Bioactivation and genotoxicity of the herbal constituents safrole, estragole and methyleugenol

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ABSTRACT

The herbal constituents safrole, estragole, and methyleugenol, belonging to the chemical class of the alkenylbenzenes, are genotoxic and carcinogenic compounds. The genotoxicity of these alkenylbenzenes proceeds via electrophilic metabolites generated by cytochrome P450 enzymes (P450) and sulfotransferases (SULT). Carcinogenicity of these compounds was demonstrated in animal experiments using relatively high doses of single compounds. Human exposure to these compounds is much lower than the doses used in the animal experiments and humans are exposed to alkenylbenzenes in a complex food matrix. The first aim of this thesis was to identify the human P450 enzymes that are responsible for the bioactivation of the alkenylbenzenes into their proximate carcinogenic 1′-hydroxymetabolites. Several *in vitro* studies using recombinant P450 enzymes and human liver microsomes were undertaken to identify the main enzymes involved in the 1′-hydroxylation of the alkenylbenzenes and to determine their kinetics. These studies showed that at low substrate concentrations, P450 1A2 is the major enzyme in the bioactivation of methyleugenol, P450 1A2 and P450 2A6 are the main enzymes in the bioactivation of estragole, and P450 2A6 is the main enzyme in the bioactivation of safrole. The second objective of this thesis was to study the influence of other herbal constituents on the bioactivation and the genotoxicity of herb-based alkenylbenzenes. An on-line high-performance liquid chromatography detection system was developed for the detection of P450 1A2 inhibitors in herbal extracts. The presence of P450 1A2 inhibitors in basil, a herb that contains methyleugenol and estragole, was demonstrated using this on-line system. In addition to these P450 1A2 inhibitors, also the alkenylbenzenes themselves may act as inhibitors competing for the active site of P450 1A2 (estragole and methyleugenol) or P450 2A6 (estragole and safrole). Furthermore it was demonstrated that basil extract is able to strongly inhibit sulfation and subsequent DNA adduct formation of 1′-hydroxyestragole in incubations with rat and human S9 homogenates and in the human hepatoma HepG2 cell line. These *in vitro* results suggest that P450- and SULT-catalyzed bioactivation of methyleugenol and/or estragole and subsequent adverse effects may be lower in a matrix of other herbal components than what would be expected on the basis of experiments using single compounds. *In vivo* experiments have to point out whether the protective effects that are found in these *in vitro* studies can be extrapolated to the *in vivo* situation. It may turn out that rodent carcinogenicity data on estragole and methyleugenol considerably overestimate the risks posed when humans are exposed to these compounds in a herbal matrix.

TABLE OF CONTENTS

1

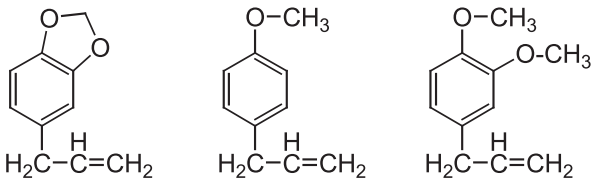
General Introduction

Background

Herbs and spices have been used for culinary and medical purposes throughout the world for thousands of years. At present, the market for herb-based products such as herbal teas and herbal extracts and the use of herb-based ingredients as food supplements, food flavours, and bioactive ingredients in functional foods is still growing. Also the use of medicinal herbs (phytotherapy) increases. In spite of the common perception among consumers that 'natural equals safe', herbs and herbal extracts may contain individual ingredients that are toxic and even genotoxic and carcinogenic. This is even true for some herbs that are used since prehistory.

The research in this thesis focuses on one class of herb-based ingredients, the alkenylbenzenes, and in particular on safrole, estragole, and methyleugenol (Fig. 1.1). Combinations of these compounds occur naturally in among others nutmeg, mace, cinnamon, tarragon, basil, star anise, and fennel, and methyleugenol and estragole are also used as food flavoring agents (*1-3*). Safrole, methyleugenol, and estragole are carcinogenic and genotoxic according to the Scientific Committee on Food of the European Union (EU-SCF) (*1-3*), and this will lead to restrictions in the use of these compounds in the European Union. Safrole is already banned as a food additive by the US Food and Drug Administration (Federal Register of December 3, 1960, 25 FR 12412) and by the Council of the European Communities (*4*). On the other hand, an industrial expert panel from the Flavor and Extract Manufacturers' Association (FEMA) published that present exposure to methyleugenol and estragole, resulting from consumption of food, mainly spices and added as such, does not pose a significant cancer risk (*5*). For June 2007, a re-evaluation of the safety of estragole is scheduled by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

These different expert judgements mainly result from the absence of adequate scientific data to support unequivocal translation of carcinogenicity data of rodent animal experiments to the human situation. Since carcinogenicity is demonstrated in animal experiments using high doses of single compounds, and since these compounds are naturally present in various herbs and spices, new mechanistic insight in the adverse effects of these ingredients and the possible interaction with other herb-based ingredients is required for a better extrapolation of the animal carcinogenicity data to the human situation.



Safrole Estragole Methyleugenol

Figure 1.1. Structural formulas of the alkenylbenzenes safrole, estragole, and methyleugenol.

Exposure to alkenylbenzenes

Occurrence of alkenylbenzenes

Safrole occurs naturally in a number of herbs and spices such as nutmeg, mace, cinnamon, anise, black pepper, and sweet basil. Safrole is also present in cola drinks. The most important dietary sources are nutmeg, mace, and their essential oils (*3*). In addition, in some countries areca (betel nut) quid is chewed, and this can give rise to safrole exposure (420 μM was measured in saliva during chewing (*6*)) and is associated with the presence of safrole-DNA adducts in human peripheral blood (*7*).

Methyleugenol exposure from food occurs predominantly from a number of herbs and spices such as nutmeg, pimento, lemongrass, tarragon, basil, star anise, and fennel. Methyleugenol is used as a flavoring agent in jellies, baked goods, non-alcoholic beverages, chewing gum, relish, and ice-cream (*1*) and also occurs at low levels in oranges, bananas, and grapefruit juice (*5*). Furthermore, it is applied as fragrance in cosmetic products such as creams, lotions, soaps, detergents, and perfumes (*8*). Methyleugenol is also used as an insect attractant and as an anaesthetic in rodents (*1,8*). Measurement of blood plasma levels of methyleugenol in a subset of the American population revealed that most people are exposed to methyleugenol. In 98% of the serum samples, methyleugenol was detected at concentrations ranging from < 3.1 (detection limit) up to 390 pg/g wet weight (Table 1.1) (*9*). When comparing these levels with the peak plasma levels measured in a study in which human volunteers were exposed to methyleugenol via consumption of ginger snap cookies (Table 1.1), it appeared that more than 5% of the population had higher plasma levels than the peak levels after exposure to methyleugenol in the experimental setting (*10*).

Estragole exposure mainly results from consumption of a variety of foods including tarragon, sweet basil, sweet fennel, anise vert, and star anise (*2*). It can be added to a wide variety of dietary products (*2*) and occurs at low levels in oranges, bananas, and grapefruit juice (*5*). To give an impression of the amounts of alkenylbenzenes in various products, Table 1.2 shows the average levels of safrole, estragole, and methyleugenol measured in different brands of various food products by Siano *et al.* (*11*). Especially in pesto sauce and fresh basil,

Table 1.1. Human serum levels of methyleugenol

Number of subjects	Dose and source	Route of administration	Plasma peak concentration (pg/g wet weight)		Ref.
213	Fasting	-	mean median 5th percentile 95th percentile range	24 16 5 78 3.1 - 390	(*9*)
20	Fasting	-	16.2 ± 4.0		(*10*)
20	216 μg methyleugenol (in gingersnap cookies)	oral	53.9 ± 7.3		(*10*)

Table 1.2. Average levels (mean and range) of safrole, estragole, and methyleugenol in some food products (*11*)

product	safrole (mg/kg)	estragole (mg/kg)	methyleugenol (mg/kg)
Tomato sauce	ND[a]	0.81 (0.22-2.81)	0.07 (0.01-0.33)
Pesto sauce	ND	5.26 (0.05-19.30)	0.12 (0.01-0.52)
Cola beverages	0.02 (0.01-0.04)	ND	0.04 (0.03-0.06)
Bologna sauce	2.09 (0.02-2.23)	ND	ND
Fresh basil	ND	13.17 (10.21-16.05)	0.62 (0.49-0.75)
Vienna sausage	0.18 (0.07-0.37)	ND	0.12 (0.10-0.14)

[a] ND = not detected

high amounts of estragole were observed. The alkenylbenzene content of herbs and spices varies significantly and depends among others on the plant maturity at harvest, harvesting techniques, storage conditions, processing conditions (e.g. drying), the geographical origin of the plant, and the distribution of the alkenylbenzenes in the plant (*5*). For example, for the average estragole concentration in the essential oil from basil, values ranging from 20 to 89% are reported (*2,12,13*).

Exposure assessment

The Scientific Committee on Food of the European Union (EU-SCF) made conservative intake estimates for safrole and estragole using food consumption data, based on seven days dietary records of adult individuals, in combination with maximum limits for flavoring substances in industrially prepared food. The exposure assessment was based on a selection of 28 categories of food, identified by industry, to which safrole and estragole can be added. Using this method, the EU-SCF estimated the average intake for safrole (consumers only) to 0.3 mg/day and the 97.5th percentile to 0.5 mg/day and for estragole (consumers only) to 4.3 mg/day and the 97.5th percentile to 8.7 mg/day (*2,3*).

For methyleugenol, food consumption data, based on seven days dietary records of adult individuals, were used in combination with maximum use levels of methyleugenol obtained from source materials, i.e. essential oil, provided by the International Organisation of the Flavour Industry. Using this method, the average intake (for consumers only) amounted to 13 mg/day and the 97.5th percentile was 36 mg/day (*1*). The Food and Extract Manufacturers Association (FEMA) made intake estimates for methyleugenol and estragole using a different method. Mean concentrations of volatile oil in methyleugenol- and estragole containing plants, mean concentrations of methyleugenol and estragole in their essential oils, and reported annual production volumes of those herbs, their essential oils, and estragole and methyleugenol to be used as flavouring substances are used. By calculating the annual production in the US and assuming that only 10% of the population consumed all of the food containing methyleugenol and estragole (‘eaters only’), the FEMA estimated the daily per capita intake for the US population to be less than 10 µg/kg bw/day (i.e. < 0.6 mg/day for a

60 kg person) (*5*). Of this daily per capita intake, 65% was derived from food that naturally contains methyleugenol, 21% was derived from essential oils containing methyleugenol, and 14% was from methyleugenol added as such. For estragole these figures were, respectively, 70%, 30%, and 11% (*5*).

Although the intake estimates of the FEMA are lower than the intake estimates of the EU-SCF (for estragole < 0.6 versus 4.3 mg/day and for methyleugenol < 0.6 versus 13 mg/day) also the FEMA indicated that certain 'specialized eaters' might be exposed to higher levels of these alkenylbenzenes. As an example, the exposure via pesto consumption was calculated by the FEMA. Pesto is traditionally prepared with basil that is 10-12 cm in height, when methyleugenol predominates in the essential oil (> 40%) (*14*). Assuming that 0.5% of the basil used consists of essential oil and that a portion of pesto contains 10 g basil (*14*), the methyleugenol intake from a portion pesto will be 20 mg (which is higher than the average daily intake calculated by the EU-SCF), equal to 333 µg/kg bw for a person of 60 kg.

Toxicity of safrole, estragole, and methyleugenol

Acute toxicity

Safrole has an oral LD_{50} of 1950 mg/kg bw for rats and 2350 mg/kg bw for mice (*15*). For methyleugenol, oral LD_{50} values of 810 to 1560 mg/kg bw for rats and 540 mg/kg bw for mice were reported (*16*) and methyleugenol (98%) was neither an eye irritant nor a skin irritant (*1*). For estragole, no acute toxicity data are available.

Sub-chronic toxicity

Sub-chronic toxicity of safrole was demonstrated by Hagan *et al.* (*17*) in male and female Osborne-Mendel rats exposed to safrole by oral intubation at doses of 0, 250, 500, and 750 mg/kg bw/day for up to 105 days and in Swiss mice exposed to 250 and 500 mg/kg bw/day for 60 days. In rats in the highest treatment group, high lethality (90%) was observed. In both species, safrole induced liver changes, including hepatic cell enlargement, adenomatoid hyperplasia, cystic necrosis, fatty metamorphosis, and bile duct proliferation (*17*). In addition, safrole caused adrenal enlargement and yellow discoloration of the adrenals (*17*).

For methyleugenol, Abdo *et al.* (*18*) described a 14-week toxicity study in which male and female F344/N rats and $B6C3F_1$ mice were exposed to methyleugenol by gavage, five days per week. Methyleugenol caused reductions in body weight gain after exposure to 100 mg/kg bw/day and higher (female rat) or 300 mg/kg bw/day and higher (male rats and male and female mice), increased mortality (mice only), and adverse effects in the liver and the glandular stomach (*18*). These adverse effects included hepatocellular damage, cholestasis, altered hepatic functions, increase in liver weight, and increase in incidences of atrophy, necrosis, oedema, mitotic alteration, and cystic glands of the glandular stomach (*18*). Based on the results of this study, a no observed adverse effect level (NOAEL) was estimated at 30 mg/kg bw/day for rats and at 10 mg/kg bw/day for mice, resulting in a NOAEL of 10 mg/kg bw for methyleugenol. For estragole, such sub-chronic toxicity data are not available.

Carcinogenicity

Safrole

Safrole was the first alkenylbenzene that was discovered to have hepatocarcinogenic properties and this was initially shown in rats (*19,20*) and later in mice (*21,22*). 1′-Hydroxysafrole had a greater carcinogenicity than the parent compound safrole (*23,24*) (for a detailed description of the metabolic pathways and metabolites of the alkenylbenzenes see below). Newborn mice were more susceptible than adults for the carcinogenic effects of 1′-hydroxysafrole (*23*). Female adult mice were more susceptible for the hepatotoxicity and hepatocarcinogenicity of safrole and 1′-hydroxysafrole than male adult mice (*23*), but opposite data were found in preweanling CD-1 mice (*25*). Besides safrole and 1′-hydroxysafrole, also 1′-hydroxysafrole-2′,3′-oxide induced liver hepatomas in mice (*25*). In rats, no hepatic carcinomas were found with 1′-hydroxysafrole-2′,3′-oxide (*25*).

In addition to hepatocarcinomas, also low incidences of carcinomas and papillomas of the forestomach, mammary carcinomas, angiosarcomas, mesenchymal kidney tumors, and lymphomas were found in rats and/or mice after exposure to safrole and/or 1′-hydroxysafrole (*23*). In infant Swiss mice, pulmonary adenomas and pulmonary adenocarcinomas were found (*22*) and also in preweanling CD-1 mice exposed to safrole and 1′-hydroxysafrole (non significant) increases in lung adenomas were observed (*24*). In adult female A/J mice exposed to 1′-hydroxysafrole-2′,3′-oxide, significant increases in lung adenomas were found (*25*). Low incidences of papillomas of the forestomach and sarcomas were observed in rats fed 1′-hydroxysafrole (*24*). Skin papillomas were found in mice treated topically with 1′-hydroxysafrole-2′,3′-oxide (*23,25*) and safrole-2′,3′-oxide (*25*) followed by application of the tumor promoter croton oil.

Studies by Vesselinovitch *et al.* (*26*) indicate that safrole might be a transplacental and lactational carcinogen. In transplacentally exposed mice, 3.2% of the male mice (2/63) and 0% of the female mice bore hepatomas. In lactation treated mice, 33% of the male mice (28/85) and 3% of the female mice (2/80) had hepatomas. In female mice exposed to safrole both via the placenta and via lactation, 7% bore kidney epithelial tumors (14/199), and these tumors were not found in male mice and in the controls.

Estragole

Estragole and 1′-hydroxyestragole induced hepatic tumors in male preweanling CD-1, $B6C3F_1$, and C3H/HE mice (*24,27,28*). Estragole was more hepatocarcinogenic for male than for female newborn CD-1 mice and estragole-2′,3′-oxide induced a non-significant increase in hepatocarcinomas, when administered during the preweanling period (*24*). Estragole and 1′-hydroxyestragole induced hepatic tumors when administered via the diet to adult female CD-1 mice (*24*). The formation of hepatomas in male newborn $B6C3F_1$ mice after intraperitoneal exposure to estragole was significantly inhibited by previous treatment with pentachlorophenol, a sulfotransferase inhibitor (*29*). This indicates that a sulfate metabolite is involved in the process of carcinogenesis induced by estragole (*28*).

Besides hepatic carcinomas, also a few incidences of lungadenomas, lymphomas, and sarcomas were observed in the experiments performed by Miller *et al.* (*25*). In adult female A/J mice exposed to 1′-hydroxyestragole-2′,3′-oxide, significant increases in lung adenomas were found (*25*). Skin papillomas were induced in mice treated topically with estragole-2′,3′-oxide and 1′-hydroxyestragole-2′,3′-oxide followed by treatment with the tumor promoter croton oil (*25*). The carcinogenicity of estragole and its metabolites have not be studied in rats, apart from one study in which male Fischer rats were treated subcutaneously with 1′-hydroxyestragole, estragole-2′,3′-oxide, and 1′-hydroxyestragole-2′,3′-oxide, but in which no significant increases in hepatic carcinomas or sarcomas at the injection site were found (*24*).

Methyleugenol

Miller *et al.* (*25*) showed that methyleugenol and 1′-hydroxymethyleugenol induced hepatic neoplasms in male B6C3F$_1$ mice after preweanling exposure to in total, respectively, 4.75 and 2.85 μmol (= 0.93 and 0.55 mg), via intraperitoneal injections. A two-year toxicity and carcinogenicity study was performed by the National Toxicology Program (NTP) in which F344/N rats and B6C3F$_1$ mice were exposed by gavage to a suspension of methyleugenol in 0.5% methylcellulose at doses of 0, 37, 75, or 150 mg methyleugenol/kg bw/day (*30,31*). In rats, increased incidences of among others hepatic neoplasms and neuroendocrine tumors of the glandular stomach were observed in both sexes, and increased incidences of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma, and subcutaneous fibroma or fibrosarcoma were observed in male rats. In B6C3F$_1$ mice, among others increased incidences of hepatic neoplasms (in both sexes) and neuroendocrine tumors of the glandular stomach (in males) were observed. Under the conditions of this study, there was clear evidence of carcinogenicity of methyleugenol in male and female F344/N rats and B6C3F$_1$ mice and the results from the study indicate that methyleugenol is a multisite, multispecies carcinogen. Neoplasms were observed in all exposure groups.

Summary

In summary, all three alkenylbenzenes are multisite carcinogens that exert their major carcinogenic effects in the liver. The hepatocarcinogenicity proceeds mainly via the 1′-hydroxymetabolites, but also estragole-2′,3′-oxide and 1'-hydroxysafrole-2′,3′-oxide have been shown to cause hepatocarcinogenic effects in some studies. The epoxides from the parent compounds or their 1′-hydroxymetabolites are to some extent involved in the formation of lung and skin carcinomas. Safrole and methyleugenol are proven to be multispecies carcinogens, but for estragole, rat carcinogenicity data are lacking. However, since the effects observed in mice are similar for all three alkenylbenzenes, it is likely that estragole will also exert carcinogenic effects in rats.

Genotoxicity

Genotoxicity in various in vitro and in vivo tests

In bacterial mutagenicity tests such as the Ames test in which various strains of *Salmonella typhimurium* and *Escherichia coli* were used, generally negative or weakly positive results were obtained for safrole, estragole, and methyleugenol in the absence of metabolic activation. Adding S9 homogenates and the cofactor(s) NADPH and/or PAPS to the test systems led in some assays to increased mutagenicity of these compounds (reviewed in refs *1-3*). Safrole induced intra-chromosomal recombination in *Saccharomyces cerevisiae* with and without metabolic activation (*32*). Safrole was positive in various mammalian cell genotoxicity assays such as chromosomal aberration assays and sister chromatid exchange assays (*3*). In the Drosophila wing somatic mutation and recombination (SMART) test, safrole only exerted a positive response in the presence of metabolic activation (*34*). *In vivo* genotoxicity of safrole was proven by a sister chromatid exchange assay in F344 rats (*33*). Estragole did not induce chromosomal aberrations in V79 hamster cells (with and without S9) and in rat hepatocytes (*2*). Methyleugenol was not mutagenic in a chromosomal aberrration assay in chinese hamster ovary (CHO) cells and a micronucleus assay in mice (*1,31*). Methyleugenol induced sister chromatid exchanges (SCEs) in CHO cells but only in the presence of S9 homogenate (*1*). Furthermore, methyleugenol induced intra-chromosomal recombination in *Saccharomyces cerevisiae* with and without metabolic activation (*32*). The most convincing evidence for the genotoxicity of the alkenylbenzenes safrole, estragole, and methyleugenol, however, comes from results obtained in the unscheduled DNA synthesis test and from the detection of DNA adduct formation, discussed in the next sections.

