*	0*
2.Methyleugenol	4/5 (80)	2.4 ± 2.5	204
3.PCP	1/5 (20) *	0.2 ±0.5*	102*
4.Nevadensin	0/5 (0) *	0.0 ± 0.0*	0*
5.Methyleugenol /nevadensin	1/5 (20) *	0.2 ± 0.5*	102*
6.Methyleugenol/PCP	1/5 (20) *	0.2 ± 0.5*	102*

PCP, pentachlorophenol.
[a] Percent incidence of rats with at least one GST-P$^+$ HAF per liver sample (2 sections per slide).
[b] Multiplicity per group, expressed as GST-P$^+$ HAF per liver sample.
[c] The greatest diameter (size) of GST-P$^+$ HAF in µm.
* indicates values statistically significant at $p<0.05$ compared to methyleugenol group.

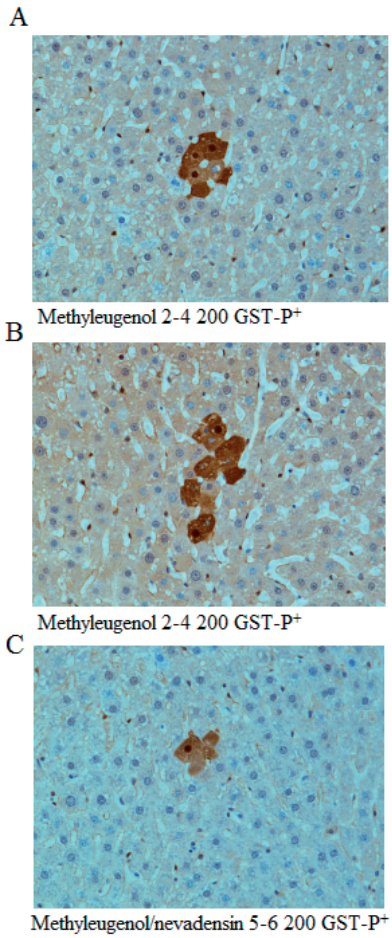


Figure 6. Microscopic GST-P$^+$ hepatocellular altered foci (HAF) at a 200x magnification from A) group 2 (methyleugenol only) rat liver (number 2-4) which has the smallest diameter of about 102 µm; B) group 2 (methyleugenol only) rat liver (number 2-4) with the greatest diameter of about 204 µm; and C) group 5 (nevadensin and methyleugenol) rat liver (number 5-6) with the smallest diameter of 51 µm.

reduction in early markers for liver carcinogenesis, as reported in other studies of anticarcinogenesis (Williams and Iatropoulos, 1996; Williams *et al.*, 2007a, b).

In a previous study we already demonstrated that nevadensin was able to inhibit significantly formation of DNA adducts in the liver of rats orally exposed simultaneously to a single dose of nevadensin and the related alkenylbenzene estragole (Alhusainy *et al.*, 2013). Since DNA adduct formation is considered a biomarker of exposure but generally not a biomarker of the ultimate carcinogenic effect (Paini *et al.*, 2010), in the present study exposure to methyleugenol and nevadensin was extended to 8 weeks to enable investigation of whether the nevadensin-mediated inhibition in alkenylbenzene DNA adduct formation in vivo would be accompanied by an inhibition of early markers of liver carcinogenesis in vivo. The results of the present study revealed that co-exposure to methyleugenol and nevadensin at a molar ratio reflecting their natural occurrence in basil resulted not only in a significant ($p<0.001$) 79 % inhibition in methyleugenol DNA adduct formation (measured by LC–ESI–MS/MS), but also in a significant ($p<0.05$) 92 % inhibition of the formation of HAF, which are markers for initiation of neoplasia as shown in numerous studies in which HAF precede the development of neoplasms and are quantitatively related to them (Williams, 1980; Bannasch, 1986; Williams, 1989; Enzmann *et al.*, 1998; Williams, 1998; Williams, 1999). On the other hand, RF values, which reflect hepatocellular proliferation, were only marginally increased (1.4 times) in group 2 (methyleugenol only) compared to group 1 (vehicle group). This magnitude of increase (e.g. 1.4) is half the previous value (e.g. 2.8) reported in a previous study at 8 weeks exposure to methyleugenol (Williams *et al.*, 2013). We consider this PCNA increase in the present study to be part of the biological variability that cannot be explained. Also, in the same study (Williams *et al.*, 2013), similar methyleugenol (high dose) doses for 8 weeks increased hepatocellular proliferation (2.8 times) compared to controls, but by week 16 the RF values of methyleugenol did not increase further, indicating that chronic compensatory proliferation is not the critical event in the induction of pre- and neoplasia. The numerical RF values were somewhat lower in the liver of rats dosed with methyleugenol/nevadensin and methyleugenol/PCP compared to rats dosed with methyleugenol only. The absence of statistical significance could reflect the fact that we need only 5 rats per group (Eldridge and Goldsworthy, 1996). Unlike HAF, RFs are not indicators of initiation of neoplasia but rather a marker of epigenetic (trophic/promotional) events. It is of interest to note that this marginal RF increase was also observed for the model SULT inhibitor PCP which did significantly reduce methyleugenol DNA adduct formation and HAF formation, and was previously shown able to also inhibit hepatic tumour formation in the liver of mice upon long-term dietary administration of safrole or its metabolite, 1'-hydroxysafrole together with PCP (Boberg *et al.*, 1983). This indicates that effects on DNA adduct and HAF formation may present better indicators of initiation of neoplasia.

Given the role of the 1'-sulfooxy metabolites of alkenylbenzenes in their hepatotoxicity and carcinogenicity (Boberg *et al.*, 1983), and the established quantitative relationship between HAF formation and neoplasm (Williams, 1980;

Bannasch, 1986; Williams, 1989; Enzmann *et al.*, 1998; Williams, 1998; Williams, 1999), the results of the present study suggest that a reduction in tumour formation would be expected in liver of rodents when nevadensin is dosed simultaneously with methyleugenol compared to studies where methyleugenol is tested alone.

So far, the effect of nevadensin in reducing the alkenylbenzene bioactivation in vivo has been established for the alkenylbenzenes estragole and methyleugenol only. However, the results of the present work can also be extended to other alkenylbenzenes. This is because the SULT inhibition by nevadensin is non-competitive in nature (Alhusainy *et al.*, 2010, 2013), and thus by definition should be evident regardless of the substrate (Simmons, 1996).

In the risk assessment of methyleugenol, an important aspect is how to integrate the current results on nevadensin-mediated modulation of methyleugenol bioactivation. In our previous work we have refined the lower confidence limit of the benchmark dose that gives 10% extra cancer incidence ($BMDL_{10}$) and subsequently calculated margin of exposure (MOE) values for both estragole (Alhusainy *et al.*, 2012) and methyleugenol (Al-Subeihi *et al.*, 2013) taking into account the possible reduction in tumour formation when nevadensin would have been co-administered with estragole or methyleugenol in the rodent carcinogenicity studies for the two alkenylbenzenes. As a first approximation we assumed that predicted reduction in alkenylbenzene DNA adduct levels using our binary methyleugenol-nevadensin physiologically based biokinetic (PBBK) model in male rat would be proportional to reduction in the incidence of hepatocellular neoplasia and then we calculated a refined $BMDL_{10}$ value which was subsequently used to estimate a refined MOE. For methyleugenol the refined MOE values increased from 3,500 and 5,300 for female rats and from about 1,100 and 2,400 for male rats based on data from studies testing the pure compound to about 11,000 and 18,000 for female rats and about 9,200 and 10,000 for male rats when the rodent study would have been performed in the presence of nevadensin (Al-Subeihi *et al.*, 2013). Although this linear relation between DNA adduct formation and hepatoma formation remains to be established, a previous study showed a correlation between the dose response curve for the formation of ME-3'-N^2-dGuo as the major methyleugenol DNA adduct with the dose response curve for mutagenicity of methyleugenol (Herrmann *et al.*, 2012). Moreover, the results of the present study showed that the nevadensin-mediated reduction in methyleugenol DNA adduct formation is accompanied by a proportional reduction in levels of HAF, the later are established to precede the development of neoplasms and are quantitatively related to development of such neoplasms (Bannasch, 1986; Enzmann et al, 1998; Williams, 1980, 1982).

An important question remaining is the relevance of these combination effects at daily human dietary exposure levels of estragole or methyleugenol and nevadensin, an objective that can be tested in rodent bioassays given that DNA adduct levels formed at dietary levels of methyleugenol for example can be readily quantified in mice and in human (Herrmann *et al.*, 2013). However, results from our previous research (Alhusainy *et al.*, 2010, 2012) using PBBK modeling indicate that these combination effects may

be limited at daily human dietary exposure levels of estragole or methyleugenol and nevadensin resulting from basil consumption, since at these levels the plasma nevadensin levels may not reach the *K*i value of nevadensin of 4 nm (Alhusainy *et al.*, 2012). Also, matrix effects may be limited for other SULT inhibiting dietary flavonoids even at high exposure levels of these flavonoids coming from supplements which is contributed to higher *K*i values for SULT inhibition by these flavonoids that are 2-3 orders of magnitude higher than the *K*i for nevadensin (Alhusainy *et al.*, 2010, 2012). This indicates that the importance of a matrix effect for risk assessment of individual compounds requires case-by-case analysis of dose dependency of the interactions detected.

Overall, the results of the present study indicate that the likelihood of bioactivation and subsequent adverse effects in rodent bioassays could be lower when the alkenylbenzene is dosed in a matrix containing SULT inhibitors such as nevadensin compared to experiments using pure alkenylbenzenes as single compounds. This is expected to result in increases $BMDL_{10}$ and resulting MOE values in a subsequent risk assessment.

ACKNOWLEDGMENT

This work was supported by a grant from the International Organization of the Flavour Industry (IOFI), Genève, Switzerland.

REFERENCES

1. Al-Subeihi, A. A. A., Alhusainy, W., Paini, A., Punt, A., J, V., Van Den Berg, J. H. J., and Rietjens, I. M. C. M. (2013). Inhibition of methyleugenol bioactivation by the herb-based constituent nevadensin and prediction of possible in vivo consequences using physiologically based kinetic modeling. *Food and Chemical Toxicology* **59**, 564-571.
2. Alhusainy, W., Paini, A., Punt, A., Louisse, J., Spenkelink, A., Vervoort, J., Delatour, T., Scholz, G., Schilter, B., Adams, T., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). Identification of nevadensin as an important herb-based constituent inhibiting estragole bioactivation and physiology-based biokinetic modeling of its possible in vivo effect. *Toxicology and Applied Pharmacology* **245**, 179-190.
3. Alhusainy, W., Paini, A., Van Den Berg, J. H. J., Punt, A., Scholz, G., Schilter, B., van Bladeren, P. J., Taylor, S., Adams, T. B., and Rietjens, I. M. C. M. (2013). In vivo validation and physiologically based modeling of the inhibition of SULT-mediated estragole DNA adduct formation in the liver of male Sprague-Dawley rats by the basil flavonoid nevadensin. *Molecular Nutrition and Food Research* **DOI 10.1002/mnfr.201300144**, http://eproof.aptaracorp.com/powerproof2/adp.do?aid=1357984731012469844.
4. Alhusainy, W., van den Berg, S. J. P. L., Paini, A., Campana, A., Asselman, M., Spenkelink, A., Punt, A., Scholz, G., Schilter, B., Adams, T. B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2012). Matrix modulation of the bioactivation of estragole by constituents of different alkenylbenzene-containing herbs and spices and physiologically based biokinetic modeling of possible in vivo Effects. *Toxicological Sciences* **129**, 174-187.
5. Bannasch, P. (1986). Preneoplastic lesions as end points in carcinogenicity testing. I. Hepatic preneoplasia. *Carcinogenesis* **7**, 689-695.
6. Beland, F. A., Doerge, D. R., Churchwell, M. I., Poirier, M. C., Schoket, B., and Marques, M. M. (1999). Synthesis, characterization, and quantitation of a 4-aminobiphenyl-DNA adduct standard. *Chemical Research in Toxicology* **12**, 68-77.
7. Bernard, B. K., Hoberman, A. M., Brown, W. R., Ranpuria, A. K., and Christian, M. S. (2002). Oral (gavage) two-generation

(one litter per generation) reproduction study of pentachlorophenol (penta) in rats. *International Journal of Toxicology* **21**, 301-318.

8. Boberg, E. W., Miller, E. C., and Miller, J. A. (1983). Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1'-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1'-hydroxysafrole in mouse liver. *Cancer Research* **43**, 5163-5173.
9. Eldridge, S.R., and Goldsworthy, S.M. (1996) Cell proliferation rates in common cancer target tissues of B6C3F1 mice and F344 rats: Effects of age, gender, and choice of marker. *Fund Appl Toxicol* **32**, 159-167.
10. Enzmann, H., Bomhard, E., Iatropoulos, M., Ahr, H. J., Schlueter, G., and Williams, G. M. (1998). Short- and intermediate-term carcinogenicity testing - A Review. Part 1: The prototypes mouse skin tumour assay and rat liver focus assay. *Food and Chemical Toxicology* **36**, 979-995.
11. European Commission (EC) (2008). Regulation (EC) No 1334/2008 of the Europian Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC.
12. Herrmann, K., Engst, W., Appel, K.E., Monien, B. H., and Glatt, H. (2012). Identification of human and murine sulfotransferases able to activate hydroxylated metabolites of methyleugenol to mutagens in Salmonella typhimurium and detection of associated DNA adducts using UPLC-MS/MS methods. *Mutagenesis* **27**, 453-462.
13. Herrmann, K., Schumacher, F., Engst, W., Appel, K. E., Klein, K., Zanger, U. M., and Glatt, H. (2013). Abundance of DNA adducts of methyleugenol, a rodent hepatocarcinogen, in human: Liver samples. *Carcinogenesis* **34**, 1025-1030.
14. Iatropoulos, M. J., and Williams, G. M. (1996). Proliferation markers. *Experimental and Toxicologic Pathology* **48**, 175-181.
15. Jeffrey, A. M., Luo, F. Q., Amin, S., Krzeminski, J., Zech, K., and Williams, G. M. (2002). Lack of DNA binding in the rat nasal mucosa and other tissues of the nasal toxicants roflumilast, a phosphodiesterase 4 inhibitor, and a metabolite, 4-amino-3,5-dichloropyridine, in contrast to the nasal carcinogen 2,6-dimethylaniline. *Drug and Chemical Toxicology* **25**, 93-107.
16. Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Research* **43**, 1124-1134.
17. Morden, B. H., Müller, C., Engst, W., Frank, H., Seidel, A., and Glatt, H. (2008). Time course of hepatic 1-methylpyrene DNA adducts in rats determined by isotope dilution LC-MS/MS and 32P-postlabeling. *Chemical Research in Toxicology* **21**, 2017-2025.
18. NTP (2000). National Toxicology Program on toxicology and carcinogenesis studies of Methyleugenol (CAS NO. 93-15-12) in F344/N rats and B6C3F1 mice (Gavage Studies). DRAFT NTP-TR-491; NIH Publication No. 98-3950., 1-412.
19. Paini, A., Punt, A., Viton, F., Scholz, G., Delatour, T., Marin-Kuan, M., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). A physiologically based biodynamic (PBBD) model for estragole DNA binding in rat liver based on in vitro kinetic data and estragole DNA adduct formation in primary hepatocytes. *Toxicology and Applied Pharmacology* **245**, 57-66.
20. Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. *Cancer Research* **41**, 176-186.
21. Phillips, D. H., Reddy, M. V., and Randerath, K. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. *Carcinogenesis* **5**, 1623-1628.
22. Punt, A., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2007). Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3'-yl)-2'-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen

1′-hydroxyestragole. *Chemical Research in Toxicology* **20**, 991-998.

23. Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis* **5**, 1613-1622.
24. Reddy, M. V., and Randerath, K. (1986). Nuclease p1-mediated enhancement of sensitivity of p-postlabeling test for structurally diverse dna adducts. *Carcinogenesis* **7**, 1543-1551.
25. Sato, K., Kitahara, A., and Satoh, K. (1984). The placental form of glutathione S-transferase as a new marker protein or preneoplasia in rat chemical hepatocarcinogenesis. *Gann, The Japanese Journal of Cancer Research* **75**, 199-202.
26. Simmons, J. E. (1996). Application of physiologically based pharmacokinetic modelling to combination toxicology. *Food and Chemical Toxicology* **34**, 1067-1073.
27. Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - Methyl eugenol and estragole. *Food and Chemical Toxicology* **40**, 851-870.
28. Williams, G. M. (1980). The pathogenesis of rat liver cancer caused by chemical carcinogens. *Biochimica et Biophysica Acta* **605**, 167-189.
29. Williams, G. M. (1982). Phenotypic properties of preneoplastic rat liver lesions and applications to detection of carcinogens and tumor promoters. *Toxicologic Pathology* **10**, 3-10.
30. Williams, G. M. (1989). The significance of chemically-induced hepatocellular altered foci in rat liver and application to carcinogen detection. *Toxicologic Pathology* **17**, 663-672.
31. Williams, G. M. (1998). Phenotypic properties of preneoplastic rat liver lesions and applications to detection of carcinogens and tumor promoters. *Toxicologic Pathology* **26**, 452-453.
32. Williams, G. M. (1999). Chemically induced preneoplastic lesions in rodents as indicators of carcinogenic activity. *IARC scientific publications*, 185-202.
33. Williams, G. M., and Iatropoulos, M. J. (1996). Inhibition of the hepatocarcinogenicity of aflatoxin B1 in rats by low levels of the phenolic antioxidants butylated hydroxyanisole and butylated hydroxytoluene. *Cancer Letters* **104**, 49-53.
34. Williams, G. M., and Mattia, A. (2009). Alkoxy-substituted allylbenzenes present in foods and essntial oils and used as flavouaring agents. In: WHO Food Additives Series 60, 69th meeting of the Joint FAO/ WHO Expert Committee on Food Additives (JECFA), Geneva, Switerland, pp 351-480.
35. Williams, G. M., Iatropoulos, M. J., Jeffrey, A. M., and Duan, J. D. (2007a). Inhibition by dietary hydroquinone of acetylaminofluorene induction of initiation of rat liver carcinogenesis. *Food and Chemical Toxicology* **45**, 1620-1625.
36. Williams, G. M., Iatropoulos, M. J., Jeffrey, A. M., and Duan, J. D. (2013). Methyleugenol hepatocellular cancer initiating effects in rat liver. *Food and Chemical Toxicology* **53**, 187-196.
37. Williams, G. M., Iatropoulos, M. J., Jeffrey, A. M., Duan, J. D., and Perrone, C. E. (2007b). Inhibition by acetaminophen of neoplastic initiation elicited in rat liver by the DNA-reactive hepatocarcinogen N-acetyl-2-aminofluorene. *European Journal of Cancer Prevention* **16**, 528-534.
38. Williams, G. M., Iatropoulos, M. J., Wang, C. X., Jeffrey, A. M., Thompson, S., Pittman, B., Palasch, M., and Gebhardt, R. (1998). Nonlinearities in 2-acetylaminofluorene exposure responses for genotoxic and epigenetic effects leading to initiation of carcinogenesis in rat liver. *Toxicological Sciences* **45**, 152-161.
39. Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F1 mice. *Cancer Research* **47**, 2275-2283.
40. Zhou, G. D., Moorthy, B., Bi, J., Donnelly, K. C., and Randerath, K. (2007). DNA adducts from alkoxyallylbenzene herb and spice constituents in cultured human (HepG2) cells. *Environmental and Molecular Mutagenesis* **48**, 715-721.

SUPPLEMENTARY MATERIAL

Table 1.Proliferating Cell Nuclear Antigen (PCNA) [a] Immunohistochemically Stained Liver Microscopic Slide Evaluation

		PCNA Evaluation	
Group Identification	Rat Number	RF	Pos/Total
1. Vehicle	1-1	3.13	32/815
	1-2	3.01	25/830
	1-3	2.90	29/1000
	1-4	3.04	29/955
	1-5	3.26	29/890
	Mean ± SD	3.23 ± 0.41	
2. Methyleugenol	2-3	4.13	33/800
	2-4	3.56	29/815
	2-5	4.65	36/775
	2-7	3.59	30/835
	2-8	6.21	54/870
	Mean ± SD	4.43 ± 1.09	
3. PCP [b]	3-1	3.72	35/940
	3-4	2.84	26/915
	3-5	3.77	30/795
	3-6	2.40	23/960
	3-7	3.91	36/920
	Mean ± SD	3.33 ± 0.67	
4. Nevadensin	4-1	3.66	30/820
	4-4	3.97	29/730
	4-5	4.32	38/880
	4-6	3.31	27/815
	4-7	4.94	38/770
	Mean ± SD	4.04 ± 0.62	
5.Methyleugenol/nevadensin	5-1	3.35	29/865
	5-4	3.16	27/855
	5-5	3.45	25/725
	5-6	4.00	32/800
	5-7	3.92	31/790
	Mean ± SD	3.58 ± 0.37	
6.Methyleugenol/PCP	6-1	3.78	28/740
	6-4	3.46	28/810
	6-5	3.54	28/790
	6-6	3.78	28/740
	6-7	4.16	32/770
	Mean ± SD	3.74 ± 0.27	

[a] PCNA immunohistochemistry evaluation reflecting the percent replicating fraction (RF), which is derived from total PCNA positive nuclei counted over total nuclei counted from ~ 5 graticule areas with the highest (most intense) PCNA staining at a 200x total magnification.
[b] PCP, pentachlorophenol.
There are no statistically significant differences among any of the groups were present.

Table 2. Incidence of GST-P[a] Positive Hepatocellular Altered Foci (HAF) per cm^2 of Microscopically and Immunohistochemically Stained and Evaluated Liver Tissue

			Evaluation	
Group Identification	Rat Number		GSTP+ HAF	/cm^2 of liver tissue
1.Vehicle	1-1		0	/3.0
	1-2		0	/3.6
	1-3		0	/2.1
	1-4		0	/3.1
	1-5		0	/2.0
		Mean ± SD	0	/2.8 or 0/cm^2
2.Methyleugenol	2-3		1	/3.5
	2-4		6 [b]	/3.7
	2-5		0	/3.8
	2-7		4	/3.3
	2-8		1	/2.4
		Mean ± SD	2.4 ± 2.5*	/2.8 or 0.86/cm^2
3.PCP [c]	3-1		0	/2.4
	3-4		0	/3.9
	3-5		0	/2.5
	3-6		0	/3.6
	3-7		1 [d]	/2.4
		Mean ± SD	0.2 ± 0.5	/3.0 or 0.07/cm^2
4. Nevadensin	4-1		0	/2.6
	4-4		0	/2.0
	4-5		0	/2.8
	4-6		0	/3.1
	4-7		0	/2.8
		Mean ± SD	0	/2.7 or 0/cm^2
5.Methyleugenol/nevadensin	5-1		0	/3.5
	5-4		0	/3.3
	5-5		0	/3.2
	5-6		1 [e]	/4.6
	5-7		0	/4.4
		Mean ± SD	0.2 ± 0.5	/3.8 or 0.07/cm^2
6.Methyleugenol/PCP	6-1		0	/3.8
	6-4		0	/2.5
	6-5		0	/3.1
	6-6		0	/4.6
	6-7		1 [e]	/2.6
		Mean ± SD	0.2 ± 0.5	/3.3 or 0.07/cm^2

[a] GST-P immunohistochemically stained and evaluated liver microscopic slides reflecting hepatocellular altered foci (HAF), a preneoplastic lesion.
[b] the greatest diameter of a GST-P+ HAF was ~ 204µ.
[c] PCP, pentachlorophenol.
[d] the greatest diameter was ~ 102µ.
[e] the greatest diameter was ~ 51µ; the total microscopic magnification was 100x;
* the mean value of this group is statistically significant from all other groups at $p<0.05$.

7 General discussion and future perspectives

GENERAL DISCUSSION

Alkenylbenzenes such as estragole and methyleugenol are common components of spices and herbs such as tarragon, basil, fennel, mace, allspice, star anise and anise and their essential oils (Smith *et al.*, 2002). There is an interest in the safety evaluation of alkenylbenzenes because some of these compounds can induce hepatic tumours in rodents when dosed orally at high dose levels (Miller *et al.*, 1983; NTP, 2000). In 2000, the National Toxicology Program (NTP) has further investigated the carcinogenicity of methyleugenol and concluded that the compound is carcinogenic in both rats and mice (NTP, 2000). Based on the rodent studies with estragole, methyleugenol and the structurally related alkenylbenzene safrole the hepatocarcinogenicity of alkenylbenzenes is ascribed to their bioactivation by cytochrome P450 enzymes leading to the formation of the proximate carcinogen, the 1′-hydroxy metabolite, which is further bioactivated to the ultimate carcinogen, the 1′-sulfooxy metabolite (Miller *et al.*, 1983; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Smith *et al.*, 2010). The 1′-sulfooxy metabolite is unstable and binds via a presumed reactive carbocation intermediate covalently to different endogenous nucleophiles including DNA (Phillips *et al.*, 1981; Boberg *et al.*, 1983; Miller *et al.*, 1983; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Fennell *et al.*, 1985; Wiseman *et al.*, 1987; Smith *et al.*, 2002). Because of their genotoxicity and carcinogenicity, the addition of estragole and methyleugenol as pure substances to foodstuffs has been prohibited within the European Union since September 2008 (European Commission, 2008). In 2008, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) re-evaluated the safety of six alkenylbenzenes and indicated that although evidence of carcinogenicity in rodents given high doses of various alkenylbenzenes exists, further research is needed to assess the potential risk to human health at relevant dietary exposure levels (JECFA, 2008).

A significant difficulty in evaluating the toxicological data for alkenylbenzenes is that human exposure to these substances results from exposure to a complex mixture of food, spice, and spice oil constituents. Other compounds in these mixtures may influence the biochemical fate and toxicological risk of the alkenylbenzenes. Such a combination effect can take place when the complex matrix, or one constituent in the matrix, alters the absorption, metabolism, distribution and/or excretion (ADME), or alters the dynamics (mode of action) (Dybing *et al.*, 2002) of the alkenylbenzenes. For example slow or incomplete release of alkenylbenzenes from the food matrix may result in a low bioavailability of these compounds from the mixture compared with their bioavailability when dosed as pure compounds. An example of an important toxicokinetic interaction for alkenylbenzenes is an influence on the sulfotransferase (SULT) activity involved in formation of their ultimate 1′-sulfoooxy metabolite. In this regard, it was shown that a methanolic extract of basil inhibited the formation of estragole DNA adducts in human HepG2 cells exposed to the proximate carcinogen 1′-hydroxyestragole (Jeurissen *et al.*, 2008). This inhibition occurred at the level of SULT-mediated bioactivation of 1′-hydroxyestragole into 1′-sulfooxyestragole (Jeurissen *et al.*, 2008).

The objective of this PhD research was to study the inhibitory action of components in alkenylbenzene-containing herbs and spices on SULT-mediated alkenylbenzene

DNA adduct formation and the consequences of this combination effect for risk assessment using estragole and methyleugenol as the model alkenylbenzenes. To achieve this objective, an integrated approach of in vitro, in vivo and physiologically based biokinetic (PBBK) models was applied to investigate how the SULT inhibition influences the bioactivation and thus potentially also the toxicity and risk assessment of estragole and methyleugenol.

The major lines of discussion in this chapter are depicted in figure 1 and include the following aspects; First the identification and presence of dietary SULT inhibitors in the alkenylbenzene-containing herbs and spices as well as in the diet and their estimated daily intake (EDI) levels will be discussed based on results from chapter 2 and 3.

Second, based on these EDIs possible exposure scenarios to the alkenylbenzenes estragole and methyleugenol and the different dietary SULT inhibiting flavonoids will be discussed based on results from chapter 2, 3, 4, 5 and 6. Third, in vitro activity of dietary SULT inhibitors will be discussed based on results from chapter 2, 3 and 5. Fourth, the in vivo effect of dietary SULT inhibitors on alkenylbenzene DNA adduct formation in the liver of exposed rodents will be discussed based on results from chapter 4 and 6 using respectively estragole and methyleugenol and in both studies nevadensin as the model compounds. Also, the anticipated in vivo activity of dietary SULT inhibitors at realistic intake levels in humans will be discussed based on their reported plasma levels and predictions made by our PBBK models in male rat and human (chapter 2 and 3). Fifth, the association between the different

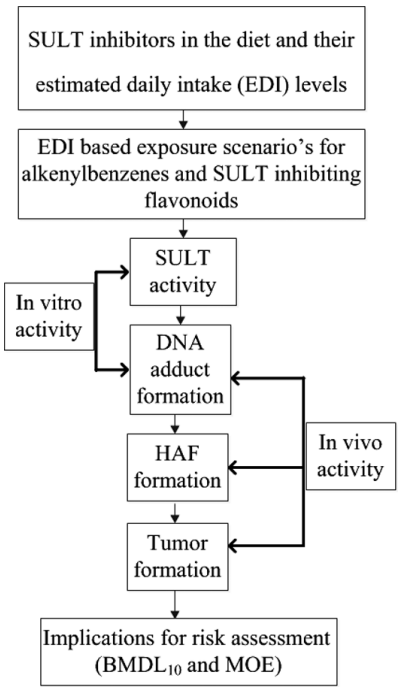


Figure 1. Schematic representation of major lines of discussion.

endpoints detected in the present thesis and their relation to the carcinogenicity of alkenylbenzenes will be discussed based on results from chapter 6. Sixth, an updated risk assessment for the alkenylbenzenes estragole and methyleugenol taking the matrix effect into account will be discussed based on results from chapter 4 and 6. Finally, future research perspectives based on issues raised by this research will be discussed and an overall conclusion will be presented.