Unscheduled DNA synthesis test

Howes *et al.* (*35*) reported an excellent correlation between results from *in vitro* unscheduled DNA synthesis (UDS) assays with known rodent carcinogenicity for safrole, estragole, and methyleugenol. In the UDS assay, DNA excision repair is measured after chemical damage to the DNA by a genotoxic compound. For this test, most often freshly isolated rat or mouse hepatocytes are used, and these contain the relevant metabolic enzyme systems involved in the metabolic activation of the alkenylbenzenes to their carcinogenic metabolites. In rat hepatocytes, safrole, estragole, and methyleugenol all induced dose-related increases in UDS (*35,36*). Furthermore, the 1′-hydroxymetabolites of safrole, estragole, and methyleugenol all exerted a greater UDS response than the parent compounds (*36*). The dose-response curves for UDS induction obtained for both the parent compounds and their 1′-hydroxymetabolites were not linear, but relatively higher UDS responses were observed at higher concentrations. Possible explanations given by the authors for these observations were saturation of detoxification, relative increase in 1′-hydroxylation at higher doses, or overloading of DNA repair mechanisms (*36*). Also in mouse hepatocytes, methyleugenol and safrole induced UDS, and the responses were higher than in rat hepatocytes. These UDS responses were inhibited when the sulfotransferase inhibitor pentachlorophenol was

coincubated (*37*). Estragole and basil oil containing 88% estragole gave positive responses in both an *in vitro* and an *ex vivo* UDS test and had a similar potency. *In vitro*, estragole and basil oil were positive in the 10^{-5} – 10^{-3} M (based on estragole content) range. *Ex vivo*, positive responses were obtained only at 2 g/kg bw, the highest dose tested (*12*).

DNA adducts

In mice given cola drinks instead of drinking water for up to 8 weeks, significant levels of covalent liver DNA adducts were observed which were not found in mice given drinking water or non-cola beverages. Small amounts of these DNA adducts were derived from safrole (*38*). Areca (betel nut) quid contains safrole, and chewing of areca quid is associated with the presence of safrole-DNA adducts in human peripheral blood (*7*).

Safrole, estragole, and methyleugenol DNA adducts were found in samples from livers of adult female CD-1 mice that were isolated 24 h after intraperitoneal administration of the test compound (2 or 10 mg/mouse). Two major adducts which appeared to be guanine adducts were found for all three alkenylbenzenes and were comparable to the two major adducts formed *in vitro* by the electrophile 1′-acetoxysafrole with mouse liver DNA. The structures of the adducts formed in these experiments were elucidated and the structures of the five main DNA adducts are shown in Fig. 1.2 (*39-42*). The adducts shown are estragole-DNA

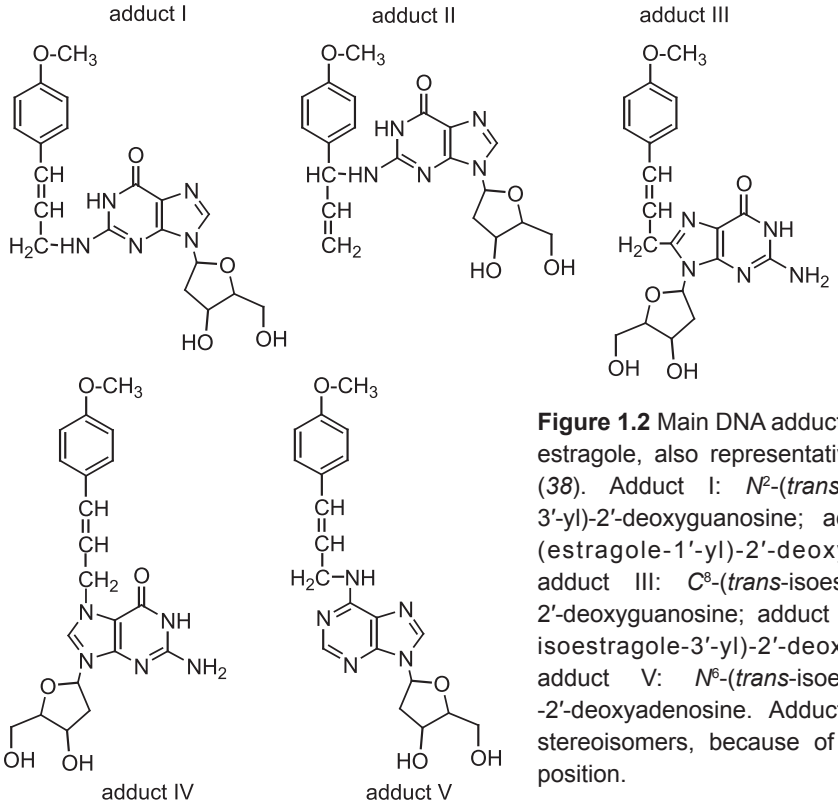


Figure 1.2 Main DNA adducts formed with estragole, also representative for safrole (*38*). Adduct I: N^2-(*trans*-isoestragole-3′-yl)-2′-deoxyguanosine; adduct II: N^2-(estragole-1′-yl)-2′-deoxyguanosine; adduct III: C^8-(*trans*-isoestragole-3′-yl)-2′-deoxyguanosine; adduct IV: N^7-(*trans*-isoestragole-3′-yl)-2′-deoxyguanosine; adduct V: N^6-(*trans*-isoestragole-3′-yl)-2′-deoxyadenosine. Adduct II has two stereoisomers, because of the chiral 1′ position.

adducts but for safrole, analogous adducts were found (*41*) and for methyleugenol probably the same adducts are relevant. The major adduct formed was N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine (Fig. 1.2, adduct I), which can arise after an allylic shift of the carbocation prior to the reaction with the exocylic amino group of 2′-deoxyguanosine (*41,42*). Also in safrole-treated F344 rats, two major and two minor adducts were found (*33*). Pentachlorophenol inhibited the binding of safrole with mouse liver DNA, indicating that the cytochrome P450 and sulfotransferase catalyzed bioactivation pathway leading to 1′-hydroxymetabolites and 1′-sulfooxymetabolites leads to the genotoxicity of the alkenylbenzenes (*39*). In addition, safrole-2′,3′-oxide and estragole-2′,3′-oxide formed DNA adducts *in vitro*, but *in vivo* these adducts were not found after exposure to these epoxides or their parent compounds. This indicates that these epoxides are not related with the *in vivo* genotoxic properties of safrole and estragole (*43*).

In newborn male $B6C3F_1$ mice, DNA binding was quantified for methyleugenol (72.7 pmol/mg DNA), estragole (30 pmol/mg DNA), and safrole (17.5 pmol/mg DNA) after exposure to similar amounts of all three alkenylbenzenes (*40*). Safrole DNA adducts were also found in fetal tissues 24 h after oral exposure of the mothers to 600 μmol safrole. Both in maternal and in fetal tissues, safrole preferentially bound to DNA of the liver (*44*).

In addition to DNA adducts, Gardner *et al.* (*45*) immunochemically detected covalently modified protein adducts in livers of rats treated with methyleugenol (10 – 300 mg/kg bw/day for 5 days intraperitoneally). A 44 kDa adduct was the only adduct detected at 10 mg/kg bw/day and the major protein adduct detected at higher concentrations. This adduct was similar to the adduct formed in hepatocytes exposed to 1′-hydroxymethyleugenol, indicating that methyleugenol protein adduct formation also proceeds via the 1′-hydroxymetabolite (*45*). Similar experiments were performed by Wakazono *et al.* (*46*) with estragole. A 155 kDa and a 44 kDA adduct were most abundant in livers of rats treated with estragole (300 mg/kg bw/day for 5 days by gavage), and also adducts of 25, 100, and 170 kDa were observed. The pattern of adducts was similar to that found in hepatocytes and recombinant V79 cells treated with 1′-hydroxyestragole (*46*).

Summary

Safrole, estragole, and methyleugenol are genotoxic carcinogens, and most evidence for their genotoxicity arises from results from UDS tests and by quantification and identification of the DNA adducts formed in liver tissue or *in vitro*. In several other genotoxicity assays, the response of the alkenylbenzenes was highly dependent on the presence and extent of adequate bioactivation in the test system used. Based on the outcomes of these experiments, it is clear that their genotoxicity proceeds via P450 catalyzed 1′-hydroxylation followed by sulfation by sulfotransferases.

Absorption, distribution, metabolism, and excretion

General

Safrole, estragole, and methyleugenol are rapidly absorbed, metabolized, and excreted after oral administration (*10,30,47-52*). Small amounts of orally administered safrole were rapidly absorbed and metabolized and were almost entirely excreted within 24 h in the urine in rat (0.6 mg/kg bw; 88% excreted) and man (0.165 mg and 1.655 mg per person; 92% excreted) (*51*). When the dose was increased from 0.6 to 750 mg/kg bw for the rat, a delay in metabolism and elimination was observed, since only 25% of the dose was excreted in the urine within 24 h, but still 90% of the dose was excreted within 4 days via urine and faeces (*51*). Safrole is also eliminated via the bile, but reabsorption of safrole or safrole metabolites in the intestine via enterohepatic circulation probably caused the low amount of safrole metabolites (< 3% of the dose) found in faeces (*51*). Safrole can also cross the placenta and can be excreted in breast milk (*26,44*).

For methyleugenol, the time to achieve maximum concentrations in blood in rats and mice after dosing by oral gavage (37 mg/kg bw up to 300 mg/kg bw) was 5-15 min (*30*). This indicates that methyleugenol is absorbed directly from the stomach and/or forestomach, since this time is shorter than the time necessary to empty the stomach (*30*). Target organs for methyleugenol include the liver, glandular stomach, forestomach, kidney, mammary gland, and subcutaneous tissue (*30*). In man exposed to methyleugenol via consumption of ginger snap cookies, the plasma peak level was reached 15 min after exposure and a half-life of elimination of 90 min was derived (*10*).

After oral administration of 100 μg of radioactive labelled estragole to two human male volunteers, ≈ 45% of the radioactive dose was recovered after 8 h and ≈ 68% of the radioactive dose could be recovered within 48 h (*52*). In the urine, ≈ 56% was found and in the expired air ≈ 12%. No radioactivity was found in the faeces (*52*). The measurement of the radioactivity in the expired air was stopped after 8 h, and that probably explains why not all radioactivity was recovered, since the results indicated that the excretion via exhalation was not yet finished after 8 h (*52*).

Main metabolic routes

In the early 1970s, it was postulated by Miller *et al.* (*53*) that the ultimate carcinogenic forms of most, if not all, chemical carcinogens are strong electrophilic reactants. Also for safrole, methyleugenol, and estragole, compounds that have only little, if any, electrophilic reactivity, metabolic activation is required to exert their carcinogenic effects. The three principle routes of metabolism start with 1′-hydroxylation, *O*-demethylation, and side-chain epoxidation (Fig. 1.3) (*5,54*) and are explained in the next sections.

1′-Hydroxylation

For safrole, the main bioactivation pathway was unraveled first. 1′-Hydroxysafrole was present in urine of β-glucuronidase treated rats, mice, hamsters, and guinea pigs exposed to

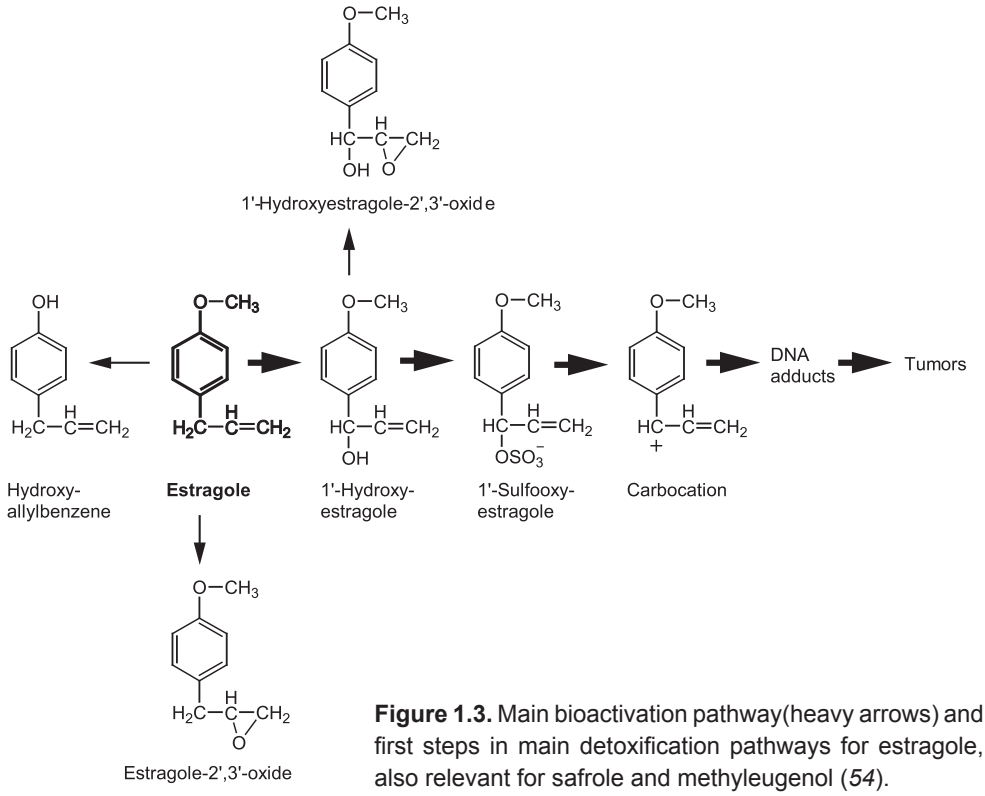


Figure 1.3. Main bioactivation pathway(heavy arrows) and first steps in main detoxification pathways for estragole, also relevant for safrole and methyleugenol (*54*).

safrole and was established as proximate carcinogenic metabolite of safrole (i.e. closer to the ultimate carcinogenic form than safrole) from the much greater hepatocarcinogenicity of 1′-hydroxysafrole as compared to safrole, for rats and mice (*25*). From the finding that 1′-acetoxysafrole reacts electrophilically with nucleosides and methionine and is carcinogenic at the site of administration (*25,55*) it was postulated that an electrophilic ester might be the ultimate carcinogenic metabolite of safrole. 1′-Sulfooxysafrole was the principal ester formed in rat and mouse liver cytoplasms (*56*). Studies with brachymorphic mice, which have a deficiency in the hepatic synthesis of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the cofactor for sulfotranferase reactions, and studies using pentochlorophenol, an inhibitor of sulfotransferase activity (*29*), showed that 1′-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1′-hydroxysafrole in mouse liver (*57*). The carbocation that is formed upon breakdown of 1′-sulfooxysafrole can (with or without isomerization to the 3′-position) bind to proteins and DNA (*58*). Also estragole was excreted by mice, rats, and humans as conjugates of 1′-hydroxyestragole in urine (*50,52,59,60*) and was found in bile of rats (*50*) and it was shown that the 1′-hydroxymetabolite of estragole is more hepatocarcinogenic in mice than the parent compound (*27*). Also 1-2% of 200 mg methyleugenol was excreted by rats as urinary and biliary conjugates of 1′-hydroxymethyleugenol (*47*) and rats treated intraperitoneally with methyleugenol for 5 days exhibited significant auto-induction of 1′-

hydroxylation of the compound from doses of 30 mg/kg bw/day onwards (*61*). Gardner *et al.* (*61*) showed that P450 2E1 and another enzyme, most likely P450 2C6, are important enzymes in this bioactivation step in rats, but the human P450 enzymes involved have not yet been described. This P450- and sulfotransferase- catalyzed bioactivation route is considered to be the most important bioactivation pathway for safrole, estragole, and methyleugenol and the genotoxicity of these compounds depends greatly if not solely on this route.

Glucuronidation of the proximate carcinogenic 1′-hydroxymetabolites instead of sulfation leads to detoxification of those proximate carcinogens and Iyer *et al.* (*62*) reported that UGT2B7 and UGT1A9 are responsible for 1′-hydroxyestragole glucuronidation in humans. In addition, the 1′-hydroxymetabolites can also isomerize to their more stable 3′-hydroxymetabolites, yielding primary alcohols that can be oxidised to produce cinnamic acid derivatives that may undergo β-oxidation and cleavage to yield benzoic acid derivatives. These metabolites may be excreted as glycine conjugates (*47,50*). Oxidation of the 1′-hydroxymetabolites leads to the corresponding ketones that may be excreted or may first react with endogenous amines and may be excreted in the urine as tertiary amino-propiophenones (*47,50,63*).

O-Demethylation

A second important pathway for all three alkenylbenzenes is P450 catalyzed *O*-demethylation, leading to phenolic derivatives that may be excreted as the sulphate or glucuronic acid conjugate in the urine or may be further metabolised to the final metabolite CO_2 and exhaled (*50,59*). In rats and mice *O*-demethylation is the principle route of metabolism for estragole (*50,59,60*) and also in man estragole *O*-demethylation is an important metabolic pathway (*52*). 1′-Hydroxylation and *O*-demethylation of estragole are reported to be dose dependent in rats and mice (see Table 1.3), with the relative contribution

Table 1.3. Influence of dose on the elimination of ^{14}C after administration of ^{14}C-estragole[a] to rats and mice. (Adjusted from (*60*))

dose (mg/kg bw)	% dose in 0-24h urine	% dose in 0-24h $^{14}CO_2$	% dose as 1′-hydroxyestragole in urine	1′-hydroxyestragole formation[b] (nmol/kg bw/24h)
			Rat[c]	
0.05	25	34	0.9	3.1
5	25	33	3.6	1224
1000	52	20	8.0	544000
			Mouse[c]	
0.05	27	38	1.3	4.4
5	25	38	1.5	510
1000	59	22	9.5	646000

[a] ^{14}C-estragole = 4-[^{14}C-methoxyl]-allylbenzene

[b] 1′-hydroxyestragole formation calculated solely from the amount excreted in the urine.

[c] figures represent means of at least 3 animals

of the (bioactivating) 1′-hydroxylation route increasing and the relative contribution of the (detoxifying) O-demethylation route decreasing when the dose increased from 0.05 to 1000 mg/kg bw (*59,60*). This relative decrease in conversion of estragole to its proximate carcinogenic metabolite at lower doses was one of the important arguments for the FEMA to conclude that exposure to estragole and methyleugenol at the low levels resulting from intake of herbs and flavor uses does not pose a significant cancer risk for humans (*5*).

For safrole, O-dealkylation leads to 4-allylcatechol and conjugated forms of this metabolite were the main metabolites excreted in the urine of rat and man (*51*). 4-Allylcatechol can also be oxidised to 4-allyl-o-quinone (Fig. 1.4) (*64,65*). This oxidation generates an electrophilic toxic metabolite (Fig. 1.4) but the role of this quinone in the carcinogenicity of safrole remains to be investigated.

Epoxidation

The third main pathway is epoxidation of the alkenylbenzenes or their 1′-hydroxymetabolites and this leads to metabolites that are genotoxic *in vitro* (*24,28,66*). In several studies, the absence of genotoxicity *in vivo* of these epoxides is shown, and therefore it was previously concluded that this pathway is unlikely to contribute to the genotoxic effects of estragole *(36,37,42,67-70)*. The lack of genotoxicity of the epoxides *in vivo* may be explained by their rapid metabolic inactivation by both glutathione-S-transferases and epoxide hydrolases (*68,71*). Epoxidation may play a role in the cytotoxicity of the alkenylbenzenes at high doses and/or when epoxide detoxification pathways are impaired (*36,37*).

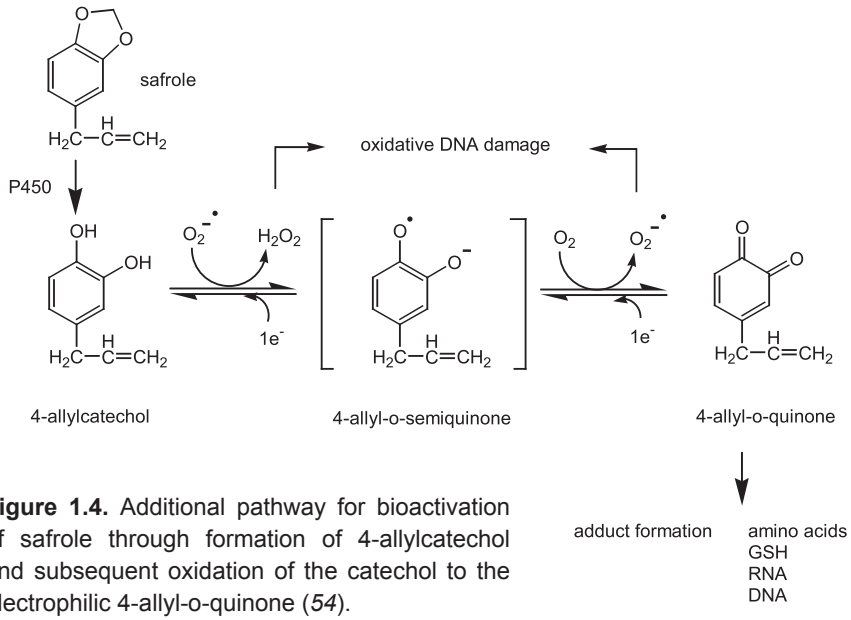


Figure 1.4. Additional pathway for bioactivation of safrole through formation of 4-allylcatechol and subsequent oxidation of the catechol to the electrophilic 4-allyl-o-quinone (*54*).

Biotransformation enzymes involved in the bioactivation of the alkenylbenzenes.

General

A major physiological function of biotransformation in the human body is to convert xenobiotics into hydrophilic metabolites that can be excreted easily in order to maintain homeostasis. To this aim, xenobiotics are modified by Phase I biotransformation enzymes such as cytochrome P450 enzymes or FAD-monooxygenases into more polar metabolites with a functional group by means of hydrolysis, reduction, or oxidation. This enables conjugation with endogenous hydrophilic moieties catalyzed by phase II enzymes such as sulfotransferases, glutathione-S-transferases, N-acetyltransferases, and UDP-glucuronosyl-transferases, which greatly increases the hydrophilicity of the xenobiotics and thus facilitates their excretion. In addition, many toxic and carcinogenic compounds do not cause adverse effects by themselves, but are converted to toxic electrophilic metabolites by the same biotransformation enzymes as described above, a process called bioactivation (*72*). In this section, P450 enzymes and sulfotransferases will be discussed since they are involved in the main bioactivation pathway of safrole, estragole, and methyleugenol. Besides their functions, nomenclature, and tissue distribution, intra- and interspecies differences in these bioactivation enzymes and interactions of dietary ingredients with these bioactivation enzymes will be addressed shortly in this section.

Cytochrome P450 enzyme system

General

Cytochromes P450 are monooxygenase enzymes that incorporate one atom of oxygen into a wide range of substrates. The name cytochrome P450 refers to the absorbance maximum around 450 nm of the complex between ferrous heme iron (Fe^{2+}) of cytochrome P450 and carbon monoxide (*73*). P450 enzymes are ubiquitously expressed in animal and plant species and have various functions. Detoxification of xenobiotics by increasing the hydrophilicity and enabling phase II conjugation reactions is an important task for P450s, but they also play key roles in the biosynthesis or catabolism of steroid hormones, bile acids, fat-soluble vitamins, fatty acids, and eicosanoids (*74*). Furthermore, P450s are involved in the bioactivation of promutagens (*53,72,74*).

Nomenclature and tissue distribution of the human cytochrome P450 superfamily

The human cytochrome P450 gene superfamily is divided into families and subfamilies based on sequence homology (*75*). P450 families (indicated with a number e.g. P450 1) consist of P450 enzymes that share at least 40% sequence identity and P450 subfamilies (indicated with a capital letter (e.g. P450 1A) share at least 55% identity (*75*). Individual genes are indicated with an additional number (e.g. P450 1A1) and allylic variants are indicated with an asterix and another additional number (e.g. P450 1A1*1). At the Home Page of the Human

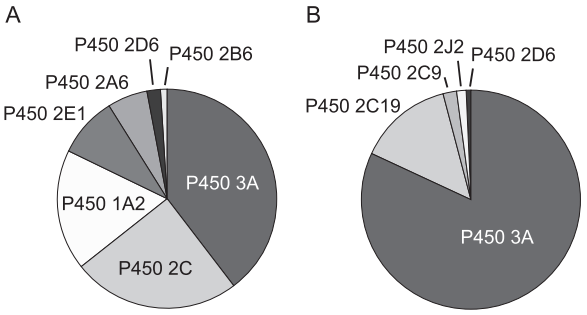


Figure 1.5. The P450 enzyme content of the liver and the intestine as determined by Shimada *et al.* (*77*) and Paine *et al.* (*76*), respectively. The contributions of individual P450 enzymes are based on total immunoquantified P450 content.

Cytochrome P450 Allele Nomenclature Committee (http://www.cypalleles.ki.se/) allele nomenclature for least 8 gene families and at least 26 isoforms is described. The majority of drugs and xenobiotics are metabolized in the human liver by enzymes of only 3 P450 gene families (P450 1, P450 2, P450 3). The liver is the organ which not only expresses most of the different cytochrome P450 genes, but also contains the highest P450 amounts, and is thereby the major site of the metabolism of xenobiotics, drugs, and endogenous compounds. However, P450 enzymes are present in virtually all tissues. After the liver, the duodenum is the second important site of phase I metabolism, and together the duodenum and the liver are responsible for the so-called first-pass metabolism of drugs and xenobiotics, and they reduce the amount of unchanged drugs or xenobiotics that reaches the systemic circulation (*76*). In Fig. 1.5, the P450 enzyme content as determined by Shimada *et al.* (*77*) and Paine *et al.* (*76*) for liver and intestine, respectively, is shown. In both organs, P450 3A is the most abundant subfamily, followed by P450 2C. In this thesis, the roles of P450 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and P450 3A4 will be studied in the bioactivation of the three herb-based alkenylbenzenes, since these enzymes are most abundant in the human liver (see Fig. 1.5).

Sulfotransferases

General

In mammals, two classes of sulfotransferases can be distinguished. The cytosolic sulfotransferases (SULT) catalyze the sulfonation of a wide range of drugs, xenobiotics, and endogenous compounds, and will be discussed in this section. The other class of sulfotransferases is mainly membrane-bound and located in the Golgi apparatus, and metabolizes macromolecular endogenous structures. No xenobiotic-metabolizing activities are reported for this second class of sulfotransferases (*78*). Sulfonation involves the transfer of a sulfonate group (SO_3^-) of the endogenous cofactor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to an oxygen, nitrogen, or sulphur atom of acceptor molecules thereby forming sulfates/sulphuric acid esters, sulfamates, or thiosulfates, respectively (*79*). In this thesis, only *O*-sulfonation (sulfation) will be discussed, since sulfation is involved in the bioactivation of safrole, estragole, and methyleugenol (as described previously). Sulfation serves various biological functions in mammalian species (*80*). Sulfation is an important

detoxification process since it leads to more hydrophilic metabolites that can be readily excreted from the body. However, since the sulfate group is an electron-withdrawing group in some molecules, including the 1′-sulfooxymetabolites of the alkenylbenzenes, it may be cleaved of heterolytically. This leads to the formation of a strongly electrophilic cation, resulting in a role for sulfation in carcinogenesis. Sulphuric esters are the major ultimate carcinogenic metabolites of many procarcinogens derived from benzylic/allylic alcohols and hydroxyl amines (*81*). Other roles of sulfation are the formation of the precursors dehydroepiandrosterone sulfate and estrone sulfate for the biosynthesis of androgens and estrogens (*82*) and the inactivation and excretion of endogenous compounds such as thyroid hormones and catechols (*80*). In addition, some pharmaceuticals are converted by means of sulfation into their pharmacologically active forms (*80*). Substrates for SULTs can often also be glucuronidated by UDP-glucuronosyltransferases. Sulfation is considered to be a high-affinity, low-capacity pathway whereas the UDP-glucuronosyltransferases catalyzed route is regarded as a low-affinity, high-capacity pathway (*74,83*). The limitations in SULT metabolism are partly caused by the relatively low intracellular concentration of PAPS (~ 75 μM) compared to the concentration of UDP-glucuronic acid (~ 350 μM), the cofactor for UDP-glucuronosyltransferases (*74*). This suggests that SULTs may be more important than UDP-glucuronosyltransferases in the metabolism of low-dose exogenous compounds (*83*).

Nomenclature and tissue distribution

All human cytosolic sulfotransferases belong to one superfamily named SULT (*84*). A classification of the SULT superfamily into families (indicated by a number, e.g. SULT1), subfamilies (indicated by an additional capital letter, e.g. SULT1A), individual genes (indicated by an additional number, e.g. SULT1A1), and different enzyme proteins due to splice variants (indicated by an asterix and another additional number, e.g. SULT1A1*1) has been developed (*85*). To date, five distinct gene families of SULT have been identified in mammals, SULT1, SULT2, SULT3, SULT4, and SULT5. In humans, at least 13 SULT forms, belonging to three SULT families, are known and are shown in Table 1.4 (*78,83,86*). The main enzymes that are present in the liver are SULT1A1, SULT2A1, and to some extent SULT1B1, SULT1E1, and SULT1A2 (as reviewed by Glatt and Meinl in refs *78* and *86* and summarized in Table 1.4).

Interspecies differences

Metabolism data obtained in animal experiments or from *in vitro* experiments using animal tissue fractions may not always reflect the metabolism in man, due to possible interspecies differences in the biotransformation enzymes involved. This may also be the case for the alkenylbenzenes safrole, estragole, and methyleugenol, since P450 enzymes and sulfotransferase enzymes exhibit interspecies differences. A rough classification for the reliability of extrapolation of P450 catalytic activities across mammalian species was described in a review on interspecies differences by Guengerich (*87*) and later experiments by Bogaards *et al.* (*88*) strengthened this classification. According to this classification,

Table 1.4. Overview of human sultotransferases based on three reviews *(78,83,86)*

Name	Trivial names/synonyms	Tissues expression	
		Hepatic	Extrahepatic
SULT1A1	Phenol-preferring phenol sulfotransferase (P-PST), P-PST 1, thermostable phenol sulfotransferase (TS PST), human aryl sulfotransferase (HAST) 1 or 2, ST1A3	High	Lower levels in many other tissues
SULT1A2	P-PST 2, TS PST2, ST1A2, HAST4, HAST4v	Low	Low
SULT1A3	Catecholamine-preferring phenol sulfotransferase (M-PST), hm-PST, thermolabile phenol sulfotransferase (TL-PST), ST1A5, HAST3, HAST5, hEST,	Negligible	High in the gut, detectable in many other tissues
SULT1A4	-	?	?
SULT1B1	Thyroid hormone sulfotransferase, ST1B2	Low	Gastro-intestinal tract, spleen, leukocytes, highest levels in colon mucosa
SULT1C2	ST1C2, SULT1C1, HAST5	Low/ND	Kidney, stomach, thyroid gland, ovary, brain, various fetal tissues
SULT1C4	ST1C3, SULT1C2, hSULT1C	Low/ND	Various fetal tissues, adult ovary, kidney, spinal cord
SULT1E1	Estrogen sulfotransferase (EST), ST1E4, hEST, hEST-1	Detectable	Small intestine, endometrium, cultured mammary cells
SULT2A1	HST, ST2A3, hydroxysteroid or dehydro-epiandrosterone sulfotransferase (DHEA-ST), DHEA-ST8, hSTa	High	High in fetal adrenal gland, adult adrenal gland, adult small intestine
SULT2B1_v1	hSULT2B1a	Low/ND	Placenta, prostate, lung
SULT2B1_v2	hSULT2B1b	Low/ND	Placenta, prostate, lung
SULT4A1_v1	SULT X3, brain SULT-like protein, ST5A1	Low/ND	High in brain
SULT4A1_v2	hSULT4A1, SULTX3	Low/ND	High in brain

extrapolation across mammalian species holds well for P450 2E1, has to be done with some caution for P450 1A2 and with more caution for P450 2D and 3A, and gives major problems with respect to P450 2A, 2B, and 2C. For extrapolation of animal data on SULT catalyzed bioactivation, also caution is required. Several SULT enzymes are present in humans and absent in rodents (e.g. in humans, four members of the SULT1A subfamily exist, whereas in rodents the SULT1A family has just a single member), but also the opposite is known

(humans have just one member of the SULT2A family whereas in rodents four members of the SULT2A family exist). Also, in humans, SULTs are widely distributed in the human body (see Table 1.4) whereas in rodents, the SULTs are mainly expressed in the liver. In addition, in rodents, several SULT forms exhibit sexual dimorphisms (*89*).

Interindividual differences

Besides these interspecies differences, also intraspecies differences, also referred to as interindividual differences, occur in P450 enzymes and SULTs. Interindividual differences in expression and activity of biotransformation enzymes might alter the individual's susceptibility towards carcinogens or other toxic compounds that are bioactivated and/or detoxified by these enzyme systems. For P450 enzymes and sulfotransferases, involved in the bioactivation and detoxification of safrole, estragole, and methyleugenol, the expression and activity of different enzymes varies in the human population due to genetic differences (mutations) and lifestyle factors (exposure to inducers/inhibitors).

For the different P450 enzymes, numerous allelic variants are described (http://www.cypalleles.ki.se/), and for most hepatic P450 enzymes, except P450 2E1 and P450 3A4, polymorphisms that are of importance *in vivo* are reported (*90,91*). These mutations can lead to enzymes with abolished, reduced, altered, or increased activity and can correspond with for example poor metabolizer phenotypes or ultra-rapid metabolizer phenotypes (*90*). The latter phenotype can also be caused by gene duplication or multiplication, which has been described for P450 2D6 and P450 2A6 (*90*). Genetic polymorphisms in P450 1A2, 2C9, 2C19, 2D6, and 3A4 are linked with adverse drugs reactions, due to altered metabolic clearance of certain drugs (*92*). For sulfotransferases, less data on genetic polymorphisms are available. Genetic polymorphisms are described for SULT1A1 and SULT1A2 (*93*,*94*) and a bimodal distribution of dehydroepiandrosterone sulfotransferase (SULT2A1) activities in 94 individual liver biopsies is described, which indicates that also for this enzyme a genetic polymorphism is likely to exist (*95*).

Besides these genetic influences on expression and activity of biotransformation enzymes, also lifestyle factors such as dietary and drinking habits and use of drugs are important causes for interindividual differences (*96*). Examples of such lifestyle factors are smoking (*97*) and the consumption of charbroiled food and cruciferous vegetables (*98*) which induce P450 1A enzymes, and the induction of P450 2E1 by consumption of alcohol (*99,100*).

Interactions of dietary ingredients with P450 enzymes and sulfotransferases

Dietary components may interfere with P450 catalyzed bioactivation and detoxification and with sulfation. This may lead to modulation of the bioactivation and subsequent adverse effects caused by dietary pro-carcinogens or pro-mutagens (*101,89*). Numerous polyphenols and ingredients of amongst others red wine, green tea, and coffee are reported to inhibit various SULT enzymes (*89*). Herbal interactions with P450 enzymes are reviewed by Zhou *et al.* (*102*), and competive, non-competitive, and mechanism based inhibition of several P450 forms are described for various herbal ingredients belonging to the chemical classes

of triterpenoids, anthraquinones, polyphenols, and alkaloids. For herb-based exposure to alkenylbenzenes, these possible P450 and SULT mediated modulating matrix effects may influence the risks associated with the consumption of herb-based alkenylbenzenes, but these effects are generally not well described and as such not (yet) taken into account in the risk assessment.

To study such matrix effects, assays to screen herbal extracts and extracts from other dietary products for the presence of bioactive compounds that are able to inhibit the enzymes of interest are necessary. Especially for P450 inhibition, many assays are described in literature. These assays include well plate assays to determine inhibition of P450 enzymes based on conversion of suitable substrates in corresponding fluorescent metabolites (*103-106*). These assays are suitable for high throughput screening of single compounds on their inhibition of P450 activity. For complex mixtures such as herbal extracts, such fluorescence-based assays are less suitable. Measurements may be disturbed by intrinsic fluorescence or quenching of fluorescence by compounds present in the mixture (*107*). In addition, also P450 assays that screen single compounds or mixtures for the inhibiting effect on different enzymes simultaneously, using cocktails of enzyme-selective substrates in combination with LC-MS analyses, have been reported (*108, 109*). However, bioassay guided fractionation remains necessary to identify individual active compounds in a complex mixture, and this is difficult and time-consuming.

Systems in which a separation technique is coupled on-line with a biochemical detection method, also called high resolution screening (*110*), detect bioactive compounds in a faster way. By including diode array detection (DAD), evaporative light scattering detection (ELSD), and/or mass spectrometry (MS) together with an on-line assessment of activity in a bioassay, identification and quantification of bioactive compounds can be done simultaneously. The coupling of high performance liquid chromatography (HPLC) separation of herbal extracts with post-column assays for radical scavenging activity has been developed and applied successfully (*111,112*) and hyphenated systems are also described for amongst others acetylcholine esterase inhibitors (*113*), estrogen receptor α and β ligands (*114*), and phosphodiesterase inhibitors (*115*). In this thesis, an on-line system will be developed that can be used to study the presence of inhibitors of the P450 enzymes in herbal and other dietary extracts.

Objective and outline of this thesis

Safrole, estragole, and methyleugenol are genotoxic carcinogens and their genotoxicity proceeds via electrophilic metabolites generated by P450 enzymes and sulfotransferases. Their carcinogenicity is demonstrated in animal experiments using high doses of single compounds. Human exposure to these compounds is lower and arises mainly from consumption of food that naturally contains these compounds (65% and 70% of the daily intake for respectively methyleugenol and estragole), but also from the use of essential oils containing these alkenylbenzenes, and from food to which estragole and methyleugenol have been added as such. Different expert judgements about the risks associated with human

exposure to these alkenylbenzenes indicate that more insight in the bioactivation of these compounds in humans is necessary to facilitate extrapolation of animal experimental data to the human situation. Such insight enables prediction of intraspecies and interindividual differences in the bioactivation of these alkenylbenzenes.

Furthermore, a risk assessment for herb-based exposure to low doses of alkenylbenzenes should take into account the potential modulating effects of other bioactive ingredients present in herbs at the level of the P450 and SULT catalyzed bioactivation steps. Compounds present in herbs that inhibit the P450 enzymes or sulfotransferases involved in the bioactivation of estragole might decrease the chances on the adverse effects of estragole present in the same herbs.

The first aim of this thesis was to identify the human cytochrome P450 enzymes involved in the bioactivation of safrole, methyleugenol, and estragole to their proximate carcinogenic 1′-hydroxymetabolites and to unravel the interindividual differences in the chances on this bioactivation caused by lifestyle factors or genetic polymorphisms. The second aim of this thesis was to study the influence of other herbal ingredients on the bioactivation and the genotoxicity of herb-based alkenylbenzenes.

Chapter 1 gives an overview on the literature on these three alkenylbenzenes especially regarding their metabolism and their carcinogenic and genotoxic properties. In addition, this chapter gives background information on the biotransformation enzymes involved in the bioactivation of these compounds. **Chapters 2-4** present the identification of the human P450 enzymes involved in respectively safrole, methyleugenol, and estragole 1′-hydroxylation by means of a combination of *in vitro* approaches. In addition, in **Chapter 4**, the enzyme specificities for the three compounds and the lifestyle-factors and genetic polymorphisms influencing the bioactivation of the three compounds are compared. In **Chapter 4**, dietary interactions between alkenylbenzenes at the level of the P450 catalyzed bioactivation were modelled using the kinetic data obtained in **Chapters 3** and **4.** Besides the competitive interactions between the different alkenylbenzenes at the P450 level, also the possible presence of P450 and SULT inhibitors in herbs which might decrease the bioactivation and the genotoxicity of the alkenylbenzenes was investigated in this thesis. **Chapters 3** and **4** indicate that P450 1A2 is an important enzyme in both methyleugenol and estragole 1′-hydroxylation. Therefore, in **Chapter 5** the presence of possible P450 1A2 inhibitors in basil was studied. Basil was chosen as a model herb, since this herb is consumed frequently and contains relatively high levels of both methyleugenol and estragole (*13,14*). To identify possible P450 1A2 inhibitors in basil, an HPLC on-line system for the detection of inhibitors of P450 1A2 was developed based on the methoxyresorufin *O*-demethylation assay and the development and application to basil extract of this system is described in **Chapter 5**. In **Chapter 6**, the influence of a basil extract on the SULT mediated DNA adduct formation of 1′-hydroxyestragole was investigated by measuring the formation of estragole-DNA adducts by rat and human liver S9 homogenates *in vitro* and in the human HepG2 liver cell line. **Chapter 7** summarizes the data presented in this thesis and comments on the implications of these findings for risk assessment for the use of safrole, methyleugenol, and estragole as food additives and for the use of these compounds in their natural matrixes.

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Human cytochrome P450 enzyme specificity for bioactivation of safrole to the proximate carcinogen 1′-hydroxysafrole.

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Abstract

In the present study, the cytochrome P450 mediated bioactivation of safrole to its proximate carcinogenic metabolite, 1′-hydroxysafrole, has been investigated for the purpose of identifying the human P450 enzymes involved. The 1′-hydroxylation of safrole was characterized in a variety of *in vitro* test systems, including Supersomes, expressing individual human P450 enzymes to a high level, and microsomes derived from cell lines expressing individual human P450 enzymes to a lower, average human liver level. Additionally, a correlation study was performed, in which safrole was incubated with a series of 15 human liver microsomes, and the 1′-hydroxylation rates obtained were correlated with the activities of these microsomes towards specific substrates for nine different enzymes. To complete the study, a final experiment was performed in which pooled human liver microsomes were incubated with safrole in the presence and absence of coumarin, a selective P450 2A6 substrate. On the basis of the results of these experiments, important roles for P450 2C9*1, P450 2A6, P450 2D6*1, and P450 2E1 were elucidated.

The possible consequences of these results for the effects of genetic polymorphisms and life style factors on the bioactivation of safrole are discussed. Polymorphisms in P450 2C9, P450 2A6, and P450 2D6, leading to poor metabolizer phenotypes, may reduce the relative risk on the harmful effects of safrole. Life style factors, such as the use of alcohol, an inducer of P450 2E1, and barbiturates, inducers of P450 2C9, and polymorphisms in P450 2D6 and P450 2A6, leading to ultraextensive metabolizer phenotypes, may increase the relative risk.

Introduction

Alkenylbenzenes such as safrole, methyleugenol, and estragole are important constituents of herbs such as nutmeg, cinnamon, anise star, tarragon, sweet basil, sweet fennel, and anise vert. Recently, the EU Scientific Committee on Food (EU-SCF) launched scientific evaluations on these three alkenylbenzenes *(1-3)*. The EU-SCF concluded that safrole, methyleugenol, and estragole are genotoxic and carcinogenic and indicated restrictions in use. Methyleugenol and estragole are used as flavoring agents in a variety of consumer dietary products (baked goods, nonalcoholic beverages, condiments, ice cream, and chewing gum, as well as hard and soft candies). Safrole, however, has already been banned as an additive in food by the U.S. Food and Drug Administration (Federal Register of December 3, 1960, 25 FR 12412) and by the Council of the European Communities *(4)*.