SULT inhibitors in the diet and their estimated daily intake (EDI) levels

Chapter 2 and 3 report that methanolic extracts from different alkenylbenzene-containing herbs and spices such as basil, nutmeg, mace and others are able to inhibit the SULT activity involved in the formation of the proximate hepatocarcinogen, 1'-sulfooxyestragole. Based on our analytical analysis and literature data, the active constitutes in herbs and spices responsible for SULT inhibition were identified. Flavonoids including nevadensin, quercetin, kaempferol, myricetin, luteolin and apigenin were the major constituents responsible for this inhibition of SULT activity. These flavonoids are all present in alkenylbenzene-containing herbs and spices, as well as throughout the diet, and they are all reported to have SULT inhibiting potency. Table 1 presents an overview of the EDI levels of these dietary flavonoids as well as the EDI levels of other important flavonoids that are also reported in the literature to exert SULT inhibiting activity including the flavonoids hesperetin and naringenin and the isoflavones daidzein and genistein (Justesen *et al.*, 1997; Ghazali and Waring, 1999; Mesía -Vela and Kauffman, 2003; Mullie *et al.*, 2008). As presented in table 1, the total EDI values of these SULT inhibiting flavonoids (excluding isoflavonoids) as recorded by different studies vary between 0.18 and 0.73 mg/kg bw/day. The main dietary source for quercetin, representing the flavonoid with the highest intake levels are black tea (61% of flavonoid intake), onions (13%) and apples (10%) (Hertog *et al.*, 1993) which is in agreement with results from other studies (De Vries *et al.*, 1997; Arai *et al.*, 2000).

Interestingly, the mean intake levels of isoflavonesin in Asian countries ranges between 0.42-0.67 mg/kg bw/day with a maximum isoflavone intake of 1.7 mg/kg bw/day day and thus may reach levels higher than the mean intake levels from all other flavonoids amounting to 0.18 and 0.73 mg/kg bw/day (Table 1). Although Americans and Europeans consume much lower levels of isoflavones, the incorporation of growing quantities of soya extracts into manufactured food products and the increasing use of isoflavone containing food supplements could result in an increase in isoflavone intake (Manach *et al.*, 2004).

Specific subpopulations may be exposed to high levels of these dietary SULT-inhibiting flavonoids such as women undergoing phytoestrogen replacement therapy for menopause consuming between 0.5 and 1.2 mg isoflavones/kg bw/day day in the form of soya extract capsules (Manach *et al.*, 2004). Some people may be exposed to even higher levels of these dietary SULT-inhibiting flavonoids from some food supplements commercially present on the market with levels of the flavonoids up to 1000 mg per tablet and with recommended use levels of one to two tablets per day resulting in intake levels of up to 33 mg/kg bw/day (assuming a 60 kg human individual consuming 2 tablets per day).

Table 1. Overview of intake levels (mg/kg bw/day) for some important dietary flavonoids.

Study population	subjects	flavonoids	intake[a] (mg/kg bw/day)	Total intake[a] (mg/kg bw/day)	reference
Hungarian	204 healthy adults (25-60 yr)	quercetin	0.11±0.11	0.31±4.70	(Lugasi *et al.*, 2003)
		kaempferol	0.02±0.03		
		myricetin	0.14±0.44		
		luteolin	0.03±0.04		
		apigenin	0.01±0.01		
Danish[b]		quercetin	0.21	0.43	(Justesen *et al.*, 1997)
		kaempferol	0.04		
		myricetin	0.03		
		hesperetin	0.11		
		naringenin	0.04		
Spanish[c]	354 control subjects	quercetin	0.14±0.15	0.18±0.16	(Garcia-Closas *et al.*, 1999)
		kaempferol	0.02±0.03		
		Myricetin	0.01±0.02		
Flemish[d]	45 female dieticians	quercetin	0.2±0.13	0.73	(Mullie *et al.*, 2008)
		kaempferol	0.08±0.07		
		myricetin	0.04±0.04		
		luteolin	0.01±0.01		
		apigenin	0.02±0.08		
		hesperetin	0.27±0.29		
		naringenin	0.13±0.20		
Dutch	805 men (65-84 yr)	quercetin	0.27±0.17	0.43±0.24	(Hertog *et al.*, 1993)
		kaempferol	0.14±0.08		
		myricetin	NR		
		luteolin	NR		
		apigenin	NR		

Japanese	115 women (29-78 yr)	quercetin kaempferol myricetin luteolin	0.16±0.12 0.10±0.05 0.01±0.01 0.01±0.01	0.27	(Arai *et al.*, 2000)
US[e]	37,886 men (<50-+70) 78,886 women (<50-+70)	quercetin kaempferol myricetin luteolin apigenin	0.25±0.17- 0.29±0.22 0.06±0.08- 0.09±0.1 0.02±0.02 NR NR	0.33±0.24- 0.39±0.29	(Sampson *et al.*, 2002)
Asian	Mean intake in population Maximum intake in population	isoflavones (daidzein and genistein)		0.42–0.67 1.7	(Coward *et al.*, 1993; Arai *et al.*, 2000) (Coward *et al.*, 1993; Arai *et al.*, 2000)
General population	Women undergoing phytoestrogen replacement therapy for menopause	isoflavones (daidzein and genistein)		0.5-1.2	(Manach *et al.*, 2004)

[a] intake levels are presented in mg/kg bw/day and were calculated using data from literature in mg/day/person assuming a human average body weight of 60 kg.
[b] based on intake data from the National statistical Office of Denmark, Household consumption survey 1987, unpublished data.
[c] the intake value is based on control group subjects.
[d] measured by a 4-day food record (4DFR).
[e] intake values in the US study were reported for different age subgroups. Intake values reported in the table represent the minimum intake value reported and the highest intake value reported across all age groups including both men and women

Apart from SULT inhibitors present in herbs and spices and identified in the present study, humans may be exposed to various other dietary SULT inhibitors including a number of food additives such as octyl gallate and tartrazine (Bamforth *et al.*, 1993), some common drugs (Bamforth *et al.*, 1992) and amines (Matsui *et al.*, 1995).

Overall, it is concluded that the diet is rich in SULT inhibiting compounds including flavonoids, food additives, drugs and amines and literature data provide good estimates for their EDI levels. The presence of such a wide range of SULT inhibitors in the regular diet offers chances on reduction of the bioactivation of the alkenylbenzenes. However, the most abundant SULT inhibitors in the diet are not necessarily the most active ones in biological systems and therefore their in vitro as well as their in vivo activity need to be evaluated.

Exposure scenarios for alkenylbenzenes and dietary flavonoids

Knowledge on the EDI levels of each dietary SULT inhibiting flavonoid and of the alkenylbenzenes is essential to define realistic exposure scenarios. One possible exposure scenario is by assuming that all abundant dietary flavonoids are consumed together at their respective EDI levels which, according to table 1, range between 0.18 and 0.73 mg/kg bw/day -excluding isoflavones-. Likewise, EDI values of 0.01 for estragole and 0.014 mg/kg bw/day for methyleugenol, resulting mainly from herbs, spices and their essential oils (Smith *et al.*, 2002) can be used.

In chapter 3, both estragole and the different dietary flavonoids were assumed to be consumed together at their EDI levels and PBBK modeling was used to evaluate the influence of this co-exposure scenario on the formation of the ultimate carcinogenic metabolite of estragole, 1'-sulfooxyestragole, in the liver. This approach serves as a first approximation because dietary flavonoids as well as alkenylbenzenes were assumed to be consumed together at the same time and in one portion. However, in reality the alkenylbenzenes and the dietary flavonoids from foods may not necessarily be consumed together in one dose and their consumption may occur in proportions all over the day.

Another possible exposure scenario could be at levels of the dietary flavonoids present in food supplements. In chapter 3, quercetin was assumed to be consumed from a tablet containing 500 mg of quercetin and thus resulting in a daily intake of 8.3 mg/kg bw/day (for a 60 Kg individual) together with 0.01 mg/kg bw/day of estragole.

In addition, a realistic exposure scenario could be based on a food item in which the alkenylbenzene and the SULT inhibitor(s) are present together. Therefore, knowledge on the levels of alkenylbenzenes and SULT inhibitor(s) in alkenylbenzene-containing herbs and spices is essential. Such data are available in the literature, however, the composition and levels of flavonoids and alkenylbenzenes in a herb or a spice can vary significantly based on different factors such as plant maturity at harvest, harvesting techniques, storage conditions, processing (e.g. drying), and method of measurement (e.g. extraction) (Smith *et al.*, 2002). Therefore, present state-of-the-art intake estimates for ingredients from botanicals and botanical preparations are not refined to such an extent that the actual human exposure levels to alkenylbenzenes and SULT inhibiting ingredients can be determined in an accurate way.

Nevertheless, a model alkenylbenzenes-containing herb or a spice can be selected and different scenarios can be investigated ranging between a worst case to a best case scenario for levels of the compounds of interest detected in the model herb. In chapter 2, 3, 4, 5 and 6 basil was selected as the model alkenylbenzene-containing herb. This is because basil is the herb that contributes the most to the consumption of estragole by a value that amounts to 50% of the EDI of estragole (Smith *et al.*, 2002). Basil may also contribute to some of the highest levels of intake of methyleugenol in some specialised eating groups, such as for example pesto eaters since fresh pesto is prepared from a large quantity of fresh sweet basil (Smith *et al.*, 2002). These special eating groups may have exposure levels of methyleugenol that are 10 times higher (up to 0.1 mg/kg bw/day methyleugenol) than normal exposure (Smith *et al.*, 2002). Also, nevadensin, a potent SULT inhibiting flavonoid, has been reported as a major flavone constituent in basil and may reach up to 58% of the total flavones present in almost all varieties of basil (Grayer *et al.*, 2004).

In chapter 2 and 3 we investigated two possible exposure scenarios resulting from consumption of basil using EDI levels of estragole reported in literature and ranging between 0.01-0.07 mg/kg bw/day (SCF, 2001a; Smith *et al.*, 2002) together with nevadensin at a molar ratio of nevadensin to estragole of 0.06 or 0.25 reflecting the molar ratio at which the two compounds are present in basil assuming a worst case and best case scenario respectively. A similar approach was also used in chapter 5 with the alkenylbenzene methyleugenol at an EDI of 0.014 mg/kg bw/day (Smith *et al.*, 2002) and nevadensin at a molar ratio of nevadensin to methyleugenol of 0.2 reflecting their presence in basil.

Another possible exposure scenario includes the dose levels of the alkenylbenzenes used in the rodent carcinogenicity studies assuming that the flavonoid would be dosed to rodents at a molar ratio of the alkenylbenzene to the SULT inhibitor reflecting their presence in an alkenylbenzene-containing herb or a spice. Since dose levels of the alkenylbenzene used in these carcinogenicity studies are high, also the dose of the SULT inhibitor will be high. This situation reflects testing the material of commerce itself instead of the pure compound. This exposure scenario was used in the PBBK modelling of chapter 4 and 5 and also in the in vivo experiments of chapter 4 and 6 to evaluate the effect of nevadensin on bioactivation of estragole and methyleugenol at a molar ratio of nevadensin to estragole or to methyleugenol reflecting their presence in basil and at dose levels used in rodent bioassays. This approach allowed us, as reported in chapter 4 and 6 to derive refined $BMDL_{10}$ values for the alkenylbenzenes estragole and methyleugenol and thus update the risk assessment for the two compounds taking the matrix effect into account (see below).

Finally exposure scenarios are not only restricted to external doses of the compounds based on their EDI values from foods but may also be based on internal doses of the compounds present in plasma or in the target tissue. Using PBBK modeling, the effect of SULT inhibitor(s) on the bioactivation of the alkenylbenzenes can be evaluated at different scenarios of internal doses in the target tissue, the liver. In chapter 2 and 3 for example the different SULT inhibiting flavonoids were assumed to follow the same

kinetics as estragole in the liver and different scenarios of uptake ranging between 0% (no uptake of flavonoid) to 100 % (complete uptake of flavonoid) were also modelled.

Overall, given that humans in reality are exposed to complex mixtures of food components and have wide variation in dietary habits, different exposure scenarios need to be investigated. This requires a good knowledge about levels of the compounds of interest (e.g. alkenylbenzenes and dietary SULT inhibiting flavonoids) in the different food matrices and the range of variation in levels of these active components.

In vitro inhibition by selected flavonoids

Table 2 presents kinetic data for the inhibition of SULT activity by different dietary flavonoids measured in vitro either in the present study (in chapters 2 and 3) or reported in the literature using different SULT-containing systems. Based on these kinetic data, nevadensin seems to be the most potent food-borne SULT inhibitor identified with a *K*i value of 4 nM for SULT inhibition that is 2-3 orders of magnitude lower than the *K*i values for the other dietary flavonoids tested in the present thesis or reported in literature (Table 2).

The type of inhibition exerted by nevadensin, quercetin, kaempferol, myricetin, and apigenin on SULT activity was shown to be non-competitive (Alhusainy *et al.*, 2010, 2012) (chapters 2 and 3). A non-competitive type of inhibition has been also reported for genistein and daidzein on human phenol SULTs (Ghazali and Waring, 1999). The non-competitive nature of the SULT inhibition by the different flavonoids

Table 2. overview of IC_{50} or *K*i values for SULT inhibition by different dietary flavonoids measured in vitro using different SULT-containing systems based on different reports.

Flavonoid	IC_{50} (µM)	*K*i (µM)	Fraction/enzyme	reference
Quercetin		1.5	pooled male rat liver S9	(Alhusainy *et al.*, 2012)
Kaempferol		0.6	pooled male rat liver S9	(Alhusainy *et al.*, 2012)
Myricetin		11.9	pooled male rat liver S9	(Alhusainy *et al.*, 2012)
Apigenin		0.7	pooled male rat liver S9	(Alhusainy *et al.*, 2012)
Nevadensin		0.004	pooled male rat and human liver S9	(Alhusainy *et al.*, 2012)
Genistein		0.21	human phenol SULTs	(Ghazali and Waring, 1999)
	0.8		rat liver cytosol	(Mesía -Vela and Kauffman, 2003)
Daidzein		0.34	human phenol SULTs	(Ghazali and Waring, 1999)
	2.2		rat liver cytosol	(Mesía -Vela and Kauffman, 2003)
Hesperetin	0.2 3.0		SULT 1A1 SULT1E1	(Harris *et al.*, 2004)
Naringenin	4 >10		SULT 1A1 SULT1E1	(Harris *et al.*, 2004)

means by definition that the inhibition and its *K*i are independent of the nature or concentration of the substrate (such as for example the 1′-hydroxy metabolites of the alkenylbenzenes) but rather only dependent on the inhibitor (for example the flavonoid) and its concentration (Simmons, 1996).

The non-competitive type of inhibition also suggests that the different flavonoids are poor substrates for SULTs. This was evident in our studies where we have shown the catalytic efficiency for nevadensin sulfonation in male rat and human liver fractions to be low and absent in intestinal fractions of both species. This is also supported by results from a previous study reporting lack of sulfonation for quercetin, kaempferol, apigenin and myricetin by human liver cytosols (Eaton *et al*., 1996) which altogether explains that most circulating flavonoid conjugates detected in vivo are glucuronides (Manach *et al*., 2004).