In contrast to the EU-SCF panel, an industrial expert panel from the Flavor and Extract Manufacturers' Association (FEMA) published that exposure to methyleugenol and estragole, resulting from spice consumption, does not pose a significant cancer risk for humans *(5)*. These opposite expert judgments mainly result from a general problem in risk assessment studies. This is the absence of adequate scientific data to support unequivocal translation of carcinogenicity data of rodent animal experiments to the human situation. One of the important issues that needs to be solved to enable better judgment of the risk posed by these alkenylbenzenes, is the identification of the cytochrome P450 enzymes involved in the bioactivation of these alkenylbenzenes to their proximate carcinogenic 1′-hydroxymetabolites.

Some data from animal experiments exist, especially for methyleugenol *(6)*, showing that phenobarbitone, isosafrole, and dexamethasone induced rat liver microsomes are especially active in the 1′-hydroxylation of this alkenylbenzene. In combination with the outcomes of studies with specific inhibitors for individual P450 enzymes, the authors suggested P450 2E1 and another enzyme, most probably P450 2C6, to be the P450 enzymes involved, whereas they excluded P450 3A, 1A2, 2D1, and 2C11 from being involved. However, more specific studies seem to be required to unequivocally identify the P450 enzymes responsible for the 1′-hydroxylation of these alkenylbenzenes. In the present study, safrole was chosen as the model alkenylbenzene and human microsomal preparations were used. The aim of the present study was to identify the human P450 enzymes responsible for the conversion of safrole to its proximate carcinogenic metabolite 1′-hydroxysafrole. 1′-Hydroxylation of safrole is believed to be the first step in the major bioactivation pathway giving rise to the genotoxicity of safrole *(7-9)*. This pathway, which is the same as the main bioactivation route for the structurally related alkenylbenzenes methyleugenol and estragole, is shown in Fig. 2.1 and consists of P450 dependent 1′-hydroxylation of safrole, followed by the conversion to 1'-sulfooxysafrole by sulfotransferase enzymes. Cleavage of the sulfate moiety will lead to an electrophilic carbocation, which can covalently bind to macromolecules and cause DNA damage *(7-9)*.

In the present study, the role in the 1′-hydroxylation of safrole of the individual P450 enzymes P450 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, which are the most

important enzymes, present in the human liver and involved in drug and xenobiotics metabolism, was studied. Safrole was incubated with Supersomes, expressing these enzymes to a high level and Gentest microsomes, prepared from lymphoblast cell lines expressing the same enzymes to an average liver level. Additionally, safrole was incubated with microsomes obtained from 15 individual human livers. The rates of 1′-hydroxylation found in these microsomes were correlated with the activities towards specific substrates for each enzyme. The results obtained with the Supersomes will give information about the intrinsic capacity of each P450 enzyme to 1′-hydroxylate safrole. The results from the incubations with Gentest microsomes and the correlation experiment will give information on the contribution of each individual P450 enzyme in the 1′-hydroxylation of safrole, taking into account the relative concentrations of the enzymes in the liver. The data of these experiments are especially required to enable a better definition of genetic human polymorphisms, but also life style factors that may influence human cancer risks as a result of exposure to safrole.

Materials and Methods

Chemicals

$MgCl_2 \cdot 6H_2O$, ascorbic acid, and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid and ammonium chloride (NH_4Cl) were from Acros (Geel, Belgium). Glucose-6-phosphate, $NADP^+$, and NADPH were obtained from Boehringer (Mannheim, Germany). Glucose-6-phosphate dehydrogenase and HCl (37%) were purchased from Roche Diagnostics (Mannheim, Germany). Safrole and coumarin were obtained from Sigma (St Louis, MO). Tris(hydroxymethyl)aminomethane was obtained from Gibco BRL Life Technologies (Paisley, Scotland). Acetonitrile and methanol were high performance liquid chromatography (HPLC) grade from Lab-Scan, Analytical Sciences (Dublin, Ireland). Piperonal, vinylmagnesium bromide (1 M solution in THF), diethyl ether, magnesium sulphate, and tetrahydrofuran (THF) were purchased from Aldrich Chemie (Steinheim, Germany). All other chemicals were from the highest quality available.

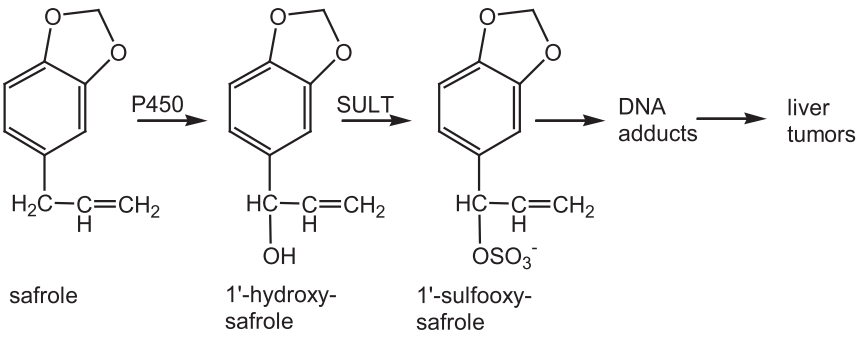


Figure 2.1. Bioactivation of safrole

Microsomal preparations

Supersomes, prepared from baculovirus-infected insect cells expressing the human individual P450 enzymes P450 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, and 3A4, and Gentest microsomes, prepared from lymphoblastoid cell lines expressing the same human individual P450 enzymes, were obtained from BD Gentest (Woburn, MA). In Gentest microsomes, the activities towards enzyme-selective substrates, expressed as pmol min^{-1} mg $protein^{-1}$, are on the same order as the mean activities found in human liver microsomes. In Supersomes, the enzymes are expressed to a much higher level than in the human liver (described in the Gentest Catalog). Microsomes from 15 individual human livers were obtained from Human Biologics (Phoenix, AZ). Pooled human liver microsomes were purchased from BD Gentest (Woburn, MA).

Synthesis of 1′-hydroxysafrole

1′-Hydroxysafrole was synthesized on the basis of the method developed by Tamayo and Ossorio *(10)* and adapted by Suga *et al*. *(11)* and Borchert *et al*. *(12)*. In short, 1′-hydroxysafrole was synthesized via the Grignard reaction, starting from piperonal and vinylmagnesium bromide (Grignard reagent, 1M solution in THF). The two reagents were used in a molar ratio of 0.5 and the reaction took place under anhydrous conditions in a nitrogen atmosphere. In detail, 0.0165 mol of piperonal was dissolved in 10 mL of dry THF and the solution was added dropwise over a period of 30 min, with stirring at 50 °C, to 0.035 mol of the Grignard reagent. The reaction was allowed to continue for another 90 min and was then poured into a solution of 4.5 g of ammonium chloride in 200 mL of ice water. The emulsion was stirred for several minutes and 1′-hydroxysafrole was extracted with diethyl ether. The organic solution was dried over magnesium sulfate and the desired compound was isolated. Structural confirmation was acquired by the UV (λ_{max} 238 and 286 nm) and MS data [*m/z* = 178 (M^+), 161 ($M^+ - OH$), 149, 135, 131] of the compound (*12*). The purity of 1′-hydroxysafrole was estimated to be more than 98%, according to GC and HPLC analysis.

In vitro incubations

Microsomal incubations using recombinant enzymes

Incubations with Supersomes were performed in a final volume of 100 µL and an NADPH-generating system was used. The NADPH generating system [1 mM $NADP^+$, 10 mM glucose-6-phosphate, and 0.05 µg/mL glucose-6-phosphate dehydrogenase in 0.2 M Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$] was preincubated during 10 min at 37 °C. After 9 min, ice-cold Supersomes (0.3 nmol/mL) were added. After 10 min preincubation, the reaction was started by adding safrole (500 µM final concentration, added from a 100× concentrated stock solution in DMSO) and ascorbic acid (1 mM, to prevent any auto-oxidation of safrole). The reaction was terminated after 20 min by adding 100 µL of ice-cold methanol. Incubations were performed in triplicate. Microsomal incubations with safrole, using Gentest microsomes expressing one single P450 enzyme, were performed in a 200 µL incubation mixture containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and 1 mg/mL microsomes in 0.2 M Tris-HCl, pH 7.4. The reaction was started by adding the

substrate safrole [dissolved in DMSO (final concentration DMSO 1%)] and was performed at 37 °C. The substrate concentrations used were 500 and 200 μM (as indicated in the Results section). The reaction was terminated after 20 min by adding 50 μL of ice-cold acetonitrile. For incubations with Gentest microsomes expressing P450 2E1, the procedure was modified. Since organic solvents inhibit P450 2E1, for this incubation, safrole was dissolved in methanol, and methanol was evaporated prior to the addition of the reaction mixture. In this case, the reaction was started by adding NADPH. Incubations were performed in duplicate or triplicate (as indicated in the Results section). For both types of microsomal preparations, control incubations without NADPH or microsomes were performed and all samples were centrifuged during 5 min at 2750*g* and stored at −20 °C until HPLC analysis.

Correlation study

The human liver microsomes from Human Biologics were characterized with respect to 7-ethoxyresorufin *O*-dealkylase, coumarin 7-hydroxylase, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylase, diclofenac 4′-hydroxylase, S-mephenytoin 4′-hydroxylase, bufuralol 1′-hydroxylase, chlorzoxazone 6-hydroxylase, and testosterone 6*β*-hydroxylase activities, as described previously *(13-15)*. Paclitaxel 6*α*-hydroxylation was determined on the basis of the instructions on the Gentest P450 2C8 data sheet (See http://www.Gentest.com/tech_resources/ass_pac.shtm). Data on protein and P450 content were provided by the supplier. Incubations with the human liver microsomes from Human Biologics were identical to the incubations with Gentest microsomes described above. These incubations were performed in duplicate.

Inhibition experiment

To investigate the role of P450 2A6 in the 1′-hydroxylation of safrole in more detail, pooled human liver microsomes were incubated with safrole, in the presence and absence of coumarin, a specific substrate for P450 2A6, also used to measure P450 2A6 activity in the human liver microsomes. The incubation was similar to the incubation with the Gentest microsomes described above, but the microsomes were preincubated for 5 min with 25 μM (final concentration) coumarin, dissolved in methanol (final concentration 1%) or with 1% methanol (controls). After 5 min, safrole (final concentration 500 μM) was added, and the mixture was incubated for 20 min. The reaction was terminated by adding 50 μL of ice-cold acetonitrile. Samples were centrifuged (2750*g*, 5 min) and stored at −20 °C until analysis.

Kinetic studies using Gentest microsomes expressing P450 2A6

For Gentest microsomes expressing P450 2A6, the K_m and k_{cat} values were determined by incubating these microsomes with substrate concentrations ranging from 50 to 500 μM (in triplicate). The incubation conditions were similar to the incubation conditions with Gentest microsomes described above. The data were fitted to the standard Michaelis−Menten equation $v = k_{cat}/(1+(K_m/[S]))$, where [S] = substrate concentration, using the LSW data analysis toolbox (version 1.1.1, MDL information systems, Inc.).

Sample analysis

HPLC analysis of 1′-hydroxysafrole

Aliquots (10 µL) of each sample were analyzed using HPLC [Waters M600 liquid chromatography system, equipped with an Alltima C18 column, 150 × 4.6 mm (Alltech, Breda, The Netherlands)]. The gradient was made with ultrapure water containing 0.05% (v/v) trifluoroacetic acid and acetonitrile. The flow rate used was 0.7 mL/min. A linear gradient from 10 to 30% (v/v) acetonitrile in water was applied during 12 min. The percentage of acetonitrile was kept at 30% (v/v) for 2 min, increased to 100% in 3 min, was kept at 100% for 2 min, lowered to 10% (v/v) and calibrated at these initial conditions for 10 min. The retention time of 1′-hydroxysafrole under these conditions was 22.5 min. Detection was carried out by a Waters 996 photodiode array detector at 280 nm. Quantification of the amount of 1′-hydroxysafrole was performed by means of a calibration curve, made using synthesized 1′-hydroxysafrole. The activities of the Supersomes and the human liver microsomes were calculated in nmol min^{-1} nmol $P450^{-1}$ and the activities of the Gentest microsomes were calculated in nmol min^{-1} mg $protein^{-1}$.

GC-MS analysis of 1′-hydroxysafrole

An incubation mixture of safrole with pooled human liver microsomes and a reference mixture containing safrole and synthesized 1′-hydroxysafrole in buffer were extracted with CH_2Cl_2. The organic layer was transferred into a new vial and the solution was concentrated under a stream of nitrogen. Aliquots (2 µL) of each sample were analyzed using an HP6890 gas chromatograph, equipped with a J&W DB-5 column (30 m × 0.25 mm, 0.25 µm film) and an HP5973 mass selective detector. The GC was programmed for a 45 min run with a temperature gradient from 50 to 250 °C at 5 °C/min. The inlet temperature was 260 °C, the split ratio was 15:1, and the pressure of the helium carrier gas was 12.0 psi. The mass spectrometer was run in the electron impact mode with electron energy at 70 eV with a mass range of *m/z* 30 − 550 and a source temperature of 280 °C.

Pearson correlation

In cases where enzymatic activities are expressed as pmol min^{-1} nmol $P450^{-1}$, correlation analysis will be independent of the amount of P450 present in the various samples. Thus, for correlation between the metabolism of safrole and the P450 marker substrates, enzyme activities expressed as pmol min^{-1} nmol $P450^{-1}$ have been used. Statistical significance was determined by the Pearson correlation test using the SAS statistical software (SAS Institute Inc., Cary, NC, Software Release 8.2).

Results

Formation of 1′-hydroxysafrole by recombinant P450 enzymes

HPLC analysis of a typical incubation of pooled human liver microsomes with safrole revealed formation of one metabolite, which could be identified as 1′-hydroxysafrole on basis of the similarity of its retention time, UV spectrum, and mass spectrum to those of the

Figure 2.2. Safrole 1'-hydroxylation activity by Supersomes. Bars indicate average activities of triplicate measurements ± SD.

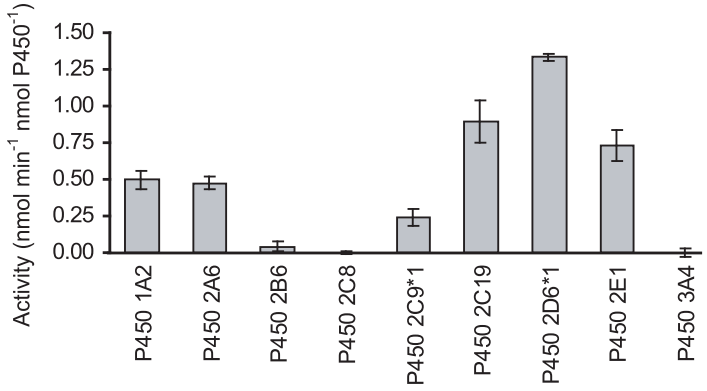
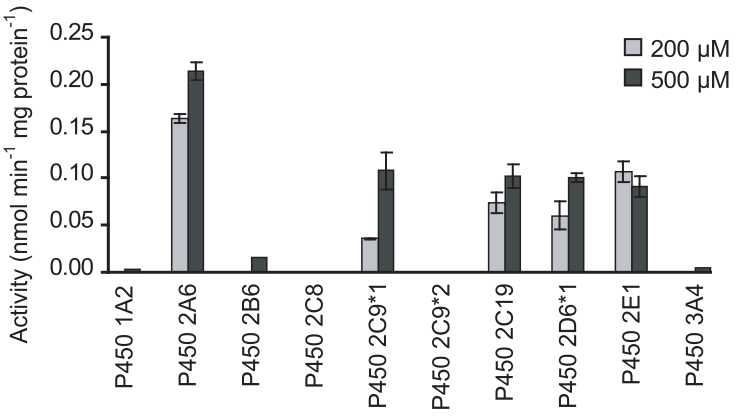


Figure 2.3. Safrole 1′-hydroxylation activity by Gentest microsomes at two different substrate concentrations. Bars indicate average activities of duplicate measurements (P450 1A2, 2B6, 2C8, 2C9*2, and 3A4) or triplicate measurements ± SD (P450 2A6, 2C9*1, 2C19, 2D6*1, and 2E1)



chemically synthesized reference compound and the data from the literature *(12)*. To define the human P450 enzymes capable of safrole 1′-hydroxylation, incubations with Supersomes were performed.

The results obtained with the Supersomes (Fig. 2.2) clearly show that many P450 enzymes are intrinsically able to catalyze the bioactivation of safrole. Of the nine enzymes tested, P450 1A2, 2A6, 2C9*1, 2C19, 2D6*1, and 2E1 were able to 1′-hydroxylate safrole. Only P450 2B6, 2C8, and 3A4 showed (almost) no 1′-hydroxylation activity. Data from the incubations with Gentest microsomes are shown in Fig. 2.3. Since in Gentest microsomes, the activities towards enzyme-selective substrates, expressed as pmol min^{-1} mg $protein^{-1}$, are

on the same order as the mean activities found in human liver microsomes, these data give an estimate of the relative contribution of each enzyme in the human liver. From these data, P450 2A6, 2C9*1, 2C19, 2D6*1, and 2E1 appear to be the most active enzymes in safrole 1′-hydroxylation at a substrate concentration of 500 μM. In contrast to the activities found for the Supersomes, P450 1A2 activity found in this experiment was very low, indicating that the contribution of this enzyme to safrole 1′-hydroxylation is negligible at average liver levels. For the enzymes most active in safrole 1′-hydroxylation, additional experiments were performed at a substrate concentration of 200 μM instead of 500 μM. P450 2E1 was saturated at the 200 – 500 μM safrole concentration range, whereas the activities of P450 2A6, 2C9*1, 2C19, and 2D6*1 were respectively 22, 68, 26, and 40% lower at a concentration of 200 μM compared to 500 μM safrole (Fig. 2.3).

Formation of 1′-hydroxysafrole by human liver microsomes

The average rate of 1′-hydroxylation of safrole in liver microsomes from 15 individuals was 1.32 ± 0.33 nmol 1′-hydroxysafrole min^{-1} nmol $P450^{-1}$. A 2.5-fold variation between different human liver samples was found (range: 0.77 − 1.95 nmol min^{-1} nmol $P450^{-1}$). Table 2.1 shows the mean ± SD activities of the panel of 15 human liver microsomes towards

Table 2.1. Correlations between the activities towards P450 enzyme selective substrates and the formation of 1′-hydroxysafrole by 15 human liver microsomal samples.

Marker activity	Mean activity ± SD in fifteen human liver microsomes (nmol min^{-1} nmol $P450^{-1}$)		P450 enzyme	Correlation coefficient
EROD[a]	0.15 ± 0.08	(range 0.02 – 0.30)	P450 1A	0.07
COUM	3.19 ± 2.64	(range 0.01 – 10.5)	P450 2A6	0.06
7-ETC	0.70 ± 0.23	(range 0.27 – 1.12)	Non specific[b]	0.09
PACL	0.55 ± 0.22	(range 0.18 – 0.91)	P450 2C8	0.18
DICLF	5.79 ± 2.82	(range 2.50 – 12.1)	P450 2C9	0.66*
MEPH	0.03 ± 0.06	(range 0.00 – 0.23)	P450 2C19	0.34
BUFU	0.38 ± 0.33	(range 0.05 – 1.22)	P450 2D6	0.44
CLZOX	6.41 ± 4.13	(range 1.74 – 15.0)	P450 2E1	0.44
TEST	10.5 ± 5.16	(range 2.85 – 20.7)	P450 3A	-0.32

[a] EROD = 7-ethoxyresorufin O-dealkylation; COUM = coumarin 7-hydroxylation; 7-ETC = 7-ethoxy-4-trifluoromethylcoumarin O-dealkylation; PACL = paclitaxel 6*α*-hydroxylation; DICLF = diclofenac 4′-hydroxylation, MEPH = S-mephenytoin 4’-hydroxylation; BUFU = bufuralol 1′-hydroxylation; CLZOX = chlorzoxazone 6-hydroxylation; TEST = testosterone 6*β*-hydroxylation.

[b] 7-ETC is mainly catalyzed by P450 2B6 and P450 1A2 *(14)*.

* Statistical significance: $P < 0.01$

specific substrates for nine different enzymes. The calculated correlation coefficients between 1′-hydroxylation of safrole and the activity towards all the specific substrates are also given in Table 2.1. A significant ($P < 0.01$) correlation between P450 2C9 activity and 1′-hydroxylation of safrole was found ($r = 0.66$). The *P*-values for the correlation between the activities of the microsomes expressing P450 2D6 ($r = 0.44$) and P450 2E1 ($r = 0.44$) towards their specific substrates and the 1′-hydroxylation of safrole were 0.1, indicating that these enzymes might also play a role in the bioactivation of safrole. P450 2A6 activity showed no correlation with the 1′-hydroxylation of safrole. This result is not in agreement with the results from the Gentest microsomes, where P450 2A6 showed the highest 1′-hydroxylation activity of all enzymes tested (Fig. 2.3).

Inhibition experiment

To investigate the role of P450 2A6 in the 1′-hydroxylation of safrole in more detail, safrole was incubated with pooled human liver microsomes in the presence and absence of coumarin. An activity of 1.21 nmol 1′-hydroxysafrole min^{-1} mg $protein^{-1}$ was found in the absence of coumarin. However, in the presence of coumarin, a specific P450 2A6 substrate, an activity of 0.68 nmol 1′-hydroxysafrole min^{-1} mg $protein^{-1}$ was found. Thus, 1′-hydroxylation of safrole in human liver microsomes was inhibited by 44% in the presence of coumarin.

K_m and k_{cat} determination for safrole 1′-hydroxylation

Because Gentest microsomes expressing individual P450 enzymes reflect the situation in the average human liver most, and P450 2A6 appears to be the most active enzyme for safrole 1′-hydroxylation in this *in vitro* model, Gentest microsomes expressing P450 2A6 were used to determine the apparent K_m and k_{cat} for the P450 2A6 mediated safrole 1′-hydroxylation. The Michaelis−Menten plot of the incubations with these microsomes revealed an apparent k_{cat} of 0.22 ± 0.01 nmol 1′-hydroxysafrole min^{-1} mg $protein^{-1}$, and an apparent K_m of 80 ± 15 μM.

Discussion

In the present study, the human cytochrome P450 enzymes involved in the bioactivation of safrole to its proximate carcinogen 1′-hydroxysafrole were studied, making use of different *in vitro* test systems. Four different kinds of experiments were undertaken to identify the human cytochrome P450 enzymes involved. Supersomes are a useful tool to obtain information about which P450 enzymes can metabolize safrole, because these Supersomes have a high activity of specific enzymes. The results from this experiment clearly show that various cytochrome P450 enzymes are able to 1′-hydroxylate safrole. Since on the basis of these results only three P450 enzymes (P450 2B6, 2C8, and 3A4) are excluded from being involved in the bioactivation of safrole, all enzymes were also tested in the experiment with the Gentest microsomes and the correlation study. In addition to the intrinsic activity of the various P450s, these *in vitro* model systems also take into account the relative amount of the various P450

enzymes present in the human liver. On the basis of the results from those two experiments, an important role for P450 2C9*1 was revealed. A significant correlation ($P < 0.01$) was found between safrole 1′-hydroxylation activity and diclofenac 4′-hydroxylation activity. In the experiment with the Gentest microsomes, P450 2C9*1 showed the second highest safrole 1′-hydroxylation activity. P450 2C9 is the main enzyme of the P450 2C subfamily in the human liver. The P450 2C subfamily accounts for 20% of the total P450 content in the human liver and is, after P450 3A (almost 30%), the most abundant P450 family *(16)*. However, the correlation coefficient obtained for P450 2C9 activity (0.66) does not approach 1.00. Obviously, P450 2C9 is not the only enzyme involved.

The results from the correlation study indicate that P450 2D6 ($r = 0.44$) and P450 2E1 ($r = 0.44$) might also add substantially to total safrole 1′-hydroxylation in the human liver. Moreover, the Gentest microsomes expressing those two enzymes also showed high safrole 1′-hydroxylation activity. These two enzymes are less prevalent in the human liver than P450 2C9. According to Shimada *et al.*, P450 2D6 accounts for 1.5% and P450 2E1 for 7% of the total P450 content *(16)*.

Gentest microsomes expressing P450 2A6 showed the highest activity (0.22 nmol 1′-hydroxysafrole min^{-1} mg $protein^{-1}$) of all microsomes tested, although no correlation between 1′-hydroxylation of safrole and coumarin 7-hydroxylation was observed. But since 44% inhibition of safrole 1′-hydroxylation activity was found in the P450 2A6 inhibition experiment, using pooled human liver microsomes and the specific P450 2A6 substrate coumarin, it is clear that P450 2A6 also plays an important role in the bioactivation of safrole. In human liver, P450 2A6 is a minor enzyme; it accounts for approximately 4% of the total P450 content *(16)*, but it is known to be involved in the bioactivation of various precarcinogens, like aflatoxin B_1 and *N*-nitrosamines *(17)*. In the human liver microsomes used in the correlation study, an approximately 1000-fold difference in coumarin 7-hydroxylation activity is present (range 0.01 − 10.5 nmol min^{-1} nmol $P450^{-1}$). For the other enzymes, the differences in activity in the 15 human liver samples are much smaller. This, in combination with the low abundance of P450 2A6 in the human liver (4%) might explain the disagreement between the results with the Gentest microsomes and the inhibition study for P450 2A6 and the correlation study.

For P450 2A6, an apparent K_m of 80 ± 15 μM was revealed. For P450 2C9*1, 2D6*1, and 2E1, no K_m and k_{cat} determination were performed, but additional incubations at 200 μM substrate concentration indicated P450 2E1 to be saturated at the 200 – 500 μM safrole concentration range, whereas the activities of P450 2A6, 2C9*1, and 2D6*1 were respectively 22, 68, and 40% lower at a safrole concentration of 200 μM compared to 500 μM. Therefore, the relative contribution of P450 2E1 to safrole 1′-hydroxylation at lower concentrations might be even higher than estimated from the *in vitro* experiments at 500 μM safrole concentration. However, at 200 μM substrate concentration, P450 2A6 still appears to be the most active enzyme in safrole 1′-hydroxylation.

Taken together, the results obtained in the present experiments indicate P450 2C9*1, 2A6, 2D6*1, and 2E1 to be the main enzymes involved in the bioactivation pathway of safrole to the proximate carcinogen 1′-hydroxysafrole in man. Gardner *et al. (6)* have published a paper

about the enzymes involved in 1′-hydroxylation of a related alkenylbenzene, methyleugenol, investigated with rat liver microsomal model systems . The authors suggested P450 2E1 and another enzyme, most probably P450 2C6, to be one of the most important enzymes for this bioactivation. These experiments were conducted with rat liver microsomes, so P450 2A6 and P450 2C9*1 were not tested. Therefore, it would be interesting to also investigate with different *in vitro* systems whether the same human P450 enzymes are involved in the bioactivation of the related alkenylbenzenes methyleugenol and estragole. Interestingly, Gardner *et al*. *(6)* found a 37-fold variation in the methyleugenol 1′-hydroxylation activity (substrate concentration 200 μM) in 13 human liver microsomes. In our study, we found a 2.5-fold difference in safrole 1′-hydroxylation activity in the 15 human liver microsomes used in the correlation study. This suggests that for methyleugenol, interindividual differences may be even more important for the risk assessment than for safrole. Interindividual variation in the bioactivation of safrole is probably caused in part by polymorphisms in the P450 enzymes involved. This could lead to“poor metabolizer” and “ultrarapid metabolizer” phenotypes. In the experiments with the Gentest microsomes, P450 2C9*2 was also tested besides the wild-type enzyme P450 2C9*1. Although the wild-type enzyme P450 2C9*1 showed a high safrole 1′-hydroxylation activity, P450 2C9*2 did not show any measurable safrole 1′-hydroxylation. P450 2C9*2 results from the ^{416}P450 2C9 C >T mutation, causing a change of ^{144}Arg to Cys. Besides the mutated allele P450 2C9*2, another variant, P450 2C9*3, with a ^{359}Iso to Leu change, has been identified. The frequencies of the alleles P450 2C9*1, P450 2C9*2, and P450 2C9*3 in the Caucasian population are 0.86, 0.08, and 0.06, respectively *(18)*.

Besides P450 2C9, also P450 2A6 and P450 2D6 are polymorphic (for a review, see ref. *19*). There is a marked interindividual variation in coumarin-7-hydroxylation activity in humans due to a genetic polymorphism that exists in the human P450 2A6 gene. At least six allelic variants have been identified in addition to the wild-type allele *(17)*. Population studies suggest that 6% of the UK population is homozygous for mutant P450 2A6 alleles, whereas in Japanese subjects the mutant allele frequency may be as high as 48% *(20,21)*. P450 2A6 polymorphism has been investigated extensively because especially the P450 2A6 whole deletion phenotype is believed to be related to a decreased risk for lung cancer, due to decreased nicotine metabolism or decreased bioactivation of *N*-nitrosamines from tobacco smoke *(17,22,23)*.

P450 2D6 is a highly polymorphic P450 isoform. At least 41 different alleles of P450 2D6 are known, leading to poor but also to increased enzyme activities *(24)*. According to Meyer *(25)*, 5.0 − 13.0% of the Caucasian population has a P450 2D6 genotype leading to a poor metabolizer phenotype. In the same population, 1 − 10 % are ultrarapid metabolizers due to P450 2D6 gene duplication or multiplication *(25)*. For a risk assessment for the use of safrole, this last category of ultrarapid metabolizers is the most important one, possibly being at higher risk for the harmful effects of safrole if exposure occurs. Besides polymorphisms as causes for interindividual differences in safrole 1′-hydroxylation, also lifestyle factors might influence one's susceptibility for safrole genotoxicity. P450 2E1 is induced in both rats and humans by ethanol *(26,27)*. Therefore, a wide variety in P450 2E1 levels will be found in the

human population. This implies that people who consume more alcoholic beverages might be at higher risk from exposure to precarcinogens bioactivated by P450 2E1. The same might be true for people who use barbiturates, which induce, amongst others, P450 2C enzymes *(28,29)*. In conclusion, our results suggest that P450 2C9*1, 2A6, 2D6*1, and 2E1 are the most important enzymes in the bioactivation of safrole. For a risk assessment for the adverse effects of safrole, special attention has to be paid to groups at higher risk in the population, due to the excessive use of alcohol or barbiturates or polymorphisms in especially the P450 2D6 gene leading to ultrarapid metabolizer phenotypes.

Acknowledgements

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Human cytochrome P450 enzymes of importance for the bioactivation of methyleugenol to the proximate carcinogen 1′-hydroxymethyleugenol

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Abstract

In vitro studies were performed to elucidate the human cytochrome P450 enzymes involved in the bioactivation of methyleugenol to its proximate carcinogen 1′-hydroxymethyleugenol. Incubations with Supersomes, expressing individual P450 enzymes to a high level, revealed that P450 1A2, 2A6, 2C9, 2C19, and 2D6 are intrinsically able to 1′-hydroxylate methyleugenol. An additional experiment with Gentest microsomes, expressing the same individual enzymes to roughly average liver levels, indicated that P450 1A2, 2C9, 2C19, and 2D6 contribute to methyleugenol 1′-hydroxylation in the human liver. A study, in which correlations between methyleugenol 1′-hydroxylation in human liver microsomes from 15 individuals and the conversion of enzyme specific substrates by the same microsomes were investigated, showed that P450 1A2 and P450 2C9 are important enzymes in the bioactivation of methyleugenol. This was confirmed in an inhibition study in which pooled human liver microsomes were incubated with methyleugenol in the presence and absence of enzyme specific inhibitors. Kinetic studies revealed that at physiologically relevant concentrations of methyleugenol P450 1A2 is the most important enzyme for bioactivation of methyleugenol in the human liver showing an enzyme efficiency (k_{cat}/K_m) that is ~ 30, 50, and > 50 times higher than the enzyme efficiencies of, respectively, P450 2C9, 2C19, and 2D6. Only when relatively higher methyleugenol concentrations are present P450 2C9 and P450 2C19 might contribute as well to the bioactivation of methyleugenol in the human liver. A 5-fold difference in activities was found between the 15 human liver microsomes of the correlation study (range, 0.89 − 4.30 nmol min^{-1} nmol $P450^{-1}$). Therefore, interindividual differences might cause variation in sensitivity towards methyleugenol. Especially lifestyle factors such as smoking (induces P450 1A) and the use of barbiturates (induces P450 2C) can increase the susceptibility for adverse effects of methyleugenol.

Introduction

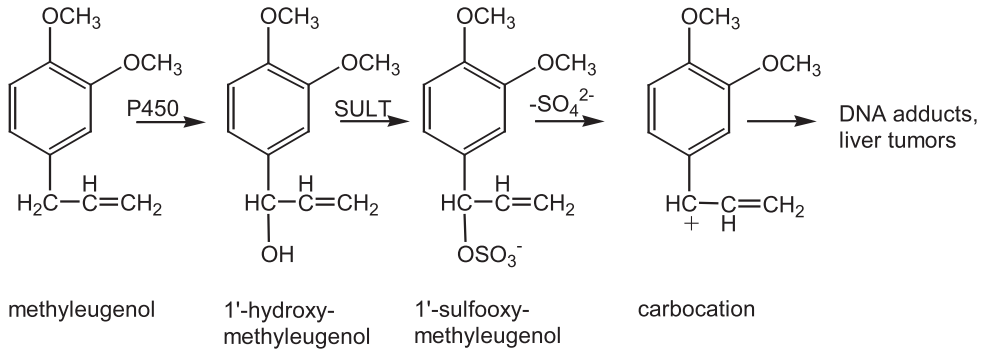
Methyleugenol is an alkenylbenzene compound that is a natural ingredient of several herbs, including basil, nutmeg, tarragon, star anise, and fennel. Furthermore, methyleugenol is used as a flavoring substance in a wide variety of dietary products such as cookies, ice cream, and nonalcoholic beverages (*1*). Dietary herb-based ingredients are natural compounds and are often regarded as safe because of their safe history of use. Methyleugenol was also classified as GRAS (Generally Recognized as Safe) by the Flavor and Extract Manufacturers Association (FEMA) in 1965 (*2*). However, the National Toxicology Program (NTP) of the United States selected methyleugenol for an investigation, because of its widespread use and its structural resemblance to safrole, a known carcinogen (*3*). The NTP reported carcinogenic activity of methyleugenol in male and female F344/N rats, and in male and female B6C3F_1 mice (*3*). In 2002, the FEMA reevaluated the data available for methyleugenol and concluded again that present exposure to methyleugenol, resulting from food consumption, does not pose a significant cancer risk (*4*). This conclusion was based on evidence of a nonlinear relationship between dose and profiles of metabolism, metabolic activation, and covalent binding of methyleugenol to protein and DNA. According to the FEMA, the harmful effects of methyleugenol are expected to be minimal at a dose rate of 1 − 10 mg/kg bw/day, which is 100 − 1000 times the average human daily intake estimated by the FEMA *(4)*. Recently, the European Union Scientific Committee on Food evaluated methyleugenol and the related herb-based alkenylbenzenes estragole and safrole. They concluded that these compounds are carcinogenic and genotoxic and that their use should be restricted (*1,5,6*). Moreover, they estimated a much higher average daily intake of methyleugenol (0.19 mg/kg bw/day) than the FEMA did (0.01 mg/kg bw/day) (*1,4*).

Fig. 3.1 shows the bioactivation pathway of methyleugenol. The cytochrome P450-based conversion of methyleugenol to the proximate carcinogen 1′-hydroxymethyleugenol is the first step in the bioactivation of methyleugenol (*7,8*). The sulfate ester of 1′-hydroxymethyleugenol is the likely ultimate carcinogenic species of methyleugenol (*9*). In an aqueous environment, 1′-sulfooxymethyleugenol is unstable. Upon loss of the sulfate group, a carbocation remains. This carbocation can cause DNA adducts and may ultimately cause liver tumors (*9*).

Insight in this bioactivation pathway in the human situation is necessary to extrapolate experimental animal data on the harmful effect of methyleugenol to the situation in man. Identification of the human P450 enzymes involved in the 1′-hydroxylation of methyleugenol is essential to gain insight into groups at a higher risk of the adverse effects of methyleugenol in the human population, due to genetic polymorphisms and life style factors that influence the P450 enzymes involved in the conversion of methyleugenol to its proximate carcinogen.

For the related alkenylbenzene safrole, important roles for the human P450 enzymes 2A6, 2C9, 2D6, and 2E1 were elucidated *(10,11)*. For methyleugenol, Gardner *et al.* indicated that P450 2E1 and another enzyme, most likely P450 2C6, are important enzymes in this bioactivation step in rats (*12*), but no human data and data from experiments using recombinant enzymes are available yet. Therefore, the aim of the present study was to identify the

Fig. 3.1. Bioactivation of methyleugenol



human P450 enzymes involved in the 1′-hydroxylation of methyleugenol. Incubations with Supersomes, expressing individual human P450 enzymes to a high level, and incubations with Gentest microsomes, derived from cell lines expressing individual human P450 enzymes to roughly average human liver levels, were performed to investigate which human P450 enzymes are able to 1′-hydroxylate methyleugenol in the human liver. Additionally, a correlation study was performed in which methyleugenol was incubated with a series of 15 human liver microsomes. The 1′-hydroxylation rates obtained were correlated with the activities of these microsomes towards specific substrates for 9 different P450 enzymes. In addition, pooled human liver microsomes were incubated with methyleugenol in the presence and absence of enzyme specific inhibitors. Finally, the kinetics for methyleugenol 1′-hydroxylation of the P450 enzymes involved were investigated.

Materials and Methods

Chemicals

Ascorbic acid, acetone, and DMSO were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid and ammonium chloride were from Acros (Geel, Belgium). NADPH was obtained from Boehringer (Mannheim, Germany). Hydrochloric acid (37%) was purchased from Roche Diagnostics (Mannheim, Germany). Methyleugenol was obtained from Aldrich (Milwaukee, WI). Tris(hydroxymethylaminomethane) was obtained from Gibco BRL Life Technologies (Paisley, Scotland). Acetonitrile and methanol were HPLC grade from Lab-Scan, Analytical Sciences (Dublin, Ireland). Dimethoxybenzaldehyde (veratraldehyde, purity 99%), tetrahydrofuran (THF), vinylmagnesium bromide (1 M solution in THF), diethyl ether, magnesium sulfate, α-naphthoflavone, coumarin, quinidine, and ketoconazole were purchased from Sigma-Aldrich (Steinheim, Germany). (*S*)-*N*-3-Benzylnirvanol, monoclonal antibody for human P450 2B6 (MAB 2B6), and monoclonal antibody for human P450 2C8 (MAB 2C8) were obtained from Gentest (Woburn, MA). Sulfaphenazole was purchased from Ultrafine Chemicals (Manchester, United Kingdom). 1′-Hydroxymethyleugenol was

synthesized as described previously for 1′-hydroxysafrole (*10,13*) with veratraldehyde as the starting material. All other chemicals were of the highest quality available.

Enzymatic preparations

Supersomes, prepared from baculovirus-infected insect cells expressing the human individual P450 enzymes 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, and 3A4 were obtained from BD Gentest. In all cells, also human P450 reductase and (except for P450 1A2) cytochrome b5 were coexpressed. Gentest microsomes, prepared from lymphoblastoid cell lines expressing the same human individual P450 enzymes, were obtained from BD Gentest. For P450 2A6, 2C8, 2C9*1, 2D6*1, 2E1, and 3A4, human P450 reductase was coexpressed. For the other enzymes, the catalytic activity was supported by reductase activity endogenous to the cell line. In general, in Gentest microsomes, the activities towards enzyme selective substrates, expressed as nmol min^{-1} mg $protein^{-1}$, are roughly at the same level as the mean activities found in human liver microsomes (the ratio between the activity of the Gentest microsomes and the human liver microsomes is approximately 1 for P450 2A6, 2B6, 2C19, 2C9, and 2E1; 0.5 for P450 1A2; 0.3 for P450 2C8; and 3.3 for P450 2D6), whereas in Supersomes, the enzyme levels are much higher than those in the human liver (described in the Gentest Catalog). Microsomes from 15 individual human livers were obtained from Human Biologics (Phoenix, AZ). Pooled human liver microsomes (mixed gender pool) were obtained from Gentest.

Synthesis of 1′-hydroxymethyleugenol

1′-Hydroxymethyleugenol was synthesized as described previously for 1′-hydroxysafrole (*10,13*), with veratraldehyde as the starting material. Structural confirmation was obtained from the UV and MS data of the compound [*m/z* (rel int. %) 194 (M^+, 99), 167 (20), 165 (30), 163 (61), 151 (32), 139 (100), 138 (22), 124 (20), 91 (21), 77 (25) and 55 (55)]. The purity of 1′-hydroxymethyleugenol was estimated to be more than 98%, according to GC and HPLC analysis.

In vitro incubations

Incubations with recombinant enzymes

Microsomal incubations with methyleugenol, using Supersomes or Gentest microsomes expressing one single P450 enzyme, were performed in a 100 µL incubation mixture containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and 0.3 nmol P450/mL Supersomes or 1 mg protein/mL microsomes in 0.2 M Tris-HCl, pH 7.4. Under these circumstances, 1′-hydroxymethyleugenol formation was linear with protein concentration, P450 concentration, and time. The reaction was started by adding the substrate methyleugenol (200 µM final concentration, added from a 20 mM stock solution in DMSO) and was performed at 37 °C. The reaction was terminated after 20 min by adding 25 µL of ice-cold acetonitrile. The substrate concentration (200 µM) is approximately the K_m for methyleugenol in human liver microsomes (K_m 0.2 ± 0.04 mM). All incubations were performed in triplicate, and control

incubations without NADPH or microsomes were performed. Samples were centrifuged for 5 min at 2750*g* and stored at −20 °C until HPLC analysis.

Correlation study

The human liver microsomes from Human Biologics were characterized with respect to 7-ethoxyresorufin *O*-dealkylase, coumarin 7-hydroxylase, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylase, diclofenac 4′-hydroxylase, *S*-mephenytoin 4′-hydroxylase, bufuralol 1′-hydroxylase, chlorzoxazone 6-hydroxylase, paclitaxel 6*α*-hydroxylase and testosterone 6*β*-hydroxylase activities, as described previously (*10*). Data on protein and P450 content were provided by the supplier. Incubations with human liver microsomes from Human Biologics were performed identical to the incubations with Gentest microsomes described above, using a substrate concentration of 200 μM and microsomes in a concentration of 1 mg protein/mL (range of 0.18 – 0.82 nmol P450/mL). Under these circumstances, 1′-hydroxymethyleugenol formation was linear with protein concentration, P450 concentration, and time. Incubations were performed in duplicate.