Interestingly, all flavonoids tested in the present study, namely, quercetin, kaempferol, myricetin, apigenin, luteolin and nevadensin were found to possess the structural requirements for effective SULT inhibitory activity linked to the presence of C5 and C7 hydroxyl substituents on the A-ring and also a C2–3 double bond in conjunction with the C4 carbonyl group on the C-ring (Morimitsu *et al*., 2004).

Given that we are exposed to a mixture of dietary flavonoids and not to only individual components we have also investigated the combined effect for a selected series of flavonoids on SULT activity using pooled male rat liver S9 fractions (chapter 3). These studies indicated that their combined effect is additive. We have also shown in chapter 3 that in the incubations with liver fractions at concentrations of the flavonoids shown to inhibit SULT activity no inhibition in glucuronidation of 1′-hydroxyestragole by any of the flavonoids tested occurs. This is important given that glucuronidation is a detoxification pathway for 1′-hydroxyestragole. On the other hand, the different flavonoids tested –with the exception of nevadensin- were able to inhibit the oxidation of 1′-hydroxyestragole with *K*i values close to *K*i values for the SULT inhibition by the same dietary flavonoids. Given these results it can be concluded that in the presence of the inhibitors the metabolic pathways for 1′-hydroxy alkenylbenzenes will shift in favour of glucuronidation at the cost of sulfonation and oxidation pointing at increased detoxification and reduced risks.

In line with these results using liver fractions, experiments using human HepG2 cells exposed simultaneously to 1′-hydroxyestragole and individual flavonoids (quercetin or kaempferol) or to a mixture of flavonoids, showed a significant inhibition in the formation of SULT-mediated DNA adduct formation and a significant inhibition in the formation of 1′-oxoestragole. The co-exposure to 1′-hydroxyestragole and the different flavonoids increased the formation of 1′-hydroxyestragole glucuronide corroborating a shift in metabolism from bioactivation (e.g. sulfonation) to detoxification (e.g. glucuronidation). These data also indicate that the different flavonoids are able to pass the cell membrane and exert an intracellular effect on SULT-mediated bioactivation of 1′-hydroxyestragole.

The effect of the different flavonoids on hydroxylation of estragole into its proximate carcinogen, 1′-hydroxyestragole, was not investigated in the present thesis. An inhibitory effect by these flavonoids on 1′-hydroxylation may reduce the ultimate levels of DNA adducts formed by estragole and methyleugenol. Kinetic

studies revealed that, at dietary relevant concentrations of estragole, P450 1A2 and 2A6 are the most important enzymes for 1′-hydroxylation in the human liver (Jeurissen *et al.*, 2007). Several reports have shown inhibitory activity for dietary flavonoids on P450 enzyme activity. For example, in an experiment on benzo(*a*)pyrene metabolism using human liver microsomes, it was shown that addition of quercetin and kaempferol results in inhibition of the hydroxylation of benzo(*a*)pyrene with IC_{50} values amounting to about 10 μM for quercetin and between 10-50 for μM kaempferol (Buening *et al.*, 1981). Also, the activity of CYP1A2 was inhibited by the presence of various flavonoids including those tested in the present study, with the most potent activity shown by chrysin with and IC_{50} value of 0.2 μM followed by apigenin (IC_{50} =1.35 μM) and luteolin (IC_{50} =13 μM) (Lee *et al.*, 1998). The study even revealed a structure-activity relationship and it was concluded that the presence of multiple hydroxyl groups (preferably two in the positions 5 and 7) on the flavone skeleton plays a crucial role in the inhibitory capacity (Lee *et al.*, 1998; Hodek *et al.*, 2002), a structural criterion that is present in all flavonoids tested in the present thesis. However, whether such an inhibitory effect of the different flavonoids on 1′-hydroxylation of estragole and methyleugenol will actually occur remains to be established.

Overall, in vitro results with the different dietary flavonoids show a potent SULT inhibition in incubations with liver fractions accompanied by a potent inhibition of DNA adduct formation in intact cellular systems and a shift of metabolism from bioactivation of estragole to detoxification. The question that may be raised subsequently is whether in vivo concentrations of these SULT-inhibiting flavonoids may reach levels high enough to inhibit SULT activity and this will be discussed in more detail in the following section.

In vivo SULT inhibition by selected flavonoids

In vivo activity of the different flavonoid-type SULT inhibitors will depend largely on their levels in the diet, their absorption, distribution, metabolism and excretion (ADME) characteristics in the body and thus their ultimate intracellular and circulating concentrations or what is usually referred to as their bioavailability.

In chapter 4 and 6, we presented data showing that nevadensin is able to inhibit SULT activity in vivo. This was demonstrated by a significant inhibition of SULT-mediated formation of DNA adducts in the liver of rats exposed to estragole (chapter 4) or to methyleugenol (chapter 6) in the presence of nevadensin dosed simultaneously at a molar ratio reflecting the natural occurrence in basil. Nevadensin may be an effective in vivo SULT inhibitor because it possesses the structural requirements for effective SULT inhibitory activity described above (Morimitsu *et al.*, 2004). Furthermore methylated flavones such as nevadensin have been demonstrated to be much more metabolically stable and have a higher intestinal absorption than their unmethylated analogues (Wen and Walle, 2006; Walle, 2007).

It is important to note that in these in vivo studies both alkenylbenzene (e.g. estragole or methyleugenol) and nevadensin were dosed to rats at high dose levels in order to allow the detection of the DNA adduct formation and its inhibition by nevadensin. This

raises the question of whether a SULT inhibiting effect can also take place at realistic dietary dose levels of the alkenylbenzenes and nevadensin or other SULT inhibiting flavonoids. In this regard, we have presented data in chapter 3 showing that different flavonoids such as quercetin, apigenin, genistein, daidzein, and hesperetin may reach levels in the plasma higher than their *K*i value detected in our in vitro experiments or reported in literature and thus may inhibit SULT activity in vivo. However, reported plasma concentrations often reflect total flavonoid concentrations including both conjugated and unconjugated forms instead of the concentrations of the aglycon, whereas the active form of the flavonoid exerting SULT inhibition tested in most in vitro experiments is the aglycone. This is important given especially that flavonoid conjugation by the three main types of conjugation; methylation, sulfonation, and glucuronidation is highly efficient, and aglycones are generally either absent in blood or present in low concentrations after consumption of nutritional doses (Manach *et al.*, 2004). Although so far most of these flavonoid conjugates have not been tested for their SULT inhibiting activity and their potential to inhibit SULT activity remains to be investigated, one study reported an inhibitory effect for daidzein-4′-O-sulfate and daidzein-7,4′-di-O-sulfate on phenol SULT activity with IC_{50} values >100 μM (Eaton *et al.*, 1996). Also, experiments investigating the activity of most dietary flavonoids on SULT activity in vivo are lacking.

In one small pilot study to determine whether ingestion of 500 mL soy milk would affect SULT1A1 activity in human it was shown that daidzein resulted in a significant reduction in the SULT1A1 activity in women "at risk" of breast cancer as compared with controls (Waring *et al.*, 2008). This has been ascribed to the fact that people "at risk" of breast cancer have a polymorphism in SULT1A1 which makes the enzyme more susceptible to inhibition by flavonoids (Waring *et al.*, 2008). Furthermore, determination of the actual bioavailability of polyphenol aglycons as well as their metabolites in tissues may be much more important than knowledge of their plasma concentrations. For example, the proportion of free aglycone in some tissues may be higher than that in blood, as has been demonstrated in the case of genistein in rats (Wang *et al.*, 2002). This may be explained by specific uptake of the aglycone or intracellular deconjugation (e.g. quercetin) (Lee-Hilz *et al.*, 2008). Still, in vivo data about intracellular levels of flavonoids are very scarce and not consistent enough to give a clear view of what occurs inside the cells, and additional studies are certainly needed (Manach *et al.*, 2004).

Therefore, to evaluate the quantitative dose and species dependent effects of SULT inhibition by the dietary flavonoids on the formation of the ultimate carcinogenic metabolite, 1′-sulfooxyestragole, the estragole PBBK models in rat and human were used as described in chapters 2 and 3. As a first approximation the SULT inhibitor(s) were assumed to follow a similar time-dependent concentration curve in the liver of rat and human as estragole and different scenarios of uptake and bioavailability ranging between 1% to 100% were simulated. PBBK model predictions suggested that inhibition of the formation of 1′-sulfooxyestragole at a realistic dietary dose of estragole of 0.01 mg/kg bw/day together with a mixture of the most common dietary flavonoids each at its EDI level does not exceed 0.2% for rat and 0.5% for human when assuming 1%

uptake of the flavonoid mixture. Even at levels of the flavonoid quercetin present in one tablet containing 500 mg and thus resulting in 8.3 mg/kg bw/day of quercetin in a 60 kg individual together with 0.01 mg/kg bw of estragole the inhibition is still limited amounting to 3% in rat and 9% for human assuming 1% uptake of quercetin.

One may ask whether the 1% uptake scenario represents a realistic uptake scenario for flavonoids. In this regard, we have shown in chapter 4 that PBBK predictions using the 1% uptake scenario for nevadensin is overestimating inhibition of DNA adduct formation at levels of estragole and nevadensin used by our in vivo study compared to the observed value by 12-fold. Obviously modelling nevadensin bioavailability and kinetics by a nevadensin PBBK sub-model as done in chapter 4 and 6 presents a better description of the in vivo situation.

Overall, given the lack of in vivo data about; i) levels of free aglycone concentrations in plasma and or in tissues after realistic dietary dose levels of flavonoids and ii) the significance of deconjugation of flavonoids in important tissues such as liver and intestine representing target organs for alkenylbenzenes and flavonoids, it remains difficult to draw a definite conclusion about SULT inhibiting potency of dietary flavonoids in vivo and thus more in vivo experiments are required to assess the effect of some dietary flavonoids on SULT-mediated pathways in vivo.

Association between 1′-sulfooxy metabolite, DNA adduct formation, hepatic foci and carcinogenicity of alkenylbenzenes

As we presented earlier, in vitro results with the different dietary flavonoids show a potent SULT inhibition in incubations with liver fractions accompanied by a potent inhibition of alkenylbenzene DNA adduct formation in intact cellular systems and a shift of metabolism from bioactivation of the alkenylbenzene to its detoxification (chapter 2 and 3). Also, a significant in vivo inhibition of SULT-mediated formation of DNA adducts occurred in the liver of rats exposed to estragole (chapter 4) or to methyleugenol (chapter 6) in the presence of nevadensin dosed simultaneously at a molar ratio reflecting the natural occurrence in basil.

The estragole DNA adduct (E-3′-N^2-dGuo) measured in our in vitro (chapter 2 and 3) and in vivo experiments (chapter 4) is the major DNA adduct formed in several in vitro and rodent studies with estragole (Phillips *et al.*, 1981; Wiseman *et al.*, 1985; Punt *et al.*, 2007). Also, the methyleugenol DNA adduct (ME-3′-N^2-dGuo) measured in our in vitro (chapter 5) and in vivo experiments (chapter 6) was the major adduct formed in in vitro studies, in mice and also in human (Herrmann *et al.*, 2012; Herrmann *et al.*, 2013). Some other studies reported E-3′-N^6-dA to be the major adduct formed in rodents (Ishii *et al.*, 2011; Suzuki *et al.*, 2012).

Given that in addition to the major adduct identified in our studies also other DNA adducts may be formed it is of interest to consider wether the adducts detected in our experiments are mutagenic adducts and thus represent a good indication for carcinogenicity of estragole and methyleugenol. In this regard, a recent study by Herrmann *et al.* (2012) has shown that different isomeric hydroxylated metabolites

of methyleugenol form the same DNA adducts in S. *typhimurium* TA100-hSULT1A1, namely, ME-3′-N^2-dGuo and ME-3′-N^6-dA. When the strain TA100-hSULT1A1 was treated with varying doses of the hydroxylated metabolites of methyleugenol the levels of these two adducts increased continuously with the dose similar to the dose response curve for mutagenicity. In addition, adduct levels correlated with the mutagenic effect induced. Moreover, ME-3′-N^2-dGuo was detected as the major DNA adduct whereas the amount of ME-3′-N^6-dA formed was only 1.5-3 % of the amount of ME-3′-N^2-dGuo detected suggesting that the dG adduct is the major adduct contributing to the mutagenic effects of methyleugenol (Herrmann *et al.*, 2012).

Overall, in the present study, as a first approximation, we assumed that nevadensin-mediated inhibition of formation of E-3′-N^2-dGuo or ME-3′-N^2-dGuo would reflect the reduction in all estragole and methyleugenol DNA adducts and that reduction in DNA adducts would be linearly proportional to the reduction in tumour incidence. This is based on the fact that all estragole or methyleugenol DNA adducts will result from a chemical reaction with their 1′-sulfooxy metabolite which is the ultimate carcinogenic metabolite and that it is the formation of these 1′-sulfooxy metabolites that is inhibited by nevadensin. If all adducts would be reduced to a similar extent one can subsequently assume that the resulting mutagenicity and carcinogencity would be reduced to a similar extent as well, even when different adducts would display different mutagenicity.

In this regard, it has been shown in rodent experiments that co-administration of the specific SULT inhibitor pentachlorophenol (PCP) resulted in a potent reduction in cytosolic SULT activity for 1′-hydroxysafrole in both mouse and rat liver, in a potent decrease in the level of adducts formed from 1′-hydroxysafrole in hepatic DNA and RNA and, more interestingly, in a potent inhibition of hepatic tumour induction in mice upon long-term dietary administration of safrole or its metabolite, 1′-hydroxysafrole together with PCP (Boberg *et al.*, 1983). This clearly proves the role of the 1′-sulfooxy metabolite and the SULT-mediated DNA adduct formation in hepatotoxicicity and hepatocarcinogenicity of alkenylbenzenes.

In addition to detecting the effects of nevadensin on DNA adduct formation, in chapter 6 we tried to move one step further towards endpoints that are even closer to initiation of carcinogenesis than DNA adduct formation, namely, hepatocellular proliferation and formation of hepatocellular altered foci (HAF). The results obtained in chapter 6 clearly show that co-exposure to methyleugenol and nevadensin resulted not only in a significant inhibition of methyleugenol DNA adduct formation in the liver but also in a significant proportional inhibition of induction of hepatocellular altered foci (HAF), representing an indicator for initiation of neoplasia. Although HAF lesions greatly exceed the number of neoplasms, they were shown to precede the development of neoplasms and are quantitatively related to neoplasms (Williams, 1980; Williams, 1982).

Overall, results of the present thesis clearly show that the potent in vivo inhibitory activity of nevadensin on SULT activity and on alkenylbenzene DNA adduct formation is accompanied by a potent in vivo inhibition in early markers of carcinogenesis. Given the role of the 1′-sulfooxy metabolite of alkenylbenzenes in the hepatotoxicity

and carcinogenicity of alkenylbenzenes (Boberg *et al.*, 1983) and the established quantitative relationship between HAF formation and neoplasm (Williams, 1980; Bannasch, 1986; Williams, 1989; Enzmann *et al.*, 1998; Williams, 1998; Williams, 1999), the results of the present thesis suggest that in high dose rodent bioassays a reduction in the incidence of liver tumour formation is expected in when alkenylbenzenes would be dosed simultaneously with nevadensin.

Implications for risk assessment

Risk assessment for the alkenylbenzenes estragole and methyleugenol can be based on applying the so-called margin of exposure (MOE) approach which is using data from carcinogenicity studies with rodents where the compounds (estragole or methyleugenol) are dosed at high levels in their pure form. The MOE approach is suggested by EFSA for the risk assessment of compounds that are both genotoxic and carcinogenic and can be used to set priorities in risk management (EFSA, 2005). 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). Using reported $BMDL_{10}$ values for estragole and methyleugenol (van den Berg *et al.*, 2011) and EDI values of 0.01 for estragole and 0.014 mg/kg bw/day for methyleugenol, representing intake resulting mainly from herbs, spices and their essential oils (Smith *et al.*, 2002) the calculated MOE value for estragole varies between 350 and 650 and that for methyleugenol between 3,500 and 5,300 for female rats and between about 1,100 and 2,400 for male rats (Al-Subeihi *et al.*, 2013).

The question that can be raised now is how to integrate the current results on nevadensin-mediated modulation of the bioactivation of estragole (chapter 4) and methyleugenol (chapter 6) in the risk assessment of these two compounds. In chapters 4 and 5 we refined the $BMDL_{10}$ and subsequently calculated MOE values for both estragole and methyleugenol taking into account what would happen when nevadensin would have been co-administered with estragole or methyleugenol in those rodent carcinogenicity studies.

To this end the estragole and methyleugenol PBBK models in male rat were connected with a newly developed nevadensin PBBK model which was validated based on our in vivo study in which DNA-adducts were quantified in the liver of rats simultaneously exposed to estragole and nevadensin in male rat. These binary PBBK models were used to calculate the possible reduction in E-3′-N^2-dGuo formation at the estragole dose levels used in the study of Miller *et al*. (1983) or in ME-3′-N^2-dGuo formation at the methyleugenol dose levels used in the NTP study, when nevadensin would be co administered with estragole or methyleugenol at a molar ratio of 1:0.25 for estragole or 1:0.20 for methyleugenol reflecting their natural occurrence in basil (Smith *et al.*, 2002; Alhusainy *et al.*, 2012). As a first approximation we assumed that reduction in alkenylbenzene DNA adduct levels would be proportional to reduction in the incidence of hepatomas and then we calculated a refined $BMDL_{10}$ value that was subsequently used to estimate a refined MOE. The refined MOE for estragole increased from 350 and 650

for data from studies testing the pure compound to 4,300 and 7,350 when the rodent study would have been performed in the presence of nevadensin. For methyleugenol the MOE values increased from 3,500 and 5,300 for female rats and from about 1,100 and 2,400 for male rats based on data from studies testing the pure compound to about 11,000 and 18,000 for female rats and about 9,200 and 10,000 for male rats when the rodent study would have been performed in the presence of nevadensin.

These updated MOE levels for estragole and methyleugenol clearly point at a lower priority for risk management when the rodent bioassays and the subsequent risk assessment would have been performed taking the matrix effect into account, than when the MOE calculation is based on the tumour data obtained with pure compounds.

FUTURE PERSPECTIVES AND CONCLUSIONS

The present thesis presents data suggesting that that the likelihood of bioactivation and subsequent adverse effects may be lower when the alkenylbenzene is consumed in a matrix containing SULT inhibitors compared to experiments using pure alkenylbenzenes as single compounds. However, a question remaining is whether an in vivo effect of SULT inhibiting flavonoids can be expected to take place at realistic dietary intake levels of these flavonoids despite the high conjugation efficiency for flavonoids in vivo. Up to date, the in vivo activity of natural SULT inhibitors has been only proven in vitro (table 2) with the exception of nevadensin which was shown by our studies to be able to inhibit SULT activity in vivo when dosed with estragole (Alhusainy *et al.*, 2013) or methyleugenol (Al-Subeihi *et al.*, 2013) at high dose levels. Therefore, it seems essential to perform in vivo experiments in animals to investigate the potency of the other dietary SULT inhibiting flavonoids at realistic dose levels of the SULT inhibitors including dose levels similar to levels present in food or in supplements, an objective that can be tested in rodent bioassays given that DNA adduct levels formed at dietary levels of methyleugenol for example can be readily quantified in mice and in human (Herrmann *et al.*, 2013).

Moreover, the present thesis focused on a single aspect of the matrix effect in which the toxicokinetics of the compound of concern (such as the alkenylbenzenes) is influenced by some components (such as dietary flavonoids) in the matrix. This interaction occurred at the level of the SULT-mediated pathway involved in formation of the ultimate carcinogenic metabolite of alkenylbenzenes, the 1'-sulfooxy metabolite, which was shown to be inhibited by the various flavonoids identified. However, constituents of the matrix might also affect the bioactivation and resulting toxicity of alkenylbenzenes through other pathways and this was not addressed in the present thesis and can be addressed in future research to further refine the risk assessment of alkenylbenzenes. Such factors are for example the effect of the complex matrix, or one of its constituent, on the absorption of both the alkenylbenzene and/or the SULT inhibitors in the gastrointestinal tract and/or on their release from the matrix.

The matrix effect might also be studied to a further extent by analysing early markers of hepatocarcinogenesis in some specialised eating groups, such as for example frequent pesto eaters since they have one of the highest levels of estragole and

methyleugenol intake (Smith *et al.*, 2002). Useful parameters that could be investigated in such a human study may include for example quantifying DNA adduct levels in white blood cells (WBC) in human blood samples. A previous study has reported an increase in WBC DNA adducts levels in rats orally dosed with methyleugenol for 16 weeks and the authors concluded that this might be a useful biomarker since the DNA of WBC can be readily obtained and monitored in humans (Williams *et al.*, 2013). Also, quantification of urine and plasma levels of alkenylbenzenes, nevadensin and their metabolites would be of used in order to judge if these compounds reach levels that match the PBBK based predictions and/or relevant *K*i values.

Not only in vivo studies are required but also in vitro studies are essential to investigate remaining issues of interest such as the effect of the different dietary flavonoids on the hydroxylation of alkenylbenzenes into their proximate carcinogen, the 1′-hydroxy metabolite. An inhibitory effect by the flavonoids on this 1′-hydroxylation may reduce the ultimate levels of DNA adducts.

Also, the inhibitory activity of flavonoid conjugates on SULT enzyme activity as well as on 1′-hydroxylation is worth further studies, especially given the fact that most flavonoid inhibitors will be metabolised to their corresponding methyl, glucuronide and/or sulfate conjugates and these forms may be the major forms in which they are present in the systemic circulation. However, up to date identification of circulating conjugates has been undertaken for only a few flavonoids (Manach *et al.*, 2004). Furthermore, such studies would also require the identification of not only the nature and number of conjugation groups but also the positions of these groups on the flavonoid structure because these positions can affect the biological properties of the conjugates (Day *et al.*, 2000). It also requires to identify the nature of the tissue metabolites which may be different from that of blood metabolites because of the specific tissue uptake or elimination or because of intracellular metabolism (Manach *et al.*, 2004).

Finally, although the addition of estragole and methyleugenol as flavouring substances in foodstuffs has been prohibited within the European Union since September 2008 (European Commission, 2008). A recent publication detected the presence of methyleugenol DNA adducts in surgical human liver samples from 30 subjects at maximum and median levels of 37 and 13 per 10^8 nucleosides which was found to be close to DNA adduct levels detected in rodents ranging between 50–5500 adducts per 10^8 nucleosides after repeated treatment by different rodent carcinogens at their TD_{50} (Herrmann *et al.*, 2013) According to the same study, similar levels of adducts of 7 and 22 per 10^8 nucleosides were detected in the liver of wild-type mice given single oral doses of 0.05 and 0.5 mg/kg bw of methyleugenol respectively. The authors conclude that it is plausible that the adducts detected in the human samples were indeed produced by methyleugenol present in the diet given that the mean daily intake of methyleugenol from food for Europe is 0.19 mg/kg bw (SCF, 2001b), a value that is in the range of dose levels used by the mice experiment. The DNA adduct levels detected in human liver were in line with the values predicted by our human PBBK model which amounted to 14.2 and 25.4 ME-3′-N^2-dGuo DNA adducts per 10^8

nucleotides at oral doses of 0.05 and 0.5 mg/kg bw of methyleugenol respectively assuming nevadensin is also present at a molar ratio of methyleugenol to nevadensin of 1:0.20 to reflect their occurrence in basil. In the absence of nevadensin our human PBBK model predicts 14.7 and 34.1 ME-3'-N^2-dGuo DNA adducts per 10^8 nucleotides at 0.05 and 0.5 mg/kg bw/day respectively. These PBBK model predictions suggest that a limited matrix effect may be relevant at realistic dietary exposure levels.

OVERALL CONCLUSION

Overall, given the role of the SULT-mediated formation of alkenylbenzene DNA adducts in the hepatocarcinogenicity of alkenylbenzenes in rodents, the results of the present study indicate that the likelihood of bioactivation and subsequent adverse effects may be lower when the alkenylbenzene is consumed in a matrix containing SULT inhibitors such as nevadensin compared to experiments using pure alkenylbenzene as a single compound. The results obtained in the present thesis suggest that the consequences of the in vivo matrix effect are significant when estragole or methyleugenol would be tested in rodent bioassays at high dose levels in the presence of nevadensin at ratios detected in basil, thereby increasing $BMDL_{10}$ and resulting MOE values substantially in a subsequent risk assessment.

However, the results also indicate that matrix effects may be lower at daily human dietary exposure levels of estragole or methyleugenol and nevadensin resulting from basil consumption. Also, matrix effects of other SULT inhibiting dietary flavonoids seem to be limited even at high exposure levels of these flavonoids upon supplement use, which can be ascribed to higher *K*i values for SULT inhibition by these flavonoids that are 2-3 orders of magnitude higher than the *K*i for nevadensin. This indicates that the importance of a matrix effect for risk assessment of individual compounds requires analysis of dose dependent effects on the interactions detected, an objective that can be achieved by using PBBK modeling.

So far, the effect of nevadensin in reducing the alkenylbenzene bioactivation in vivo has been established for the alkenylbenzenes estragole and methyleugenol only. However, the results of the present work can also be extended to other alkenylbenzenes. This is because the SULT inhibition by nevadensin is non-competitive in nature (Alhusainy *et al.*, 2010; Alhusainy *et al.*, 2013) and thus by definition should be evident regardless of the substrate (Simmons, 1996).

Overall, the present study provides an example of an approach that can be used to characterise dose- species- and inter-individual differences as well as matrix effects in the risk assessment of food-borne toxicants present in botanicals (e.g. alkenylbenzenes). In this approach the most important toxicokinetic interactions are addressed using an integrated strategy of in vitro, in vivo and PBBK modeling approaches.

REFERENCES

1. Al-Subeihi, A. A. A., Alhusainy, W., Paini, A., Punt, A., J, V., Van Den Berg, J. H. J., and Rietjens, I. M. C. M. (2013). Inhibition of methyleugenol bioactivation by the herb-based constituent nevadensin and prediction of possible in vivo consequences using physiologically based kinetic modeling. *Food and Chemical Toxicology* **59**, 564-571.
2. Alhusainy, W., Paini, A., Punt, A., Louisse, J., Spenkelink, A., Vervoort, J., Delatour, T., Scholz, G., Schilter, B., Adams, T., van Bladeren, P. J., and Rietjens, I. M. C. M. (2010). Identification of nevadensin as an important herb-based constituent inhibiting estragole bioactivation and physiology-based biokinetic modeling of its possible in vivo effect. *Toxicology and Applied Pharmacology* **245**, 179-190.
3. Alhusainy, W., Paini, A., Van Den Berg, J. H. J., Punt, A., Scholz, G., Schilter, B., van Bladeren, P. J., Taylor, S., Adams, T. B., and Rietjens, I. M. C. M. (2013). In vivo validation and physiologically based modeling of the inhibition of SULT-mediated estragole DNA adduct formation in the liver of male Sprague-Dawley rats by the basil flavonoid nevadensin. *Molecular Nutrition and Food Research* DOI 10.1002/mnfr.201300144, http://eproof.aptaracorp.com/powerproof2/adp.do?aid=1357984731012469844.
4. Alhusainy, W., van den Berg, S. J. P. L., Paini, A., Campana, A., Asselman, M., Spenkelink, A., Punt, A., Scholz, G., Schilter, B., Adams, T. B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2012). Matrix modulation of the bioactivation of estragole by constituents of different alkenylbenzene-containing herbs and spices and physiologically based biokinetic modeling of possible in vivo Effects. *Toxicological Sciences* **129**, 174-187.
5. Arai, Y., Watanabe, S., Kimira, M., Shimoi, K., Mochizuki, R., and Kinae, N. (2000). Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. *Journal of Nutrition* **130**, 2243-2250.
6. Bamforth, K. J., Dalgliesh, K., and Coughtrie, M. W. H. (1992). Inhibition of human liver steroid sulfotransferase activities by drugs: A novel mechanism of drug toxicity? *European Journal of Pharmacology - Environmental Toxicology and Pharmacology Section* **228**, 15-21.
7. Bamforth, K. J., Jones, A. L., Roberts, R. C., and Coughtrie, M. W. H. (1993). Common food additives are potent inhibitors of human liver 17α-ethinyloestradiol and dopamine sulphotransferases. *Biochemical Pharmacology* **46**, 1713-1720.
8. Bannasch, P. (1986). Preneoplastic lesions as end points in carcinogenicity testing. I. Hepatic preneoplasia. *Carcinogenesis* **7**, 689-695.
9. Boberg, E. W., Miller, E. C., and Miller, J. A. (1983). Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1'-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1'-hydroxysafrole in mouse liver. *Cancer Research* **43**, 5163-5173.
10. Buening, M. K., Chang, R. L., Huang, M. T., Fortner, J. G., Wood, A. W., and Conney, A. H. (1981). Activation and inhibition of benzo(a)pyrene and aflatoxin B 1 metabolism in human liver microsomes by naturally occurring flavonoids. *Cancer Research* **41**, 67-72.
11. Coward, L., Barnes, N. C., Setchell, K. D. R., and Barnes, S. (1993). Genistein, daidzein, and their beta-glycoside conjugates - Antitumor isoflavones in soybean foods from American and Asian diets. *J.AGRIC. FOOD CHEM.* **41**, 1961-1967.
12. Day, A. J., Bao, Y., Morgan, M. R. A., and Williamson, G. (2000). Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radical Biology and Medicine* **29**, 1234-1243.
13. De Vries, J. H. M., Janssen, P. L. T. M. K., Hollman, P. C. H., Van Staveren, W. A., and Katan, M. B. (1997). Consumption of quercetin and kaempferol in free-living subjects eating a variety of diets. *Cancer Letters* **114**, 141-144.
14. Dybing, E., Doe, J., Groten, J., Kleiner, J., O'Brien, J., Renwick, A. G., Schlatter, J., Steinberg, P., Tritscher, A., Walker, R., and Younes, M. (2002). Hazard characterisation of chemicals in food and diet: Dose response, mechanisms and extrapolation issues. *Food and Chemical Toxicology* **40**, 237-282.