Inhibition study

Microsomal incubations, using pooled human liver microsomes (1 mg/mL; 0.36 nmol P450/mL), were performed in 100 μL incubations containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and 0.2 M Tris-HCl, pH 7.4. To these incubations, 1 μL of a 100 times concentrated stock solution of one of the chemical inhibitors in methanol was added as follows: α-naphthoflavone (final concentration, 1 μM), coumarin (final concentration, 10 μM), sulfaphenazole (final concentration, 10 μM), (*S*)-*N*-3-benzylnirvanol (final concentration, 5 μM), quinidine (final concentration, 5 μM), ketoconazole (final concentration, 1 μM) and acetone (final concentration, 1 % v/v). For P450 2B6 and P450 2C8, 5 μL of their respective antibody was added (5 μL/100 μg microsomal protein). The selection of the specific chemical inhibitors and their concentrations is based on either literature data *(14-16)* or data of the manufacturer [for (*S*)-*N*-3-benzylnirvanol, MAB 2B6, and MAB 2C8, see Gentest catalog]. After 5 min of preincubation, 1 μL of 20 mM methyleugenol (final concentration, 200 μM) was added. The reactions were terminated after 20 min of incubation by adding 25 μL of acetonitrile. All incubations were performed in triplicate, and control incubations without NAPDH and without chemical inhibitor/antibody were performed. Samples were centrifuged for 5 min at 2750*g* and stored at −20 °C until HPLC analysis.

Kinetic studies

For Gentest microsomes expressing P450 1A2, 2C9, 2C19, and 2D6, the K_m and k_{cat} values were determined by incubating these microsomes with substrate concentrations ranging from 0 to 200 μM (for P450 1A2) or from 0 to 500 μM (for P450 2C9, 2C19, and 2D6) (in triplicate). The incubation conditions were similar to the incubation conditions with Gentest microsomes described above. The data were fitted to the standard Michaelis−Menten equation $v = k_{cat}/\{1+(K_m/[S])\}$, where [S] = substrate concentration, using the LSW data

analysis toolbox (version 1.1.1, MDL Information Systems, Inc.) and the parameters k_{cat}, K_m and k_{cat}/K_m were determined for all enzymes.

Sample analyses

HPLC analysis

Aliquots (10 µL) of each sample were analyzed using an HPLC (Waters M600 liquid chromatography system) equipped with an Alltima C18 column, 150 mm × 4.6 mm (Alltech, Breda, The Netherlands). The gradient was made with ultrapure water containing 0.1% (v/v) trifluoroacetic acid and acetonitrile. The flow rate used was 1.0 mL/min. A linear gradient from 10 to 30% (v/v) acetonitrile in water was applied during 12 min. The percentage of acetonitrile was kept at 30% (v/v) for 2 min, increased to 100% in 3 min and was kept at 100% for 2 min. The retention time of 1′-hydroxymethyleugenol was 16.4 min under these conditions. Detection was carried out by a Waters 996 photodiode array detector at 280 nm. The amount of 1′-hydroxymethyleugenol was quantified by means of a calibration curve made using synthesized 1′-hydroxymethyleugenol. Activities were expressed as nmol 1′-hydroxymethyleugenol min^{-1} mg $protein^{-1}$ and/or nmol 1′-hydroxymethyleugenol min^{-1} nmol $P450^{-1}$.

GC-MS analysis

GC-MS analysis was performed to identify the 1′-hydroxymetabolite of methyleugenol formed during the microsomal incubations. An incubation mixture of methyleugenol with Supersomes expressing P450 1A2 and a reference mixture containing methyleugenol and synthesized 1′-hydroxymethyleugenol in buffer were extracted with CH_2Cl_2. The organic layers were transferred into new vials and the solutions were concentrated under a stream of nitrogen. Aliquots (2 µL) of each sample were analyzed using an HP6890 gas chromatograph, equipped with a J&W DB-5 column (30 m × 0.25 mm, 0.25 µm film) and an HP5973 mass selective detector. The GC was programmed for a 45 min run with a temperature gradient from 50 to 250 °C at 5 °C/min. The inlet temperature was 260 °C, the split ratio was 15:1 and the pressure of the helium carrier gas was 12.0 psi. The mass-spectrometer was run in the electron impact mode with electron energy at 70 eV with a mass range of *m/z* 30 − 550 and a source temperature of 280 °C.

Statistical analysis

For correlations between the metabolism of methyleugenol and the metabolism of P450 marker substrates, enzyme activities expressed as nmol min^{-1} nmol $P450^{-1}$ were used, because in this way, correlation analysis will be independent of the amount of P450 present in the various samples. Pearson correlation tests were performed to investigate correlations between the metabolism of individual P450 marker substrates and the 1′-hydroxylation of methyleugenol. To investigate the relationship between the metabolism of multiple P450 marker substrates and the methyleugenol 1′-hydroxylation, multiple linear regressions were performed using an inclusion approach in which all activities were tested. These statistical analyses were performed with SPSS 10.1 for Windows (SPSS Inc, Chicago, IL).

Results

Formation of 1′-hydroxmethyleugenol by recombinant enzymes

Incubations with Supersomes were performed to define the human P450 enzymes capable of methyleugenol 1′-hydroxylation. HPLC analysis of a typical incubation of Supersomes expressing P450 1A2 with methyleugenol showed formation of 1′-hydroxymethyleugenol, which could be identified based on the similarity of its retention time (16.4 min), UV spectrum, and mass spectrum to those of the chemically synthesized reference compound. Fig. 3.2 shows the results obtained with the Supersomes. P450 1A2 showed the highest methyleugenol 1′-hydroxylating activity, followed by P450 2D6. In addition, P450 2C9 and P450 2C19 showed some methyleugenol 1′-hydroxylating activity. Hardly any activity was observed for P450 2A6 (~ 0.04 nmol min^{-1} nmol $P450^{-1}$), whereas no activity was detected for P450 2B6, 2C8, 2E1, and 3A4 (detection limit ~ 0.02 nmol min^{-1} nmol $P450^{-1}$).

Incubations of methyleugenol with Gentest microsomes were performed to investigate which enzymes show methyleugenol 1′-hydroxylation, when tested at roughly average liver levels. Fig. 3.3 shows the results of these incubations. In Fig. 3.3A, activities are expressed in nmol min^{-1} mg $protein^{-1}$. In general, in Gentest microsomes, the activities towards enzyme selective substrates, expressed in nmol min^{-1} mg $protein^{-1}$, are roughly comparable to the activities found in human liver microsomes (see Gentest Catalog). These results indicate that the P450 enzymes 1A2, 2C9, 2C19, and 2D6 may be involved in methyleugenol 1′-hydroxylation in the human liver (detection limit ~ 0.01 nmol min^{-1} mg $protein^{-1}$). In Fig. 3.3B activities are expressed in nmol min^{-1} nmol $P450^{-1}$. The different activities show the same pattern as in Fig. 3.3A.

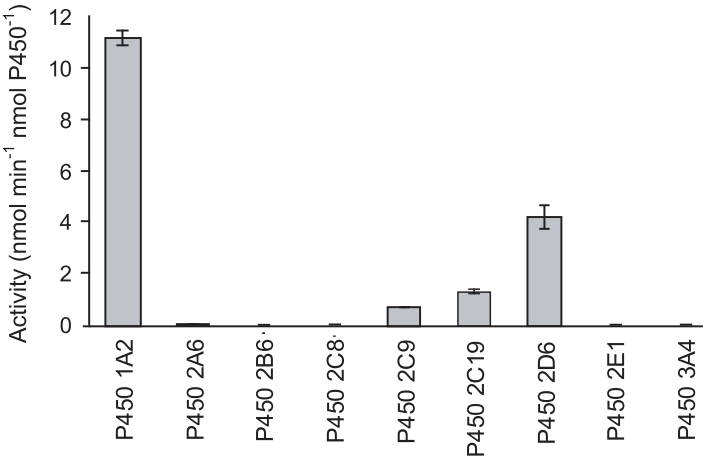
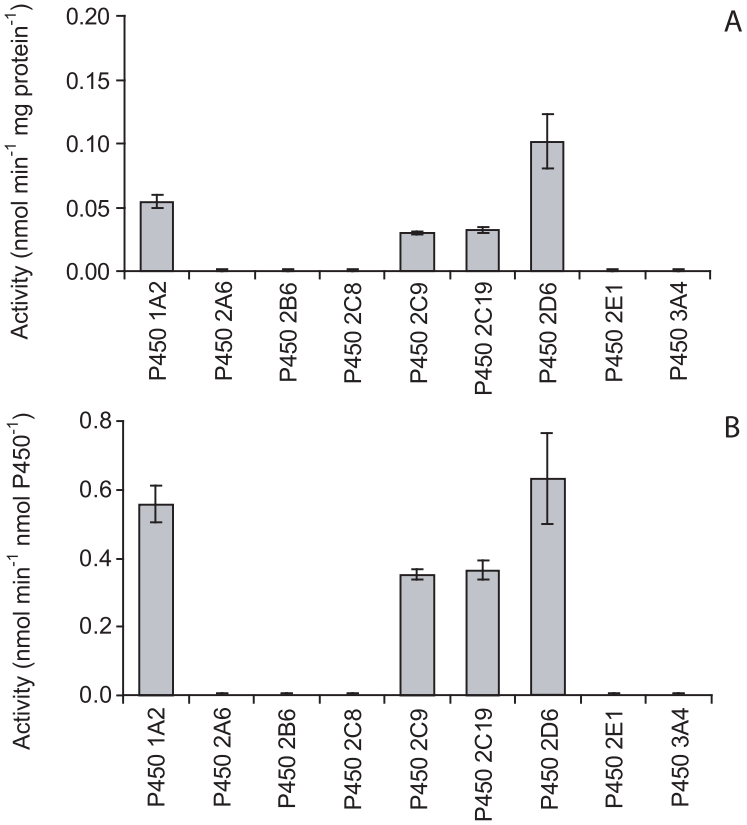


Fig. 3.2. Methyleugenol 1'-hydroxylation activity by Supersomes at a substrate concentration of 200 µM. Bars indicate average activities of triplicate measurements ± SD.

Fig. 3.3. Methyleugenol 1′-hydroxylation activity expressed in (A) nmol min^{-1} mg $protein^{-1}$ and (B) nmol min^{-1} nmol $P450^{-1}$ by Gentest microsomes at a substrate concentration of 200 µM. Bars indicate average activities of triplicate measurements ± SD.



Correlation study

The average rate of 1′-hydroxylation of methyleugenol in liver microsomes from 15 individuals was 2.45 ± 0.34 nmol 1′-hydroxymethyleugenol min^{-1} nmol $P450^{-1}$. A 5-fold variation between different human liver samples was found (range, 0.89 − 4.30 nmol min^{-1} nmol $P450^{-1}$; median value, 2.25 nmol min^{-1} nmol $P450^{-1}$). Table 3.1 shows the mean activities of the panel of 15 human liver microsomes towards specific substrates for nine different enzymes. Table 3.1 also presents the correlation coefficient for the correlation between 1′-hydroxylation of methyleugenol by the 15 human liver microsomes and their activity towards the specific substrates. Correlation analysis showed a significant correlation ($p < 0.05$) between P450 1A2 activity and methyleugenol 1′-hydroxylation. Because incubations with recombinant P450 enzymes also indicated the involvement of P450 2C9, 2C19, and 2D6 in the metabolism of methyleugenol, the results of the correlation experiment were further investigated with multiple regression analysis. By including both P450 1A2 and P450 2C9 in the model, p values < 0.01 were obtained for both enzymes, demonstrating an important

Table 3.1. Correlations between the activities towards P450 enzyme selective substrates and the formation of 1′-hydroxymethyleugenol by 15 human liver microsomal samples.

Marker activity	Mean activity ± SD (nmol min^{-1} nmol $P450^{-1}$)		P450 enzyme	Correlation coefficient
EROD[a]	0.15 ± 0.08	(range 0.02 – 0.30)	P450 1A	0.51*
COUM	3.19 ± 2.64	(range 0.01 – 10.5)	P450 2A6	0.02
7-ETC	0.70 ± 0.23	(range 0.27 – 1.12)	Non specific[b]	0.35
PACL	0.55 ± 0.22	(range 0.18 – 0.91)	P450 2C8	-0.05
DICLF	5.79 ± 2.82	(range 2.50 – 12.1)	P450 2C9	0.47
MEPH	0.03 ± 0.06	(range 0.00 – 0.23)	P450 2C19	0.45
BUFU	0.38 ± 0.33	(range 0.05 – 1.22)	P450 2D6	0.22
CLZOX	6.41 ± 4.13	(range 1.74 – 15.0)	P450 2E1	-0.04
TEST	10.5 ± 5.16	(range 2.85 – 20.7)	P450 3A	0.05

[a] EROD = 7-ethoxyresorufin O-dealkylation; COUM = coumarin 7-hydroxylation; 7-ETC = 7-ethoxy-4-trifluoromethylcoumarin O-dealkylation; PACL = paclitaxel 6*α*-hydroxylation; DICLF = diclofenac 4′-hydroxylation, MEPH = S-mephenytoin 4′-hydroxylation; BUFU = bufuralol 1'-hydroxylation; CLZOX = chlorzoxazone 6-hydroxylation; TEST = testosterone 6*β*-hydroxylation.

[b] 7-ETC is mainly catalyzed by P450 2B6 and P450 1A2 *(27)*.

* Statistical significance: $P < 0.01$

Table 3.2. Inhibition of the formation of 1′-hydroxymethyleugenol by pooled human liver microsomes by enzyme specific inhibitors

Inhibitor	Percentage of control activity ± SD	P450 enzyme
α-naphthoflavone	54 ± 13	P450 1A2
coumarin	92 ± 16	P450 2A6
MAB-2B6[a]	107 ± 17	P450 2B6
MAB-2C8	102 ± 14	P450 2C8
sulfaphenazole	70 ± 11	P450 2C9
(S)-N-3-benzylnirvanol	89 ± 11	P450 2C19
quinidine	97 ± 2	P450 2D6
acetone	122 ± 5	P450 2E1
ketoconazole	99 ± 11	P450 3A

[a]MAB = monoclonal antibody

contribution of both P450 1A2 and P450 2C9 in the 1′-hydroxylation of methyleugenol (correlation coefficient model, 0.78). No significant contribution of the other P450 enzymes regarding the metabolism of methyleugenol was found in this correlation study.

Inhibition study

The average activity of the control incubations of the pooled human liver microsomes was 0.25 ± 0.09 nmol 1′-hydroxymethyleugenol min^{-1} mg $protein^{-1}$ (0.69 ± 0.25 nmol min^{-1} nmol $P450^{-1}$). The percentage activity remaining in the microsomal incubations in the presence of the different enzyme selective chemical inhibitors/antibodies is shown in Table 3.2. The P450 1A2 inhibitor α-naphthoflavone and the P450 2C9 inhibitor sulfaphenazole were most effective in inhibiting the methyleugenol 1′-hydroxylation by human liver microsomes. In the presence of those inhibitors, activities of only 54 ± 13 and 70 ± 11% of the control 1′-hydroxylation activity were found. For all other inhibitors, the inhibition was less than 12%.

Kinetic studies

Fig. 3.4 shows plots of methyleugenol 1′-hydroxylation versus methyleugenol concentration for Gentest microsomes containing, respectively, P450 1A2, 2C9, 2C19, and 2D6, and Table 3.3 presents the parameters k_{cat}, K_m, and k_{cat}/K_m (enzyme efficiency) derived

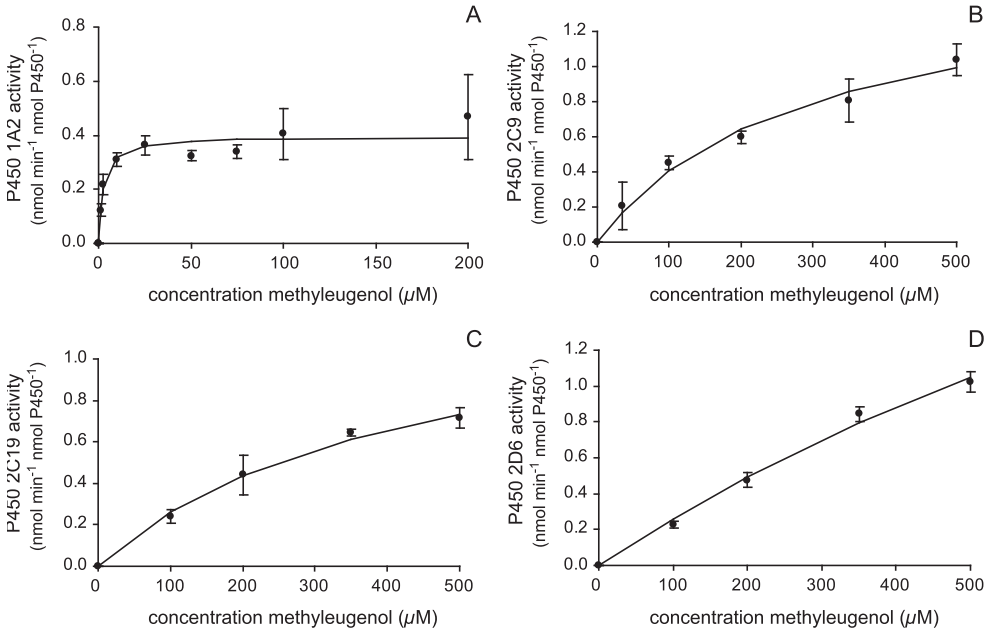


Fig. 3.4. Plots of methyleugenol 1′-hydroxylation versus methyleugenol concentration for Gentest microsomes containing (A) P450 1A2, (B) P450 2C9, (C) P450 2C19, and (D) P450 2D6.

Table 3.3. Kinetic parameters for Gentest microsomes expressing the enzymes involved in methyleugenol 1′-hydroxylation

Enzyme	k_{cat} (nmol min^{-1} nmol $P450^{-1}$)	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
P450 1A2	0.40 ± 0.03	2.4 x 10^{-3} ± 1.1 x 10^{-3}	167
P450 2C9	1.55 ± 0.32	0.28 ± 0.12	5
P450 2C19	1.34 ± 0.31	0.42 ± 0.18	3
P450 2D6	4.22 ± 1.79	> 1.5	< 3

from these studies. Although the k_{cat} for P450 1A2 is the lowest k_{cat} observed among the four enzymes, the K_m value for P450 1A2 is much lower than the K_m values of the other enzymes. As a result, the enzyme efficiency k_{cat}/K_m of P450 1A2 is ~ 30, 50, and > 50 times higher than the enzyme efficiencies of, respectively, P450 2C9, 2C19, and 2D6.

Discussion

The human P450 enzyme specificity for bioactivation of methyleugenol to its proximate carcinogen 1′-hydroxymethyleugenol was studied with different *in vitro* test systems, using either recombinant P450 enzymes or human liver micromes. The incubations with Supersomes expressing nine individual enzymes show the intrinsic capacity of especially P450 1A2 to 1′-hydroxylate methyleugenol. Furthermore, methyleugenol was also bioactivated by P450 2D6, 2C9, 2C19, and to a small extent by P450 2A6. Incubations with Gentest microsomes, which express P450 enzymes to roughly average liver levels, indicated that P450 1A2, 2C9, 2C19, and 2D6 contribute to methyleugenol 1′-hydroxylation. For Gentest microsomes expressing P450 2A6, no methyleugenol 1′-hydroxylating activity was detected. In a correlation study, using human liver microsomes, a significant correlation ($p < 0.05$) between P450 1A2 activity and methyleugenol 1′-hydroxylation was found. By including both P450 1A2 and P450 2C9 in the model, p values < 0.01 were obtained for both enzymes, demonstrating an important contribution of both P450 1A2 and P450 2C9 in the 1′-hydroxylation of methyleugenol (correlation coefficient model, 0.78). In a study investigating the effect of enzyme selective inhibitors and antibodies on methyleugenol 1′-hydroxylation in human liver microsomes, the highest inhibition was caused by the P450 1A2 inhibitor α-naphthoflavone and the P450 2C9 inhibitor sulfaphenazole.

Taken together, the different *in vitro* systems indicate that P450 1A2 and P450 2C9 are important enzymes in the bioactivation of methyleugenol. Furthermore, on the basis of the incubations with Supersomes and Gentest microsomes, P450 2D6 and P450 2C19 may also be involved in methyleugenol 1′-hydroxylation. For those four enzymes, the kinetic parameters k_{cat}, K_m and k_{cat}/K_m were determined using Gentest microsomes. From these studies, it appeared that at physiologically relevant concentrations of methyleugenol, P450 1A2 is the most important enzyme in methyleugenol 1′-hydroxylation with an enzyme efficiency

(k_{cat}/K_m) that is ~ 30, 50, and > 50 times higher than the enzyme efficiencies of, respectively, P450 2C9, 2C19, and 2D6. P450 2D6 has the highest k_{cat} value for the 1′-hydroxylation of methyleugenol, but the K_m is relatively high (> 1.5 mM), so at low levels of methyleugenol, this enzyme will not contribute to methyleugenol 1′-hydroxylation. The enzymes P450 2C9 and P450 2C19 might contribute to the bioactivation of methyleugenol only at relatively higher methyleugenol concentrations.

For the related alkenylbenzene safrole, we reported that especially the enzymes P450 2A6, 2C9, 2D6, and 2E1 are important in 1′-hydroxylation, whereas P450 2C19 and P450 1A2 also showed intrinsic safrole 1′-hydroxylating activity (*10*). Except for P450 2E1, for which no methyleugenol 1′-hydroxylation was observed, the same enzymes are intrinsically able to 1′-hydroxylate both alkenylbenzenes. However, the relative importance of the different enzymes differs for the two alkenylbenzenes.