15. Eaton, E. A., Walle, U. K., Lewis, A. J., Hudson, T., Wilson, A. A., and Walle, T. (1996). Flavonoids, potent inhibitors of the human P-form phenolsulfotransferase: Potential role in drug metabolism and chemoprevention. *Drug Metabolism and Disposition* **24**, 232-237.
16. EFSA (2005). Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. *EFSA Journal* **282**, 1-31.
17. Enzmann, H., Bomhard, E., Iatropoulos, M., Ahr, H. J., Schlueter, G., and Williams, G. M. (1998). Short- and intermediate-term carcinogenicity testing - A Review. Part 1: The prototypes mouse skin tumour assay and rat liver focus assay. *Food and Chemical Toxicology* **36**, 979-995.
18. European Commission (EC) (2008). Regulation (EC) No 1334/2008 of the Europian Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC.
19. Fennell, T. R., Wiseman, R. W., Miller, J. A., and Miller, E. C. (1985). Major role of hepatic sulfotransferase activity in the metabolic activation, DNA adduct formation, and carcinogenicity of 1′-hydroxy-2′,3′-dehydroestragole in infant male C57BL/6J x C3H/HeJ F1 mice. *Cancer Research* **45**, 5310-5320.
20. Garcia-Closas, R., Gonzalez, C. A., Agudo, A., and Riboli, E. (1999). Intake of specific carotenoids and flavonoids and the risk of gastric cancer in Spain. *Cancer Causes and Control* **10**, 71-75.
21. Ghazali, R. A., and Waring, R. H. (1999). The effects of flavonoids on human phenolsulphotransferases: Potential in drug metabolism and chemoprevention. *Life Sciences* **65**, 1625-1632.
22. Grayer, R. J., Vieira, R. F., Price, A. M., Kite, G. C., Simon, J. E., and Paton, A. J. (2004). Characterization of cultivars within species of Ocimum by exudate flavonoid profiles. *Biochemical Systematics and Ecology* **32**, 901-913.
23. Harris, R. M., Wood, D. M., Bottomley, L., Blagg, S., Owen, K., Hughes, P. J., Waring, R. H., and Kirk, C. J. (2004). Phytoestrogens are potent inhibitors of estrogen sulfation: Implications for breast cancer risk and treatment. *Journal of Clinical Endocrinology and Metabolism* **89**, 1779-1787.
24. Herrmann, K., Engst, W., Appel, K. E., Monien, B. H., and Glatt, H. (2012). Identification of human and murine sulfotransferases able to activate hydroxylated metabolites of methyleugenol to mutagens in Salmonella typhimurium and detection of associated DNA adducts using UPLC-MS/MS methods. *Mutagenesis* **27**, 453-462.
25. Herrmann, K., Schumacher, F., Engst, W., Appel, K. E., Klein, K., Zanger, U. M., and Glatt, H. (2013). Abundance of DNA adducts of methyleugenol, a rodent hepatocarcinogen, in human: Liver samples. *Carcinogenesis* **34**, 1025-1030.
26. Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B., and Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* **342**, 1007-1011.
27. Hodek, P., Trefil, P., and Stiborová, M. (2002). Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. *Chemico-Biological Interactions* **139**, 1-21.
28. Ishii, Y., Suzuki, Y., Hibi, D., Jin, M., Fukuhara, K., Umemura, T., and Nishikawa, A. (2011). Detection and quantification of specific DNA adducts by liquid chromatography-tandem mass spectrometry in the livers of rats given estragole at the carcinogenic dose. *Chemical Research in Toxicology* **24**, 532-541.
29. JECFA (2008). Sixty-ninth Meeting, Rome, Italy, 17–26 June 2008. (JECFA, Ed.), (http://www.who.int/entity/ipcs/food/jecfa/summaries/summary69.pdf).
30. Jeurissen, S. M. F., Punt, A., Boersma, M. G., Bogaards, J. J. P., Fiamegos, Y. C., Schilter, B., Van Bladeren, P. J., Cnubben, N. H. P., and Rietjens, I. M. C. M. (2007). Human cytochrome P450 enzyme specificity for the bioactivation of estragole and related alkenylbenzenes. *Chemical Research in Toxicology* **20**, 798-806.
31. Jeurissen, S. M. F., Punt, A., Delatour, T., and Rietjens, I. M. C. M. (2008). Basil extract inhibits the sulfotransferase

mediated formation of DNA adducts of the procarcinogen 1 '-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. *Food and Chemical Toxicology* **46**, 2296-2302.

32. Justesen, U., Knuthsen, P., and Leth, T. (1997). Determination of plant polyphenols in Danish foodstuffs by HPLC-UV and LC-MS detection. *Cancer Letters* **114**, 165-167.
33. Lee-Hilz, Y. Y., Stolaki, M., van Berkel, W. J. H., Aarts, J. M. M. J. G., and Rietjens, I. M. C. M. (2008). Activation of EpRE-mediated gene transcription by quercetin glucuronides depends on their deconjugation. *Food and Chemical Toxicology* **46**, 2128-2134.
34. Lee, H., Yeom, H., Kim, Y. G., Yoon, C. N., Jin, C., Choi, J. S., Kim, B. R., and Kim, D. H. (1998). Structure-related inhibition of human hepatic caffeine N3-demethylation by naturally occurring flavonoids. *Biochemical Pharmacology* **55**, 1369-1375.
35. Lugasi, A., Hóvári, J., Sági, K. V., and Bíró, L. (2003). The role of antioxidant phytonutrients in the prevention of diseases. *Acta Biologica Szegediensis* **47**, 119-125.
36. Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jiménez, L. (2004). Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition* **79**, 727-747.
37. Matsui, M., Takahashi, M., Miwa, Y., Motoyoshi, Y., and Homma, H. (1995). Structure-activity relationships of alkylamines that inhibit rat liver hydroxysteroid sulfotransferase activities in vitro. *Biochemical Pharmacology* **49**, 739-741.
38. Mesía -Vela, S., and Kauffman, F. C. (2003). Inhibition of rat liver sulfotransferases SULT1A1 and SULT2A1 and glucuronosyltransferase by dietary flavonoids. *Xenobiotica* **33**, 1211-1220.
39. Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. (1983). Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Research* **43**, 1124-1134.
40. Morimitsu, Y., Sugihara, N., and Furuno, K. (2004). Inhibitory effect of flavonoids on sulfo- and glucurono-conjugation of acetaminophen in rat cultured hepatocytes and liver subcellular preparations. *Biological and Pharmaceutical Bulletin* **27**, 714-717.
41. Mullie, P., Clarys, P., Deriemaeker, P., and Hebbelinck, M. (2008). Estimation of daily human intake of food flavonoids. *International Journal of Food Sciences and Nutrition* **59**, 291-298.
42. NTP (2000). National Toxicology Program on toxicology and carcinogenesis studies of Methyleugenol (CAS NO. 93-15-12) in F344/N rats and B6C3F1 mice (Gavage Studies). DRAFT NTP-TR-491; NIH Publication No. 98-3950., 1-412.
43. Phillips, D. H., Miller, J. A., Miller, E. C., and Adams, B. (1981). Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1'-hydroxyestragole. *Cancer Research* **41**, 176-186.
44. Phillips, D. H., Reddy, M. V., and Randerath, K. (1984). 32P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. *Carcinogenesis* **5**, 1623-1628.
45. Punt, A., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J., and Rietjens, I. M. C. M. (2007). Tandem mass spectrometry analysis of N-2-(trans-isoestragol-3'-yl)-2'-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1'-hydroxyestragole. *Chemical Research in Toxicology* **20**, 991-998.
46. Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. (1984). ^{32}P-post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis* **5**, 1613-1622.
47. Sampson, L., Rimm, E., Hollman, P. C. H., De Vries, J. H. M., and Katan, M. B. (2002). Flavonol and flavone intakes in US health professionals. *Journal of the American Dietetic Association* **102**, 1414-1420.
48. SCF (2001a). Opinion of the Scientific Committee on Food on Estragole (1-allyl-4-methoxybenzene). European Commission, Health and Consumer Protection Directorate. General, Report

Series 10, Directorate C, Scientific Opinions, Brussels, Belgium. Obtained January 10, 2008, at http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf.

49. SCF (2001b). Opinion of the Scientific Committee on Food on methyleugenol (4-allyl-1,2-dimethoxybenzene.
50. Simmons, J. E. (1996). Application of physiologically based pharmacokinetic modelling to combination toxicology. *Food and Chemical Toxicology* **34**, 1067-1073.
51. Smith, B., Cadby, P., Leblanc, J. C., and Setzer, R. W. (2010). Application of the margin of exposure (MoE) approach to substances in food that are genotoxic and carcinogenic. Example: Methyleugenol, CASRN: 93-15-2. *Food and Chemical Toxicology* **48**, S89-S97.
52. Smith, R. L., Adams, T. B., Doull, J., Feron, V. J., Goodman, J. I., Marnett, L. J., Portoghese, P. S., Waddell, W. J., Wagner, B. M., Rogers, A. E., Caldwell, J., and Sipes, I. G. (2002). Safety assessment of allylalkoxybenzene derivatives used as flavouring substances - Methyl eugenol and estragole. *Food and Chemical Toxicology* **40**, 851-870.
53. Suzuki, Y., Umemura, T., Ishii, Y., Hibi, D., Inoue, T., Jin, M., Sakai, H., Kodama, Y., Nohmi, T., Yanai, T., Nishikawa, A., and Ogawa, K. (2012). Possible involvement of sulfotransferase 1A1 in estragole-induced DNA modification and carcinogenesis in the livers of female mice. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis* **749**, 23-28.
54. van den Berg, S. J. P. L., Restani, P., Boersma, M. G., Delmulle, L., and Rietjens, I. M. C. M. (2011). Levels of Genotoxic and Carcinogenic Compounds in Plant Food Supplements and Associated Risk Assessment. *Food and Nutrition Sciences* **2**, 989-1010.
55. Walle, T. (2007). Methylation of dietary flavones greatly improves their hepatic metabolic stability and intestinal absorption. *Molecular Pharmaceutics* **4**, 826-832.
56. Wang, J., Eltoum, I. E., and Lamartiniere, C. A. (2002). Dietary genistein suppresses chemically induced prostate cancer in Lobund-Wistar rats. *Cancer Letters* **186**, 11-18.
57. Waring, R. H., Ayers, S., Gescher, A. J., Glatt, H. R., Meinl, W., Jarratt, P., Kirk, C. J., Pettitt, T., Rea, D., and Harris, R. M. (2008). Phytoestrogens and xenoestrogens: The contribution of diet and environment to endocrine disruption. *Journal of Steroid Biochemistry and Molecular Biology* **108**, 213-220.
58. Wen, X., and Walle, T. (2006). Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug Metabolism and Disposition* **34**, 1786-1792.
59. Williams, G. M. (1980). The pathogenesis of rat liver cancer caused by chemical carcinogens. *Biochimica et Biophysica Acta* **605**, 167-189.
60. Williams, G. M. (1982). Phenotypic properties of preneoplastic rat liver lesions and applications to detection of carcinogens and tumor promoters. *Toxicologic Pathology* **10**, 3-10.
61. Williams, G. M. (1989). The significance of chemically-induced hepatocellular altered foci in rat liver and application to carcinogen detection. *Toxicologic Pathology* **17**, 663-672.
62. Williams, G. M. (1998). Phenotypic properties of preneoplastic rat liver lesions and applications to detection of carcinogens and tumor promoters. *Toxicologic Pathology* **26**, 452-453.
63. Williams, G. M. (1999). Chemically induced preneoplastic lesions in rodents as indicators of carcinogenic activity. *IARC scientific publications*, 185-202.
64. Wiseman, R. W., Fennell, T. R., Miller, J. A., and Miller, E. C. (1985). Further characterization of the DNA adducts formed by electrophilic esters of the hepatocarcinogens 1′-hydroxysafrole and 1′-hydroxyestragole in vitro and in mouse liver in vivo, including new adducts at C-8 and N-7 of guanine residues. *Cancer Research* **45**, 3096-3105.
65. Wiseman, R. W., Miller, E. C., Miller, J. A., and Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F1 mice. *Cancer Research* **47**, 2275-2283.

8

Summary

SUMMARY

Alkenylbenzenes such as estragole and methyleugenol are common components of spices and herbs such as tarragon, basil, fennel, mace, allspice, star anise and anise and their essential oils (Smith *et al.*, 2002). There is an interest in the safety evaluation of alkenylbenzenes because these compounds can induce hepatic tumours in rodents when dosed orally at high dose levels (Miller *et al.*, 1983; NTP, 2000). Based on the rodent studies with estragole, methyleugenol and structurally related alkenylbenzenes like safrole the hepatocarcinogenicity of alkenylbenzenes is ascribed to their bioactivation by cytochrome P450 enzymes leading to the formation of the proximate carcinogenen, the 1′-hydroxy metabolite, which is further bioactivated to the ultimate carcinogenen, the 1′-sulfooxy metabolite (Miller *et al.*, 1983; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Smith *et al.*, 2010). The 1′-sulfooxy metabolite is unstable and binds via a presumed reactive carbocation intermediate covalently to different endogenous nucleophiles including DNA (Phillips *et al.*, 1981; Boberg *et al.*, 1983; Miller *et al.*, 1983; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Fennell *et al.*, 1985; Wiseman *et al.*, 1987; Smith *et al.*, 2002).

Because of their genotoxicity and carcinogenicity, the addition of estragole and methyleugenol as pure substances to foodstuffs has been prohibited within the European Union since September 2008 (European Commission, 2008). In 2008, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) re-evaluated the safety of alkenylbenzenes and indicated that although evidence of carcinogenicity to rodents given high doses of alkenylbenzenes exists, further research is needed to assess the potential risk to human health at relevant dietary exposure levels (JECFA, 2008).

A significant difficulty in evaluating the toxicological data for alkenylbenzenes is that human exposure to these substances results from exposure to a complex mixture of food, spice, and spice oil constituents which may influence the biochemical fate and toxicological risk of the alkenylbenzenes. In this regard, it was shown that a methanolic extract of basil inhibited the formation of estragole DNA adducts in human HepG2 cells exposed to the proximate carcinogen 1′-hydroxyestragole (Jeurissen *et al.*, 2008). This inhibition occurred at the level of sulfotransferase (SULT)-mediated bioactivation of 1′-hydroxyestragole into 1′-sulfooxyestragole (Jeurissen *et al.*, 2008).

The objective of this PhD research was to study the inhibitory action of components in alkenylbenzene-containing herbs and spices on SULT-mediated alkenylbenzene DNA adduct formation and the consequences of this combination effect for risk assessment using estragole and methyleugenol as the model alkenylbenzenes. To achieve this objective, an integrated approach of in vitro, in vivo and physiologically based biokinetic (PBBK) models was applied to investigate how the SULT inhibition influences the bioactivation and thus potentially also the toxicity and risk assessment of estragole and methyleugenol.

Chapter 1 of the thesis presents an introduction to the bioactivation, detoxification, genotoxicity and carcinogenicity of the alkenylbenzenes estragole and methyleugenol as well as a short introduction to PBBK modeling and the state-of-the-art knowledge on risk assessment strategies and regulatory status for alkenylbenzenes.

Chapter 2 of the thesis identifies nevadensin as a basil constituent able to inhibit SULT-mediated DNA adduct formation in rat hepatocytes exposed to the proximate carcinogen 1′-hydroxyestragole and nevadensin. The type of inhibition by nevadensin was shown to be non-competitive with an inhibition constant (*Ki*) of 4 nM. Furthermore, nevadensin up to 20 μM did not inhibit 1′-hydroxyestragole detoxification by glucuronidation and oxidation. The inhibition of SULT by nevadensin was incorporated into the PBBK models describing bioactivation and detoxification of estragole in male rat and human. The models thus obtained predict that co-administration of estragole at a level inducing hepatic tumours in vivo (50 mg/kg bw) with nevadensin at a molar ratio to estragole representing the molar ratio of their occurrence in basil, results in more than 83% inhibition of the formation of the carcinogenic metabolite, 1′-sulfooxyestragole, in the liver of male rat and human even at 1% uptake of nevadensin.

To extend the work to other alkenylbenzene-containing herbs and spices than basil chapter 3 presents data showing that methanolic extracts from different alkenylbenzene-containing herbs and spices such as nutmeg, mace, anise and others are able to inhibit the SULT enzyme activity. Flavonoids including nevadensin, quercetin, kaempferol, myricetin, luteolin and apigenin were the major constituents responsible for this inhibition of SULT activity with *Ki* values in the nano to sub-micromolar range. Also, the various flavonoids individually or in mixtures were able to inhibit estragole DNA adduct formation in human HepG2 cells exposed to the proximate carcinogen 1′-hydroxyestragole, and to shift metabolism in favour of detoxification (e.g. glucuronidation) at the cost of bioactivation (e.g. sulfonation).

In a next step, the kinetics for SULT inhibition were incorporated in PBBK models for estragole in rat and human to predict the effect of co-exposure to estragole and (mixtures of) the different flavonoids on the bioactivation in vivo. The PBBK-model-based predictions indicate that the reduction of estragole bioactivation in rat and human by co-administration of the flavonoids is dependent on whether the intracellular liver concentrations of the flavonoids can reach their *Ki* values. Finally, we concluded that it is expected that this is most easily achieved for nevadensin which has a *Ki* value in the nanomolar range and is, due to its methylation, more metabolically stable and bioavailable than the other flavonoids.

Chapter 4 of the thesis investigates whether the previous observation that nevadensin is able to inhibit SULT-mediated estragole DNA adduct formation in primary rat hepatocytes could be validated in vivo. Moreover, the previously developed PBBK models to study this inhibition in rat and in human liver was refined by including a sub-model describing nevadensin kinetics. Nevadensin resulted in a significant reduction in the levels of estragole DNA adducts formed in the liver of Sprague–Dawley rats orally dosed with estragole and nevadensin simultaneously at a ratio reflecting their presence in basil. Moreover, the refined PBBK model predicted the formation of estragole DNA adducts in the liver of rat with less than 2-fold difference compared to in vivo data and suggests more potent inhibition in the liver of human compared to rat due to less efficient metabolism of nevadensin in human liver and intestine.

Also, an updated risk assessment for estragole was presented taking into account the matrix effect and this revealed that the $BMDL_{10}$ and the resulting MOE for estragole increase substantially when they would be derived from rodent bioassays in which the animals would be exposed to estragole in the presence of nevadensin instead of to pure estragole.

To extend the work to other alkenylbenzenes than estragole **chapter 5** of the thesis investigates the potential of nevadensin to inhibit the SULT-mediated bioactivation and subsequent DNA adduct formation of methyleugenol using human HepG2 cells as an in vitro model. Nevadensin was able to inhibit SULT-mediated DNA adduct formation in HepG2 cells exposed to the proximate carcinogen 1′-hydroxymethyleugenol in the presence of nevadensin. To investigate possible in vivo implications for SULT inhibition by nevadensin on methyleugenol bioactivation, the rat PBBK model developed in our previous work to describe the dose-dependent bioactivation and detoxification of methyleugenol in male rat was combined with the recently developed PBBK model describing the dose-dependent kinetics of nevadensin in male rat. Similar to what was presented for estragole in chapter 4, chapter 5 presents an updated risk assessment for methyleugenol taking the matrix effect into account. This revealed that the $BMDL_{10}$ and the resulting MOE for methyleugenol increase substantially when they would be derived from rodent bioassays in which the animals would be exposed to methyleugenol in the presence of nevadensin instead of to pure methyleugenol.

In a next step, we aimed at moving one step forward towards endpoints that are closer to initiation of carcinogenesis than DNA adduct formation, namely, formation of hepatocellular altered foci (HAF). **Chapter 6** presents data showing that the potent in vivo inhibitory activity of nevadensin on SULT enzyme activity and on alkenylbenzene DNA adduct formation is accompanied by a potent in vivo reduction in early markers of carcinogenesis such as HAF. This also suggests that a reduction in the incidence of hepatocarcinogenicity is expected in liver of rodents when alkenylbenzenes would be dosed simultaneously with nevadensin.

Chapter 7 presents a discussion on the in vitro and in vivo activity of dietary SULT inhibitors and their potential in reducing the cancer risk associated with alkenylbenzene consumption. This chapter also presents some future perspectives based on the major issues raised by our research.

Altogether, the results of the present thesis indicate that the likelihood of bioactivation and subsequent adverse effects may be lower when alkenylbenzenes are consumed in a matrix containing SULT inhibitors such as nevadensin compared to experiments using pure alkenylbenzenes as single compounds. Also, the consequences of the in vivo matrix effect were shown to be significant when estragole or methyleugenol was tested in rodent bioassays in the presence of nevadensin at ratios detected in basil, thereby likely increasing $BMDL_{10}$ and resulting MOE values substantially in a subsequent risk assessment. However, the results also indicate that matrix effects may be lower at daily human dietary exposure levels of estragole or methyleugenol and nevadensin resulting from basil consumption. Also, matrix effects

seem to be limited in the presence of other SULT inhibiting dietary flavonoids even at high exposure levels of these flavonoids coming from supplements. This indicates that the importance of a matrix effect for risk assessment of individual compounds requires analysis of dose dependent effects on the interactions detected, an objective that can be achieved by using PBBK modeling.

Overall, the present study provides an example of an approach that can be used to characterise dose- species- and inter-individual differences as well as matrix effects in the risk assessment of food-borne toxicants present (e.g. alkenylbenzenes). In this approach the most important toxicokinetic interactions are addressed using an integrated strategy of in vitro, in vivo and PBBK modeling approaches.

9 Samenvatting

SAMENVATTING

Alkenylbenzenen zoals estragole en methyleugenol zijn normale bestanddelen van specerijen en kruiden zoals dragon, basilicum, venkel, foelie, piment, steranijs en anijs en de essentiële oliën daarvan (Smith *et al.*, 2002). Er bestaat belangstelling voor de veiligheidsevaluatie van alkenylbenzenen omdat deze verbindingen levertumoren veroorzaken in knaagdieren als ze in hoge doses oraal worden toegediend (Miller *et al.*, 1983; NTP, 2000). Gebaseerd op deze dierstudies met estragole, methyleugenol en structureel verwante alkenylbenzenen zoals safrole, wordt de levercarcinogeniciteit toegeschreven aan de bioactivering van deze stoffen door cytochroom P450-enzymen resulterend in de vorming van de proximale carcinogene 1'-hydroxymetaboliet, die verder wordt geactiveerd tot de ultieme carcinogene 1'-sulfooxymetaboliet (Miller *et al.*, 1983; Philips *et al.*, Randerath *et al.*, 1984; Smith *et al.*, 2010). De 1'-sulfooxymetaboliet is instabiel en bindt via een veronderstelde reactieve carbocation intermediair covalent aan verschillende endogene nucleofielen waaronder ook DNA (Philips *et al.*, 1981; Boberg *et al.*,; Miller *et al.*, 1983; Philips *et al.*, 1984; Randerath *et al.*, 1984; Fennel *et al.*, 1985; Wiseman *et al.*, 1987; Smith *et al.*, 2002).

Vanwege hun genotoxiciteit en carcinogeniciteit is het toevoegen van estragole en methyleugenol als pure stoffen aan voedingsmiddelen binnen de Europese Gemeenschap sinds september 2008 (European Commission (EC), 2008) verboden. In 2008 heeft the Joint FAO/WHO Expert Committee on Food Additives (JECFA) de veiligheid van alkenylbenzenen opnieuw geëvalueerd en aangegeven dat, ondanks het feit dat bewijs bestaat van carcinogeniciteit voor knaagdieren die hoge doses toegediend kregen, verder onderzoek noodzakelijk is om het potentiële risico voor de gezondheid van mensen te bepalen bij relatieve lage blootstellingsniveaus via het dieet (JECFA, 2008).

Een belangrijk probleem bij de evaluatie van de betekenis van de toxicologische data voor alkenylbenzenen uit dierstudies voor de mens is, dat menselijke blootstelling aan deze stoffen het resultaat is van blootstelling aan een complex mengsel van voeding, specerijen and specerij-oliebestanddelen die het biochemische lot en het toxicologische risico van de alkenylbenzenen zouden kunnen beïnvloeden. In dit verband is aangetoond dat een methanolextract van basilicum de vorming van estragole DNA-adducten remde in humane HepG2 cellen die waren blootgesteld aan het proximale carcinogene 1'-hydroxyestragole (Jeurissen *et al.*, 2008). Deze remming deed zich voor op het niveau van de sulfotransferase (SULT)-gemedieerde bioactivering van 1'-hydroxyestragole naar 1'-sulfooxyestragole (Jeurissen *et al.*, 2008).

Het doel van dit PhD-onderzoek was de remmende invloed te bestuderen van bestanddelen in alkenylbenzeen bevattende kruiden en specerijen op SULT-gemedieerde alkenylbenzeen DNA-adductvorming en de gevolgen van deze combinatie-effecten voor de risico-evaluatie waarbij estragole en methyleugenol als de model-alkenylbenzenen zijn gebruikt. Om dit doel te bereiken is een geïntegreerde benadering toegepast van in vitro, in vivo en fysiologisch gebaseerde biokinetische (PBBK) modellering, om te onderzoeken hoe de SULT-remming de bioactivering en zodoende in potentie ook de toxiciteit en risicoschatting van estragole en methyleugenol beïnvloedt.

Hoofdstuk 1 van dit proefschrift presenteert zowel een inleiding over de bioactivering, detoxificatie, genotoxiciteit en carcinogeniciteit van de alkenylbenzenen estragole en methyleugenol als ook een korte introductie van PBBK modellering en de state-of-the-art met betrekking tot strategieën voor risicoschatting en de regelgeving voor alkenlylbenzenen.

Hoofdstuk 2 van het proefschrift identificeert nevadensin als een bestanddeel van basilicum dat in staat is SULT-gemedieerde estragole DNA-adductvorming te remmen in hepatocyten van ratten die zijn blootgesteld aan het proximale carcinogeen 1'-hydroxyestragole en nevadensin. Het type remming door nevadensin bleek non-competitief te zijn met een remmingsconstante (*K*i) van 4 nM. Bovendien remde nevadensin tot 20 µM niet 1'-hydroxyestragole detoxificatie via glucuronidering en oxidatie. De remming van SULT door nevadensin werd opgenomen in de PBBK-modellen die bioactivering en detoxificatie van estragole in de mannelijke rat en in de mens beschrijven. De op deze manier verkregen modellen voorspellen dat gelijktijdige toediening van estragole op een niveau dat hepatische tumoren in vivo veroorzaakt(50 mg/kg bw) met nevadensin gedoseerd in een molaire ratio ten opzichte van estragole van 0.06 (de molaire ratio van het voorkomen in basilicum), resulteert in meer dan 83% remming van de vorming van de carcinogene metaboliet, 1'-sulfooxyestrageole, in de lever van de mannelijke rat en de mens zelfs bij 1% opname van nevadensin.

Om het werk uit te breiden naar andere alkenylbenzeen-bevattende kruiden en specerijen dan basilicum presenteert **hoofdstuk 3** data die aantonen dat methanolextracten van verschillende alkenylbenzeen-bevattende kruiden en specerijen zoals nootmuskaat, foelie, anijs en andere, in staat zijn de SULT-enzymactiviteit te remmen. Flavonoïden, inclusief nevadensin, quercetin, kaempferol, myricetin, luteolin en apigenin, waren de belangrijkste bestanddelen die verantwoordelijk waren voor deze remming van SULT-activiteit met *K*i-waarden in het nano- tot sub-micromolaire gebied. Tevens waren de verschillende flavonoïden, individueel of in mengsels, in staat estragole DNA-adductvorming te remmen in humane HepG2-cellen die waren blootgesteld aan het proximale carcinogeen 1'-hydroxyestragole, en verschoof metabolisme in het voordeel van detoxificatie (b.v. glucuronidering) ten koste van bioactivering (sulfonering). In een volgende stap werd de kinetiek voor SULT-remming geïncorporeerd in PBBK-modellen voor estragole in rat en mens om het effect te voorspellen van gelijktijdige blootstelling aan estragole en (mengsels van) de verschillende flavonoïden op de bioactivering in vivo. De op het PBBK-model gebaseerde voorspellingen wijzen er op dat de reductie van estragole bioactivering in rat en mens door gelijktijdige toediening van de flavonoïden afhankelijk is van de vaag of de intracellulaire leverconcentraties van de flavonoïden hun *K*i-waarden kunnen bereiken. Tot slot concludeerden we dat het te verwachten is dat dit het gemakkelijkst te bereiken is voor nevadensin die een *K*i-waarde heeft in het nanomolaire gebied en, dank zij zijn methylering, metabolisch stabieler is en meer biobeschikbaar dan de andere flavonoïden.

Hoofdstuk 4 van het proefschrift onderzocht of de eerdere observatie dat nevadensin in staat is SULT-gemedieerde estragole DNA-adductvorming te remmen in primaire rat-hepatocyten ook in vivo kon worden gevalideerd. De experimenten werden ook gebruikt

om de voorheen ontwikkelde PBBK-modellen om deze remming te bestuderen in ratteen in humane lever te verbeteren door een sub-model toe te voegen dat nevadensinkinetiek beschrijft. Nevadensin resulteerde in een aanzienlijke reductie in de niveaus van estragole DNA-adducten in de lever van Sprague-Dawley ratten wanneer de ratten oraal tegelijkertijd estragole en nevadensin kregen toegediend in een ratio die gelijk was aan de aanwezigheid ervan in basilicum. Het verfijnde PBBK-model voorspelde de vorming van estragole DNA-adducten in de lever van de rat met minder dan een tweevoudig verschil, vergeleken met in vivo data. De modellen voorspelden ook een potentere remming in de lever van de mens dan van de rat, dankzij het minder efficiënte metabolisme van nevadensin in humane lever en darm.