Gardner *et al.* (*12*) have studied the selectivity of P450 enzymes involved in methyleugenol 1′-hydroxylation in rats, in different rat *in vivo* and *in vitro* systems. Contrary to our results for man, they found that P450 2E1 is one of the most important enzymes in this bioactivation step in rats (*12*), whereas they excluded P450 1A2 from being involved in methyleugenol 1′-hydroxylation. We did not detect any methyleugenol 1′-hydroxylation activity in Supersomes and Gentest microsomes expressing human P450 2E1, nor did we find support for a role for human P450 2E1 in the correlation and the inhibition study. Therefore, it seems unlikely that this enzyme is an important one in methyleugenol 1′-hydroxylation in the human liver. The exclusion of P450 1A2 by Gardner *et al.* was based on the results of an inhibitor study, in which rat liver microsomes were incubated with methyleugenol in the presence of the P450 1A2 selective inhibitor furafylline. Although rat and human P450 1A2 are orthologous genes, with 80% sequence similarity (*17*), furafylline is a much weaker inhibitor of 7-ethoxyresorufin *O*-dealkylase activity in rat than in man (*14,18,19*). This might explain why Gardner *et al.* found a low inhibition of methyleugenol 1′-hydroxylation in rat microsomes in the presence of furafylline. Furthermore, Gardner *et al.* found a significant inhibition of methyleugenol 1′-hydroxylation by α-naphthoflavone *(12)*, an inhibitor of P450 1A enzymes *(20)*, so their results also indicate that P450 1A enzymes might be involved in methyleugenol 1′-hydroxylation.

In the correlation study, 5-fold (range of 0.89 − 4.30 nmol min^{-1} nmol $P450^{-1}$) differences were found in the 15 human liver microsomes. Genetic polymorphisms in the enzymes involved in methyleugenol 1′-hydroxylation but also life style factors that influence the activities of these enzymes might cause interindividual differences in the susceptibility for the adverse effects of methyleugenol. For P450 1A2, which accounts for 13% of the total P450 content of the human liver (*21*), polymorphisms are very rare (reviewed in ref *22*), and so far, no allelic variant that is associated with increased enzyme activity has been identified (www.imm.ki.se/cypalleles). Therefore, life style factors that increase the activity of P450 1A2 are more important than genetic polymorphisms for the individual risks of bioactivation of methyleugenol. Cigarette smoking is known to induce P450 1A2 (*23*) and also charbroiled food and cruciferous vegetables can do so (reviewed in ref *24*). For P450 2C9 and P450

2C19, both members of the 2C family that accounts for 20% of the total P450 content of the human liver (*21*), only allelic variants that are associated with impaired enzyme activity *in vivo* are known so far (www.imm.ki.se/cypalleles). Recently, a P450 2C9*8 allele was discovered, which leads to an increased activity *in vitro* but no *in vivo* data are available yet *(25)*. People who use barbiturates, which induce P450 2C enzymes (*26*), might have higher levels of P450 2C enzymes and higher methyleugenol 1′-hydroxylation activity, pointing at increased risk of the adverse effects of methyleugenol.

In conclusion, our results indicate that at low concentrations of methyleugenol, P450 1A2 is the main enzyme involved in the bioactivation of methyleugenol and that at higher substrate concentrations P450 2C9 and 2C19 may also contribute to the bioactivation of methyleugenol. For a risk assessment for the use of methyleugenol, special attention should be paid to interindividual differences in the bioactivation of methyleugenol. In particular, people who smoke or use barbiturates might have a higher methyleugenol 1′-hydroxylation rate. These groups of people might be at higher risk of the adverse effects of exposure to methyleugenol.

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4

Human cytochrome P450 enzyme specificity for the bioactivation of estragole and related alkenylbenzenes

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Abstract

Human cytochrome P450 enzymes involved in the bioactivation of estragole to its proximate carcinogen 1′-hydroxyestragole were identified and compared to the enzymes of importance for 1′-hydroxylation of the related alkenylbenzenes methyleugenol and safrole. Incubations with Supersomes revealed that all enzymes tested, except P450 2C8, are intrinsically able to 1′-hydroxylate estragole. Experiments with Gentest microsomes, expressing P450 enzymes to roughly average liver levels, indicated that P450 1A2, 2A6, 2C19, 2D6, and 2E1 might contribute to estragole 1′-hydroxylation in the human liver. Especially P450 1A2 is an important enzyme based on the correlation between P450 1A2 activity and estragole 1′-hydroxylation in human liver microsomal samples and inhibition of estragole 1′-hydroxylation by the P450 1A2 inhibitor α-naphthoflavone. Kinetic studies revealed that, at physiologically relevant concentrations of estragole, P450 1A2 and 2A6 are the most important enzymes for bioactivation in the human liver showing enzyme efficiencies (k_{cat}/K_m) of respectively 59 and 341 min^{-1} mM^{-1}. Only at relatively high estragole concentrations, P450 2C19, 2D6, and 2E1 might contribute to some extent. Comparison to results from similar studies for safrole and methyleugenol revealed that competitive interactions between estragole and methyleugenol 1′-hydroxylation and between estragole and safrole 1′-hydroxylation are to be expected because of the involvement of, respectively, P450 1A2 and P450 2A6 in the bioactivation of these compounds. Furthermore, poor metabolizer phenotypes in P450 2A6 might diminish the chances on bioactivation of estragole and safrole, whereas lifestyle factors increasing P450 1A2 activities such as cigarette smoking and consumption of charbroiled food might increase those chances for estragole and methyleugenol.

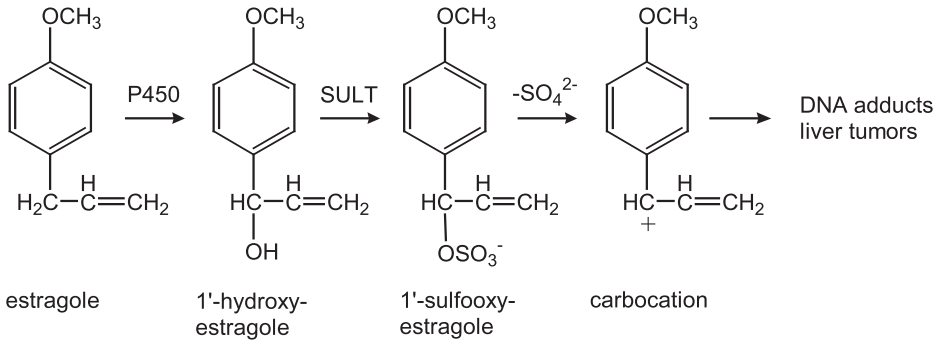
Introduction

The alkenylbenzene estragole (4-allyl-1-methoxybenzene) is a natural ingredient of herbs such as tarragon, basil, fennel, and anise (*1*). Estragole is also used as a flavoring substance in, among others, baked goods, nonalcoholic beverages, and hard and soft candy (*1,2*). The Scientific Committee on Food (SCF) of the European Union concluded that estragole is genotoxic and carcinogenic and this will result in restrictions in the use of this pure compound as a food additive in the European Union (*1,3*). The estimated average intake (for consumers only) was estimated by the SCF to amount to 4.3 mg/day, which is equal to 72 µg/kg bw/day assuming 60 kg body weight. On the other hand, the Expert Panel of the Flavor and Extract Manufacturers' Association (FEMA) of the United States concluded that the present exposure to estragole from food, mainly spices and added as such, does not pose a significant cancer risk (*4*). This conclusion was based on evidence of a nonlinear relationship between dose and profiles of metabolism, metabolic activation, and covalent binding of estragole to proteins and DNA (*4*). The FEMA Expert Panel estimated the mean daily per capita intake ("eaters only") for estragole to be less than 10 µg/kg bw/day, which is equal to less than 0.6 mg/day for a 60 kg person.

Fig. 4.1 shows the most important bioactivation pathway for estragole. This pathway is similar to the main bioactivation route of the related alkenylbenzenes safrole and methyleugenol. The bioactivation pathway starts with the conversion of estragole into its proximate carcinogen 1′-hydroxyestragole by P450 enzymes (*5,6*). The 1′-hydroxymetabolite can be sulfated by sulfotransferases to the ultimate carcinogenic species 1′-sulfooxyestragole. 1′-Sulfooxyestragole is unstable in an aqueous environment, and upon loss of the sulfate group, a carbocation remains. This carbocation may bind to DNA and proteins and may cause DNA adducts and liver tumors (*7*). The proximate carcinogen 1′-hydroxyestragole can also be glucuronidated instead of sulfated (*5,8*), and 1′-hydroxyestragole was found in β-glucuronidase-treated urine of men dosed with 100 µg of estragole (*9*) and of rats and mice (*10,11*). An important detoxification route for estragole is P450-catalyzed *O*-demethylation. In rats and mice, *O*-demethylation is the principle route of metabolism (*10,11*) and also in man estragole *O*-demethylation is an important metabolic pathway (*9*). Epoxidation of estragole or 1′-hydroxyestragole is another bioactivation route leading to metabolites that are genotoxic *in vitro* (*12,13*), but *in vivo*, these epoxides are efficiently detoxified by epoxide hydrolases and glutathione S-transferases; therefore it was previously concluded that this pathway is unlikely to contribute to the genotoxic effects of estragole *in vivo* (*14-16*).

To better estimate the risks associated with the consumption of estragole, it is important to know which enzymes catalyze the different biotransformation steps and to which extent. The P450 enzymes catalyzing the bioactivation of estragole into 1′-hydroxyestragole have not yet been identified. Knowledge of the P450 enzymes involved may identify groups of people at increased or reduced risk for the possible adverse effects of estragole, due to differences in the activities of the enzymes involved in bioactivation caused by genetic polymorphisms or lifestyle factors. Recently, the human P450 enzymes responsible for the 1′-hydroxylation

Figure 4.1. Bioactivation pathway of estragole.



of the related alkenylbenzenes safrole and methyleugenol were identified *(17-19)*. In this chapter, we describe the identification of the P450 enzymes involved in the 1′-hydroxylation of estragole in the human liver and the kinetics for those P450 enzymes. Identification of the human P450 enzymes involved in estragole 1′-hydroxylation enables comparison to the pattern of P450 enzymes previously shown to be involved in the similar bioactivation of methyleugenol *(17)* and safrole *(18,19)*. This indicates whether competitive interactions between alkenylbenzenes that are converted by the same P450 enzymes are to be expected and whether the same genetic polymorphisms or lifestyle factors may influence the chances on P450-mediated bioactivation of the related alkenylbenzenes. To enable this comparison, in the present study, the kinetics for the P450 enzymes previously identified to be important in safrole 1′-hydroxylation (*18,19)* were also determined. For methyleugenol this comparison could be made based on kinetic data taken from our previous study *(17)*.

Materials and Methods

Chemicals

Ascorbic acid, acetone, and DMSO were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid was from Acros (Geel, Belgium). NADPH was obtained from Boehringer (Mannheim, Germany). Hydrochloric acid (37%) was purchased from Roche Diagnostics (Mannheim, Germany). Tris(hydroxymethylaminomethane) was obtained from Gibco BRL Life Technologies (Paisley, Scotland). Acetonitrile and methanol were HPLC grade from Lab-Scan, Analytical Sciences (Dublin, Ireland). Estragole (4-allylanisole), *p*-anisaldehyde, α-naphthoflavone, coumarin, quinidine, ketoconazole, and safrole were purchased from Sigma-Aldrich (Steinheim, Germany). (*S*)-*N*-3-Benzylnirvanol, monoclonal antibody for human P450 2B6 (MAB 2B6), and monoclonal antibody for human P450 2C8 (MAB 2C8) were obtained from BD Gentest (Woburn, MA). Sulfaphenazole was purchased from Ultrafine Chemicals (Manchester, United Kingdom).

Microsomal preparations

Supersomes, prepared from baculovirus-infected insect cells expressing the human individual P450 enzymes 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, and 3A4, were obtained from BD Gentest. Also, in all cells, human P450 reductase and (except for P450 1A2) cytochrome b5 were coexpressed. Gentest microsomes, prepared from lymphoblastoid cell lines expressing the same human individual P450 enzymes (described in refs *20* and *21*), were obtained from BD Gentest. For P450 2A6, 2C8, 2C9*1, 2D6*1, 2E1, and 3A4, human P450 reductase was coexpressed. For the other enzymes, the catalytic activity was supported by reducase activity endogenous to the cell line. In general, in Gentest microsomes, the activities towards enzyme-selective substrates, expressed as nmol min^{-1} mg $protein^{-1}$, are roughly at the same level as the mean activities found in human liver microsomes (the ratio between the activity of the Gentest microsomes and the human liver microsomes towards typical substrates is reported by the producer to be approximately 1 for P450 2A6, 2B6, 2C9, 2C19 and 2E1; 0.5 for 1A2; 0.3 for 2C8; 3.3 for 2D6, and 0.25 for 3A4). In Supersomes, the enzyme levels are much higher than those in the human liver (the ratio between the activity of the Supersomes and the human liver microsomes varies from 1.7 to 100 according to the data provided by the producer). Microsomes from fifteen individual human livers were obtained from Human Biologics (Phoenix, USA). Pooled human liver microsomes (mixed gender pool) were obtained from Gentest.

Synthesis of 1′-hydroxyestragole

1′-Hydroxyestragole was synthesized as described previously for 1′-hydroxysafrole (*18*), starting from *p*-anisaldehyde instead of piperonal, based on the method developed by Tamayo and Ossorio (*22*), which was adapted by Suga *et al.* (*23*) and Borchert *et al.* (*24*). GC-MS analysis was performed as previously described for safrole (*18*). The purity of 1′-hydroxyestragole was estimated to be more than 98% according to GC-MS and HPLC analyses. Structural confirmation was obtained from the UV (λ_{max} 229 nm and λ_{max} 272 nm) and MS data of the compound [*m/z* (rel. int. %) 109 (100), 164 (84), 135 (70), 77 (66), 121 (65), 163 (63), 137 (61), 133 (43), 94 (42), 108 (38)] that were comparable to those reported in the literature (*5*).

In vitro incubations

Incubations with recombinant enzymes

Microsomal incubations with estragole, using Supersomes or Gentest microsomes expressing one single P450 enzyme, were performed in a 100 µL incubation mixture containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and Supersomes at 0.3 nmol P450/mL or microsomes at 1 mg protein/mL in 0.2 M Tris-HCl, pH 7.4. The reaction was started by adding the substrate estragole (500 µM final concentration, added from a 50 mM stock solution in DMSO). Incubations were performed at 37 °C and the reaction was terminated after 20 min by adding 25 µL of ice-cold acetonitrile. Product formation was linear in time under these conditions. Incubations were performed in triplicate and all samples were centrifuged for 5 min at 16.000*g* (14.000 rpm, Eppendorf Centrifuge, type

5415C, Hamburg, Germany), and the supernatant was stored at –20 °C until HPLC analysis for quantification of 1′-hydroxyestragole.

Correlation study

The human liver microsomes from Human Biologics were characterized with respect to 7-ethoxyresorufin *O*-dealkylase, coumarin 7-hydroxylase, 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylase, diclofenac 4′-hydroxylase, *S*-mephenytoin 4′-hydroxylase, bufuralol 1′-hydroxylase, chlorzoxazone 6-hydroxylase, and testosterone 6*β*-hydroxylase activities, as described previously (*25-27*). Paclitaxel 6α-hydroxylation was determined by incubating 1 mg/mL human liver microsomes at 37 °C for 15 min in a 200 µL incubation mixture containing 0.1 M potassium phosphate pH 7.4, 3 mM NADPH, and 50 µM paclitaxel. The reaction was terminated by the addition of 100 µL of ice-cold acetonitrile. After centrifugation for 5 min at 2750*g*, the supernatant was analyzed by HPLC using UV-detection (230 nm). HPLC analysis was performed using a 250 x 4.6 mm Inertsil ODS-3 column, a gradient of water and acetonitrile, and a flow rate of 1.0 mL/min. The formation of the product was quantified using a calibration curve of 6α-paclitaxel. Data on protein and P450 content were provided by the supplier. Incubations with human liver microsomes from Human Biologics were performed identically to the incubations with Gentest microsomes described above, using an estragole concentration of 500 µM and microsomes in a concentration of 1 mg protein/mL (range 0.18 – 0.82 nmol P450/mL). Incubations were performed in duplicate. Samples were centrifuged for 5 min at 16.000*g* and the supernatant was stored at –20 °C until HPLC analysis for quantification of 1′-hydroxyestragole.

Inhibition study

Microsomal incubations, using pooled human liver microsomes (1 mg protein/mL; 0.36 nmol P450/mL), were performed in 200 µL incubations containing (final concentrations) 3 mM NADPH, 1 mM ascorbic acid, and 0.2 M Tris-HCl, pH 7.4. To these incubations, 2 µL of a 100 times concentrated stock solution of one of the chemical inhibitors in methanol were added as follows: α-naphthoflavone for P450 1A2 (final concentration, 1 µM), coumarin for P450 2A6 (final concentration, 10 µM), sulfaphenazole for P450 2C9 (final concentration, 10 µM), (*S*)-*N*-3-benzylnirvanol for P450 2C19 (final concentration, 5 µM), quinidine for P450 2D6 (final concentration, 5 µM), acetone for P450 2E1 (final concentration, 1 % v/v), and ketoconazole for P450 3A4 (final concentration, 1 µM). For P450 2B6 and P450 2C8, 5 µL of their respective antibody was added (5 µL/100 µg microsomal protein). The selection of the specific chemical inhibitors and their concentrations was based on either literature data (*25,26,28*) or data of the manufacturer [for (*S*)-*N*-3-benzylnirvanol, MAB 2B6, and MAB 2C8, see the Gentest catalog]. After 5 min of preincubation, 2 µL of 10 mM estragole (final concentration 100 µM) was added. The reactions were terminated after 20 min of incubation by adding 50 µL of acetonitrile. All incubations were performed in triplicate, and control incubations without NAPDH and without chemical inhibitor/antibody were performed. Samples were centrifuged for 5 min at 16.000*g* and the supernatant was stored at –20 °C until HPLC analysis for quantification of 1′-hydroxyestragole.

Kinetic studies

K_{cat} and K_m determination for safrole and estragole

For Gentest microsomes expressing P450 1A2, 2A6, 2C19, 2D6, and 2E1, the k_{cat} and K_m values for estragole 1′-hydroxylation were determined by incubating these microsomes with estragole concentrations ranging from 0 to 500 µM (for P450 1A2, 2A6, and 2E1) or from 0 to 1000 µM (for P450 2C19 and P450 2D6 in triplicate and for P450 1A2 in quadruplicate). The incubation conditions were similar to the incubation conditions with Gentest microsomes described above. For the related compound safrole, the kinetics for the enzymes involved in safrole 1′-hydroxylation were determined similarly (18). For Gentest microsomes expressing P450 2A6, 2C9, 2C19, 2D6, and 2E1, the k_{cat} and K_m values for safrole 1′-hydroxylation were determined by incubating these microsomes with safrole concentrations ranging from 0 to 1000 µM (for P450 2C9 from 0 to 5000 µM) in triplicate.

Possible P450 based interactions between estragole and methyleugenol

To investigate possible interactions between estragole and methyleugenol at the level of the P450 1A2-catalyzed 1′-hydroxylation, incubations with Gentest microsomes expressing P450 1A2 were performed as described above with estragole (50, 100, or 200 µM), methyleugenol (50, 100, or 200 µM), and combinations of methyleugenol and estragole (concentration of both substrates 50, 100, or 200 µM).

Sample analysis

HPLC analysis of 1′-hydroxyestragole

Aliquots (50 µL) of each sample were analyzed on an Alltima C18 5 µm column, 150 x 4.6 mm (Alltech, Breda, The Netherlands) using an HPLC (Waters Alliance 2695 Separations Module) coupled to a Waters 2996 photodiode array detector. The gradient was made with ultrapure water containing 0.1% (v/v) trifluoroacetic acid and acetonitrile. The flow rate used was 1.0 mL/min. HPLC analysis started for 20 min in isocratic mode with 25% (v/v) acetonitrile, followed by a linear increase from 25 to 50% (v/v) acetonitrile in another 20 min. The percentage of acetonitrile was increased to 100% in 2 min, kept at 100% for 2 min, then lowered to 0% in 2 min, kept at 0% for 2 min and then increased to 25% (v/v) in 2 min for reequilibration at these initial conditions for 10 min. The retention time of 1′-hydroxyestragole under these conditions was approximately 16 min. Quantification of 1′-hydroxyestragole was performed with a calibration curve measured at 280 nm, made using synthesized 1′-hydroxyestragole. The activities were calculated in nmol 1′-hydroxyestragole min^{-1} nmol $P450^{-1}$ and/or nmol 1′-hydroxyestragole min^{-1} mg $protein^{-1}$.

HPLC analysis of 1′-hydroxysafrole

Aliquots (80 µL) of each sample were analyzed on an Alltima C18 5 µm column, 150 x 4.6 mm (Alltech, Breda, The Netherlands) using a Waters 600 Controller HPLC equipped with a Waters 717plus autosampler and coupled to a Waters 2996 photodiode array detector. The gradient was made with ultrapure water containing 0.1% (v/v) trifluoroacetic acid and

acetonitrile. The flow rate used was 1.0 mL/min. In 12 min, the acetonitrile concentration was increased from 10 to 25% (v/v). Thereafter, it was kept at 25% (v/v) for 10 min. In 5 min, the acetonitrile concentration was increased to 30% (v/v) and in another 5 min to 50% (v/v). Then, the acetonitrile concentration was increased to 100% and kept at 100% for 2 min. Thereafter, acetonitrile was lowered to the starting conditions (10%, v/v) in 1 min and the column was equilibrated at these conditions for another 10 min. The retention time of 1′-hydroxysafrole under these conditions was approximately 26.5 min. Detection was carried out by a Waters™ 996 photodiode array detector at 280 nm. Quantification of 1′-hydroxysafrole was performed by means of a calibration curve made using synthesized 1′-hydroxysafrole (*18*).