Er werd ook een verfijnde risicoschatting voor estragole gepresenteerd waarbij rekening werd gehouden met het matrixeffect en dit toonde aan dat de $BMDL_{10}$ en de daaruit resulterende MOE voor estragole aanzienlijk zouden toenemen als zij zouden worden bepaald op basis van bioassays waarin de dieren zouden zijn blootgesteld aan estragole tegelijk met nevadensin in plaats van aan pure estragole.

Om het werk uit te breiden naar andere alkenylbenzenen dan alleen estragole, onderzoekt **hoofdstuk 5** van het proefschrift het vermogen van nevadensin om de SULT- gemedieerde bioactivering van methyleugenol te remmen en daardoor de DNA-adductvorming van methyleugenol in humane HepG2-cellen in vitro. nevadensin bleek in staat te zijn SULT-gemedieerde methyleugenol DNA-adductvorming te remmen in HepG2-cellen die waren blootgesteld aan het proximale carcinogene 1'-hydroxymethyleugenol tegelijk met nevadensin. Om mogelijke in vivo implicaties te onderzoeken voor SULT-remming door nevadensin op methyleugenol-bioactivering, werd het rat PBBK-model dat in ons eerdere werk was ontwikkeld om de dosis-afhankelijke bioactivering en detoxificatie van methyleugenol te beschrijven, gecombineerd met het recent ontwikkelde PBBK-model dat de dosis-afhankelijke kinetiek van nevadensin in de mannelijke rat beschrijft. Net als voor estragole in hoofdstuk 4, laat hoofdstuk 5 een aangepaste risicoschatting zien voor methyleugenol, waarbij rekening wordt gehouden met het matrixeffect. Dit toonde aan dat de $BMDL_{10}$ en de daarvan afgeleide MOE voor methyleugenol aanzienlijk toenemen wanneer zij zouden worden ontleend aan knaagdier-bioassays waarin de dieren zouden zijn blootgesteld aan methyleugenol tegelijk met nevadensin in plaats van aan zuivere methyleugenol.

In een volgende fase van het onderzoek werd geprobeerd een stap verder te zetten naar eindpunten die dichter bij de initiatie van carcinogenese komen dan DNA-adductvorming, namelijk vorming van zogenoemde hepatocellulaire altered foci (HAF). **Hoofdstuk 6** toont data waaruit blijkt dat de in vivo remmende activiteit van nevadensin op SULT-enzymactiviteit en op alkenylbenzeen DNA-adductvorming, samengaat met een in vivo reductie in vroege markers van carcinogenese zoals HAF. Dit suggereert dat een reductie in incidentie van hepatocarcinogeniciteit kan optreden in de lever van knaagdieren als nevadensin tegelijk met alkenylbenzenen zou zijn gedoseerd.

Hoofdstuk 7 presenteert een discussie van de in vitro en in vivo activiteit van dieet SULT-remmers en hun vermogen om het risico op kanker te reduceren, dat wordt

geassocieerd met consumptie van alkenylbenzenen. Dit hoofdstuk beschrijft ook enkele toekomstige perspectieven gebaseerd op de voornaamste onderwerpen die voortvloeiden uit ons onderzoek.

Alles bijeen tonen de resultaten van dit proefschrift aan dat de waarschijnlijkheid van bioactivering en daarop volgende nadelige effecten, mogelijk lager zijn wanneer alkenylbenzenen worden geconsumeerd in een matrix die SULT-remmers bevat, zoals nevadensin, vergeleken met experimenten die zuivere alkenylbenzenen als enige stof gebruiken. Tevens bleken de gevolgen van het in vivo matrixeffect aanzienlijk te zijn wanneer estragole of methyleugenol werd getest in knaagdier-bioassays met nevadensin in ratio's die in basilicum zijn gedetecteerd, resulterend in hogere $BMDL_{10}$ en bijbehorende MOE-waarden. De resultaten wijzen er echter ook op dat matrixeffecten lager zijn bij blootstellingsniveaus representatief voor een dagelijks humaan dieet van estragole of methyleugenol en nevadensin door basilicumconsumptie. Tevens lijken matrixeffecten beperkt te zijn bij andere SULT-remmende dieetflavonoïden, zelfs bij hoge blootstellingsniveaus aan deze flavonoïden die in supplementen voorkomen. Dit toont aan dat het belang van een matrixeffect voor risicoschatting van individuele stoffen een analyse vergt van dosis-afhankelijke effecten op de gedetecteerde interacties, een doel dat kan worden bereikt door PBBK-modellering te gebruiken. De huidige studie geeft een voorbeeld van een benadering die kan worden gebruikt bij risicoschatting van botanische toxinen (zoals alkenylbenzenen). In deze benadering worden de belangrijkste toxico-kinetische interacties in planten behandeld door gebruik van een integrale benadering van in vitro, in vivo en PBBK-modellering.

&

Appendices:

Acknowledgements
Curriculum Vitae
List of publications
Overview of completed training activities

ACKNOWLEDGEMENTS

I would like in this opportunity to express my sincere gratitude and appreciation to my promotors, colleagues, family and friends. Without you, the outcomes and quality of this work would have not been the same.

First, I gratefully thank you Ivonne Rietjens for your dedicated supervision and guidance during my masters and PhD studies. Also, for being a great example of how a scientist should be dedicated, efficient, intelligent and precise. Many thanks for your stimulating suggestions, encouragement, and for your great help in my search for a job.

I would also like to extend my extreme gratitude to my promotor Peter van Bladeren, thank you very much for your guidance, support and for helping me in exploring new job opportunities for my future career.

To my co-promotor Ans Punt who helped and guided me in the field of PBBK modeling not only in my PhD research but at earlier stage during my master research, thank you very much for your constructive instructions and help.

Furthermore, I would like to acknowledge with much appreciation the sponsors of my PhD research, namely, the International Organization of the Flavour Industry (IOFI) and Nestlé Research Centre.

I would also like to express my deepest appreciation to collaborators from the department of Pathology in New York medical college, namely Dr. Gary M. Williams, Dr. Alan M. Jeffrey , Dr. Michael J. Iatropoulos and Dr. Jiang-Dong Duan, thank you very much for the fruitful collaboration which resulted in a valuable chapter in my PhD thesis.

To Dr. Gabriele Scholz and Dr. Benoit Schilter from Nestlé Research Centre, many thanks for your valuable input in the different chapters of this PhD thesis.

Special thanks go to Dr. Sean Taylor from the International Organization of the Flavour Industry (IOFI) for organising the synthesis of nevadensin required for the in vivo studies and many thanks for Dr. Timothy B. Adams from the Flavour and Extract Manufacturers Association (FEMA) for the support and valuable input.

To Irene Keultjes and Gre Schurink-Heitkonig, many thanks for your enormous help in all administrative work which is very much appreciated.

To Bert Spenkelink, thank you very much for your valuable input and help in practical work and for the very interesting chat we had on intercultural and family related topics.

To Hans vanden Berg, many thanks for your help in practical work and for your explicit and clear explanations and for the funny remarks that made me always smiling.

To Laura de Haan, thank you very much for your help whenever was needed and for the nice atmosphere you create when you are present.

To all my PhD colleagues, I would like to thank you very much for the wonderful time we spent together at work and outside working hours. I would also like to thank my master students for their important contribution to my PhD thesis namely, Adelaine Campana, Mariska Asselman, Stella Fragki, Laura Trijsburg and Suzanne van den Berg who have become a PhD colleague at the same department and the collaboration was further extended between us on few joint projects.

A special gratitude I give to my dear colleague and friend Reiko Kiwamoto, thank you Reiko for your support and for the joyful time we spent together. To my dear friend Lisa Gkogka, many thanks for your warmth and help which started from the first days of my arrival to the Netherlands. To my dear friends Parisa Rezaie and Benyamin Houshyani, thank you very much for the support and help you gave me since my first days in the Netherlands. With All love and appreciation I thank you very much for being my friends and in my world.

Karsten I would like to thank you for all the help you provided to make my first visit to New York comfortable which eventually resulted in a valuable chapter in my PhD thesis, thank you very much.

To all my friends I met in the Netherlands, namely, Natalie Terzikhan-Barsoumian, Farah Al-delaigan, Sahar El Aidy, Kees Folkertsma and Mahmoud Al Alayli thank you very much for making my days nicer, brighter and much warmer.

To my Paranymphs Reiko Kiwamoto and Jonathan Nicolas, thank you very much for your great help and presence.

To my childhood's friend Rasha, and to my close friends in Jordan Dena Akkash and Ghadeer Aloran, thank you very much for your support. Despite the long distance you were always present and close to me.

Rose you have been the mother and the warm family for me, for Ahmad and for Josef in the Netherlands. Thank you very much because of your love, care and endless support.

To my parents, siblings Uruba, Uhood, Essa, Wasan, Beesan, Majd and Ahmad, thank you very much because you always believed in my potential and gave me all the support to make it real. I know that this degree means to you as much as it means to me, thank you very much.

&

To my uncle Abdullah, Aunt Qadar and cousins Marwan, Mohammd and Munther, thank you very much for being my family and for the love and support you always surrounded me with and for your endless encouragement.

To my uncles Ali, Fawzi, Jehad, Hussein and Fayez and to my dearest Aunts Amneh and Fatima, thank you very much for your love and support.

To my family in law, thank you very much for your love and support.

Last but not least, many thanks coated with love and appreciation go to my husband and best friend ever Ahmad Kokash and to our beloved son Josef. Without You Ahmad I wouldn't have had the same drive and motivation. Your support and love Ahmad made everything in my life easier and worth it. To our little angel Josef, despite the fact that you didn't make it easier on me at all, still you have with no doubts made our whole life happier and more meaningful, I love you my son so much.

CURRICULUM VITAE

Wasma Alhusainy was born on January 28th, 1982 in Amman, Jordan. After her secondary education in Amman in 2000, she started her undergraduate study in Biology at the University of Jordan. After receiving her BSc degree in 2004, she worked for two years for the Food Safety institute in Amman, Jordan as a lab technician in a food microbiology lab and then joined the newly established studies and follow–up department at the same institute where she was mostly involved in food safety/hygiene promotion. In 2006 she moved to the Netherlands to peruse a master degree in Food safety at Wageningen University funded by The Netherlands Organization for International Cooperation in Higher Education (NUFFIC) fellowship program. During her master, Wasma conducted a thesis at the Division of Toxicology of Wageningen University and afterwards she spent a 6 month internship at Nestlé Research Centre (NRC) Lausanne, Switzerland. After completing her master in 2008, she was appointed as a researcher at the Division of Toxicology of Wageningen University from October 2008 until December 2008. From January 2009 until May 2013, she worked as a PhD student on the project presented in this thesis, which was a collaboration between the Division of Toxicology of Wageningen University, Nestlé Research Centre (NRC) Lausanne, Switzerland and the the Flavor and Extract Manufacturers Association (FEMA) Washington, US. During her PhD study, she followed several postgraduate courses in toxicology which enabled her to register as a European toxicologist. After completing her PhD, she was appointed as a researcher at the Division of Toxicology of Wageningen University from May 2013 until January 2014.

LIST OF PUBLICATIONS

Alhusainy, W., Paini, A., Punt, A., Louisse, J., Spenkelink, A., Vervoort, J., Delatour, T., Scholz, G., Schilter, B., Adams, T., van Bladeren, P.J., Rietjens, I.M.C.M. (2010) Identification of nevadensin as an important herb-based constituent inhibiting estragole bioactivation and physiology-based biokinetic modeling of its possible in vivo effect. *Toxicology and Applied Pharmacology 245*, 179-190.

Rietjens, I.M.C.M., Alhusainy, W., Boersma M.G. (2011) Flavonoids and alkenylbenzenes: New concepts in bioactivation studies. *Chemico-biological interactions 192*, 87-95.

Alhusainy, W., van den Berg S.J.P.L., Paini, A., Campana, A., Asselman, M., Spenkelink, A., Punt, A., Scholz, G., Schilter, B., Adams, T.B., van Bladeren, P.J., Rietjens, I.M.C.M. (2012) Matrix modulation of the bioactivation of estragole by constituents of different alkenylbenzene-containing herbs and spices and physiologically based biokinetic modeling of possible *in vivo* Effects. *Toxicological Sciences 129*, 174–187.

Alhusainy,W., Paini, A., van den Berg, J.H.J., Punt, A., Scholz, G., Schilter, B., van Bladeren, P.J., Taylor, S., Adams, T.B., Rietjens, I.M.C.M. (2013) In vivo validation and physiologically based bio kinetic modelling of the inhibition of SULT-mediated estragole DNA adduct formation in the liver of male Sprague-Dawley rats by the basil flavonoid nevadensin. *Molecular Nutrition and Food Research*. DOI 10.1002/mnfr.201300144.

Al-Subeihi, A., Alhusainy, W., Paini, A., Punt, A., Vervoort, J., van Bladeren, P.J., Rietjens, I.M.C.M. (2013) Inhibition of methyleugenol bioactivation by the herb-based constituent nevadensin and prediction of possible in vivo consequences using physiologically based kinetic modeling. *Food and Chemical Toxicology 59*,564-571.

van den Berg, S.J.P.L., Klaus, V., Alhusainy, W., Rietjens, I. M.C.M. (2013) Matrix-derived combination effect and risk assessment of estragole from basil-containing plant food supplements (PFS). *Food and Chemical Toxicology 62*, 32-40.

Alhusainy, W., Williams, G.M., Jeffery, A.M., Iatropoulos, M.J., Duan, J.D., Taylor, S., Adams, T.B., Rietjens, I.M. C. M. Protective effect of nevadensin against methyleugenol induced hepatocarcinogenicity in male F344 rats (*submitted*)

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Laboratory animal science, Utrecht University, 2010
Toxicological Risk Assessment, Wageningen University, 2010
Pathobiology, Utrecht University, 2009
Organ toxicology, Utrecht University, 2009
Mutagenisis and carcinogenisi, Leiden University, 2012
Medical, Forensic and Regulatory Toxicology, Utrecht University, 2010
Reproductive Toxicology, Utrecht University, 2011
Physiologically based biokinetic (PBBK) modeling, Wageningen University, 2011

Meetings

Nederlandse Vereniging voor Toxicologie (NVT annual meetings, 2009-2012, 1 oral presentation and 3 poster presentations)
The 47th congress of the European societies of toxicology, Paris, France, 2011 (oral presentation)
The Joint meeting of the for British Toxicology Society and the Dutch Society of Toxicology (BTS), Durham, UK, 2011 (poster presentation)

General courses

VLAG PhD week, 2009
Techniques for Writing and Presenting Scientific papers, Wageningen University, 2008
Radioisotope User training, New York medical college, US
Philosophy and Ethics of Food science and Technology, Wageningen Graduate Schools, 2011
PhD competence assessment (WGS), Wageningen University,
PhD symposium, NVT, 2009-2012

Optional activities

Preparation PhD research proposal
Attending scientific presentations at toxicology, 2009-2013, Wageningen
General Toxicology, Wageningen University, 2012

Approved by graduate school VLAG

The research described in this thesis was financially supported by a grant from the International Organization of the Flavor Industry (IOFI), Genève, Switzerland and from Nestlé Research Center, Lausanne, Switzerlan.

Lay out and Printing: Off Page, Amsterdam, The Netherlands
Cover design: Marian Hendriks