HPLC analysis of combinations of 1′-hydroxymethyleugenol and 1′-hydroxyestragole

Aliquots (50 µL) of each sample were analyzed on an Alltima C18 5 µm column, 150 x 4.6 mm (Alltech, Breda, The Netherlands) using a Waters 600 Controller HPLC equipped with a Waters 717plus autosampler and coupled to a Waters 2996 photodiode array detector. The gradient was made with ultrapure water containing 0.1% (v/v) trifluoroacetic acid and acetonitrile. The flow rate used was 1.0 mL/min. In 5 min, the acetonitrile concentration was increased from 0 to 25% (v/v). Thereafter it was kept at 25% (v/v) for 20 min. In 2 min, the acetonitrile concentration was increased to 100% (v/v) and was kept at 100% for 2 min. Thereafter, acetonitrile was lowered to the starting conditions (0%, v/v) in 2 min, and the column was equilibrated at these conditions for another 10 min. The retention time of 1′-hydroxymethyleugenol was approximately 18 min and the retention time of 1′-hydroxyestragole was approximately 25 min under these conditions. Detection was carried out by a Waters 996 photodiode array detector at 230 nm and quantification was performed by means of calibration curves made using synthesized 1′-hydroxyestragole and synthesized 1′-hydroxymethyleugenol (*17*).

Statistical analysis

For correlations between the 1′-hydroxylation of estragole and the metabolism of P450 marker substrates, enzyme activities expressed as nmol min^{-1} nmol $P450^{-1}$ were used, because in this way, correlation analysis will be independent of the amount of P450 present in the various samples. Pearson correlation tests were performed to investigate correlations between the metabolism of individual P450 marker substrates and the 1′-hydroxylation of estragole. These statistical analyses were performed with SPSS 10.1 for Windows (SPSS Inc, Chicago, IL, USA). To test whether the inhibition by enzyme specific inhibitors/antibodies was significant, two sample *t*-tests (one-sided, equal variances) were performed, after F tests for equal variances were done, using Excel (Microsoft Office 2000).

Kinetic analysis

K_{cat} and K_m determination for safrole and estragole

The data from the kinetic studies with Gentest microsomes were fitted to the standard Michaelis–Menten equation (equation 4.1) in which [S] = substrate concentration, using the LSW data analysis toolbox (version 1.1.1, MDL Information Systems, Inc.). The parameters k_{cat}, K_m and k_{cat}/K_m were determined.

$$v = \frac{k_{cat}}{1 + \frac{K_m}{[S]}} \qquad \text{(equation 4.1)}$$

Modeling P450 based interactions between alkenylbenzenes

Methyleugenol and estragole are both 1′-hydroxylated by P450 1A2 and safrole and estragole are both 1′-hydroxylated by P450 2A6. To illustrate the effect of co-exposure to these combinations of alkenylbenzenes that are bioactivated by the same P450 enzyme, 1′-hydroxylation was modeled assuming that the two compounds act as competitive inhibitors on the 1′-hydroxylation of the other compound. Competitive inhibition was modeled using equation 4.2 in which v_1 describes the 1′-hydroxylation of the compound regarded as the substrate, K_{m1}, k_{cat1} and S_1 are the parameters for the compound regarded as the substrate, and K_{m2} and S_2 are the parameters for the compound that is regarded as the competitive inhibitor.

$$v_1 = \frac{k_{cat_1}}{1 + \frac{K_{m_1}}{[S_1]} \times (1 + \frac{[S_2]}{K_{m_2}})} \qquad \text{(equation 4.2)}$$

This equation was derived from the equation describing competitive enzyme inhibition (*29,30*) by replacing K_i (inhibition constant) by K_{m2} and [I] (concentration inhibitor) by $[S_2]$. By switching the roles of the two interactive compounds equation 4.2 was used to predict the conversion rate of each compound in the presence of the other one. Total 1′-hydroxylation (v_{tot}) was calculated as the sum of v1 calculated by equation 4.2 for either one of the alkenylbenzenes. Also, on the basis of these formulas, the ratio between their 1′-hydroxylation at different concentrations was calculated. Furthermore, for comparison, the 1′-hydroxylation of these combinations of alkenylbenzenes was also modeled using the Michaelis-Menten equation (equation 4.1) assuming that the two compounds do not affect each others' metabolism. Also for this hypothetical situation, the total 1′-hydroxylation rate (v_{tot}) and the ratio between the 1′-hydroxylation of the two pairs of alkenylbenzenes were calculated at different concentrations for comparison to the situation where the competitive interaction was taken into account.

Results

Formation of 1′-hydroxyestragole by recombinant P450 enzymes

Incubations with Supersomes were performed to define which human P450 enzymes are able to 1′-hydroxylate estragole. The results obtained with the Supersomes (Fig. 4.2) show that many P450 enzymes are intrinsically able to catalyze the bioactivation of estragole. P450 1A2, 2A6, 2C9, 2C19, 2D6, and 2E1 were able to 1′-hydroxylate estragole, whereas for P450 2B6 and P450 3A4, almost no activity was observed, and for P450 2C8 no 1′-hydroxylation activity was observed at all. Data from the incubations with Gentest microsomes are shown in Fig. 4.3. Activities are expressed in nmol min^{-1} nmol $P450^{-1}$ and when expressing the results per mg protein, a comparable pattern of activities is obtained (data not shown). Because in Gentest microsomes, the activities towards enzyme-selective substrates (expressed per mg protein) are in the same order as the mean activities found in human liver microsomes, contrary to Supersomes in which activities in general are higher than in human liver microsomes, the data in Fig. 4.3 give an impression of the relative contribution of each enzyme in the human liver. From these data, especially P450 2A6 appears to be active in estragole 1′-hydroxylation. Furthermore, also P450 1A2, 2C19, 2D6, and 2E1 showed a moderate activity. Although some activity was observed for Supersomes expressing P450 2B6, 2C9, and 3A4, for Gentest microsomes expressing these enzymes this was not the case, so it is concluded that these enzymes will not contribute to estragole 1′-hydroxylation in the human liver.

Formation of 1′-hydroxyestragole by human liver microsomes

Table 4.1 shows the average activities of 15 human liver microsomes towards nine different P450 substrates. The average rate of 1′-hydroxylation of estragole was 1.20 ± 0.30 nmol 1′-hydroxyestragole min^{-1} nmol $P450^{-1}$. A 2.7-fold variation between different human liver samples was found (range: 0.60 - 1.63 nmol min^{-1} nmol $P450^{-1}$). The calculated correlation coefficients between 1′-hydroxylation of estragole and the activities towards all nine substrates are also shown in Table 4.1. A significant ($P < 0.01$) correlation was found between 7-ethoxyresorufin *O*-dealkylation activity and 1′-hydroxylation of estragole ($r = 0.71$),

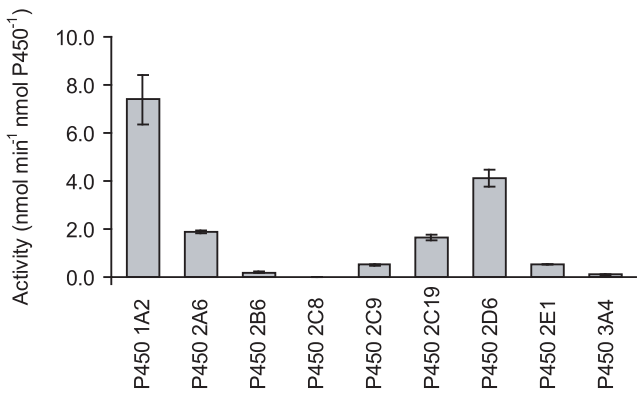


Figure 4.2. Estragole 1′-hydroxylation activity by Supersomes at a substrate concentration of 500 µM. Bars indicate average activities of triplicate measurements ± SEM (n=3).

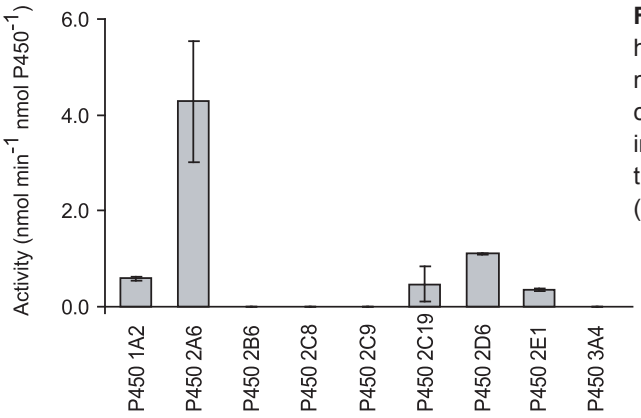


Figure 4.3. Estragole 1′-hydroxylation activity by Gentest microsomes at a substrate concentration of 500 µM. Bars indicate average activities of triplicate measurements ± SEM (n=3).

indicating that P450 1A enzymes are involved in estragole 1′-hydroxylation. Furthermore, a significant correlation between 7-ethoxy-4-trifluoromethylcoumarin *O*-dealkylation (7-ETC) and 1′-hydroxylation of estragole was found ($r = 0.78$). 7-Ethoxy-4-trifluoromethylcoumarin *O*-dealkylation is mainly catalyzed by P450 2B6 and P450 1A2 (*27*), and because (almost)

Table 4.1. Correlations between the activities towards P450 enzyme selective substrates and the formation of 1′-hydroxyestragole by 15 human liver microsomal samples.

Marker activity[a]	Mean activity ± SD (nmol min^{-1} nmol P450^{-1})		P450 enzyme	Correlation coefficient
EROD	0.15 ± 0.08	(range 0.02 – 0.30)	P450 1A	0.71*
COUM	3.19 ± 2.64	(range 0.01 – 10.5)	P450 2A6	0.41
7-ETC	0.70 ± 0.23	(range 0.27 – 1.12)	Non specific[b]	0.78*
PACL	0.55 ± 0.22	(range 0.18 – 0.91)	P450 2C8	0.01
DICLF	5.79 ± 2.82	(range 2.50 – 12.1)	P450 2C9	0.07
MEPH	0.03 ± 0.06	(range 0.00 – 0.23)	P450 2C19	0.43
BUFU	0.38 ± 0.33	(range 0.05 – 1.22)	P450 2D6	0.18
CLZOX	6.41 ± 4.13	(range 1.74 – 15.0)	P450 2E1	0.06
TEST	10.5 ± 5.16	(range 2.85 – 20.7)	P450 3A	0.28

[a] EROD = 7-ethoxyresorufin O-dealkylation; COUM = coumarin 7-hydroxylation; 7-ETC = 7-ethoxy-4-trifluoromethylcoumarin O-dealkylation; PACL = paclitaxel 6*α*-hydroxylation; DICLF = diclofenac 4′-hydroxylation, MEPH = S-mephenytoin 4′-hydroxylation; BUFU = bufuralol 1'-hydroxylation; CLZOX = chlorzoxazone 6-hydroxylation; TEST = testosterone 6*β*-hydroxylation.

[b] 7-ETC is mainly catalyzed by P450 2B6 and P450 1A2 (27).

* Statistical significance: $P < 0.01$

no intrinsic estragole 1′-hydroxylation was found in the incubations with both Supersomes and Gentest microsomes expressing P450 2B6, it is concluded that P450 2B6 is not involved in estragole 1′-hydroxylation in the human liver, and that the observed correlation with 7-ETC is most likely due to the involvement of P450 1A2 in estragole 1′-hydroxylation. The *P* values for the correlation between the activities of P450 2C19 (r = 0.43) and P450 2A6 (r = 0.41) towards their specific substrates and the 1′-hydroxylation of estragole were 0.1, indicating that these enzymes might also play a role in the bioactivation of estragole.

Inhibition experiment

Estragole 1′-hydroxylation in human liver microsomes was significantly ($P < 0.01$) inhibited with 39% (remaining activity 61%) by the P450 1A2 inhibitor α-naphthoflavone. For the other eight P450 enzyme specific inhibitors, the inhibition was less then 10% and not significant (data not shown).

Kinetic studies

Estragole 1′-hydroxylation

Fig. 4.4 shows the plots of estragole 1′-hydroxylation versus estragole concentration for Gentest microsomes containing, respectively, P450 1A2, 2A6, 2C19, 2D6, and 2E1 and

Figure 4.4. Plots of estragole 1′-hydroxylation versus estragole concentration for Gentest microsomes containing A) P450 2A6 (▲), P450 1A2 (●) and P450 2E1 (∆) and B) P450 2C19 (■) and P450 2D6 (○). Data points represent average activities ± SEM (n=4 for P450 1A2 and n=3 for all other enzymes).

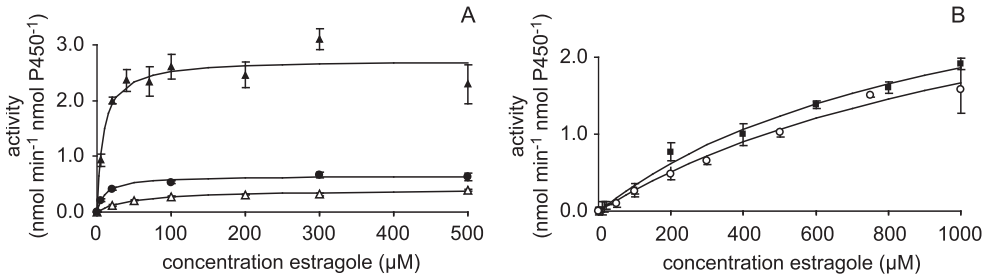


Table 4.2. Kinetic parameters for Gentest microsomes expressing the enzymes involved in estragole 1′-hydroxylation

Enzyme	k_{cat} (nmol min^{-1} nmol P450^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
P450 1A2	0.65 ± 0.03	11 x 10^{-3} ± 2 x 10^{-3}	59
P450 2A6	2.73 ± 0.12	8 x 10^{-3} ± 2 x 10^{-3}	341
P450 2C19	3.72 ± 0.47	1.0 ± 0.2	4
P450 2D6	3.89 ± 1.04	1.3 ± 0.5	3
P450 2E1	0.40 ± 0.02	49 x 10^{-3} ± 7 x 10^{-3}	8

Table 4.2 presents the parameters k_{cat}, K_m, and k_{cat}/K_m (enzyme efficiency) derived from these studies. P450 2A6 had both a high k_{cat} value (2.73 ± 0.12 nmol min^{-1} nmol $P450^{-1}$) and the lowest K_m (8 ± 2 µM) of all enzymes tested, resulting in the highest enzyme efficiency (k_{cat}/K_m = 341 min^{-1} mM^{-1}). Although the k_{cat} for P450 1A2 was - after the k_{cat} for P450 2E1 - the lowest k_{cat} observed among the five enzymes, the K_m value of P450 1A2 was much lower than the K_m values of the other enzymes (except P450 2A6). Therefore, P450 1A2 had the second highest enzyme efficiency of 59 min^{-1} mM^{-1}. The enzyme efficiencies for P450 2C19, 2D6, and 2E1 were an order of magnitude lower, respectively, 4, 3, and 8 min^{-1} mM^{-1}.

Figure 4.5. Plots of safrole 1′-hydroxylation versus safrole concentration for Gentest microsomes containing A) P450 2A6 (▲) and P450 2C19 (■), B) P450 2D6 (○) and P450 2E1 (∆) and C) P450 2C9 (♦). Data points represent average activities of triplicate measurements ± SEM (n=3).

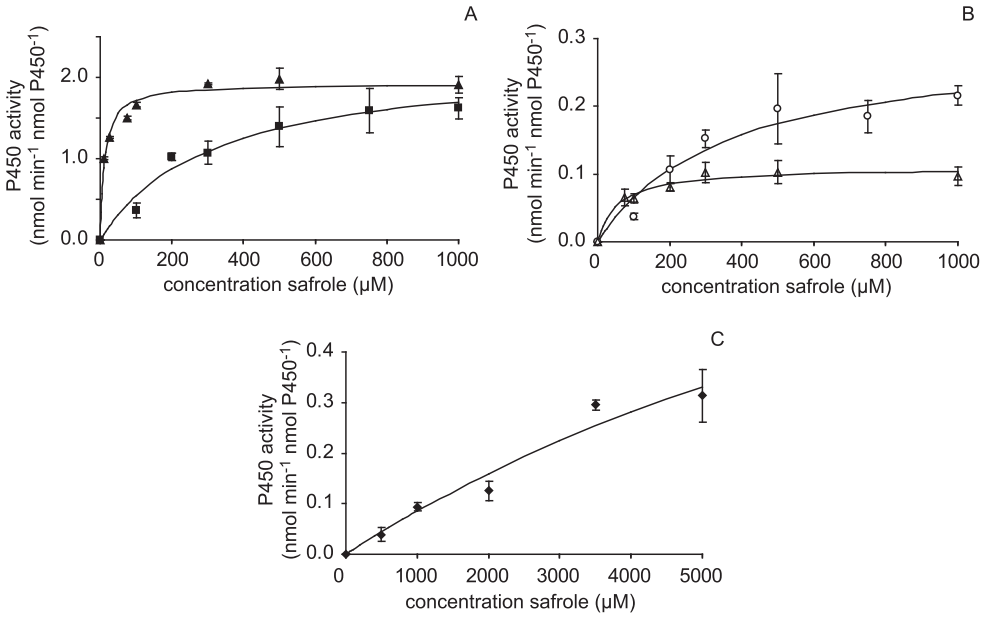


Table 4.3. Kinetic parameters for Gentest microsomes expressing the enzymes involved in safrole 1′-hydroxylation

Enzyme	k_{cat} (nmol min^{-1} nmol $P450^{-1}$)	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
P450 2A6	1.92 ± 0.04	12 x 10^{-3} ± 2 x 10^{-3}	160
P450 2C9	1.16 ± 0.79	13 ± 11	9 x 10^{-2}
P450 2C19	2.22 ± 0.33	0.31 ± 0.12	7
P450 2D6	0.30 ± 0.05	0.35 ± 0.15	1
P450 2E1	0.11 ± 0.01	57 x 10^{-3} ± 24 x 10^{-3}	2

Safrole 1'-hydroxylation

To compare the enzyme specificities of estragole and methyleugenol (*17*) to the enzyme specificities of safrole, kinetic studies were performed for the enzymes previously shown to be involved in safrole 1′-hydroxylation (*18*). Fig. 4.5 shows the plots of safrole 1′-hydroxylation versus safrole concentration for Gentest microsomes containing, respectively, P450 2A6, 2C9, 2C19, 2D6, and 2E1 and Table 4.3 presents the parameters k_{cat}, K_m, and k_{cat}/K_m (enzyme efficiency) derived from these studies. P450 2A6 showed by far the highest enzyme efficiency (k_{cat}/K_m = 160 min^{-1} mM^{-1}) and the other enzymes showed enzyme efficiencies ≤ 7 min^{-1} mM^{-1}.

P450-based interactions between alkenylbenzenes

To illustrate the effect of co-exposure to combinations of alkenylbenzenes that are bioactivated by the same P450 enzyme, 1′-hydroxylation of equimolar combinations of methyleugenol and estragole was modeled in Fig. 4.6A assuming competitive interaction at the active site of P450 1A2. In Fig. 4.6B, the 1′-hydroxylation of each of the two alkenylbenzenes is modeled for the situation in which only one of the two alkenylbenzenes is present. The sum and ratio of the 1′-hydroxmetabolites of the two alkenylbenzenes are presented in Fig.

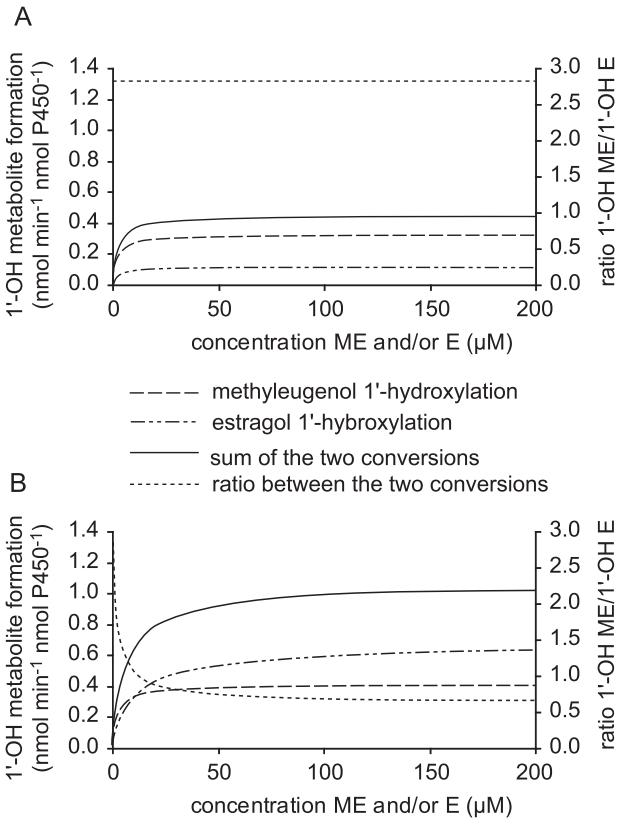


Figure 4.6. Theoretical plots of rate versus substrate concentration for the 1′-hydroxylation of methyleugenol, the 1′-hydroxylation of estragole, the sum of the two conversions, and the ratio between the two conversions assuming A) that the two compounds act as competitive inhibitors of the others 1′-hydroxylation and B) assuming no interaction between the two compounds. Concentrations plotted on the x-axis reflect substrate concentrations for both compounds (e.g. 10 μM = 10 μM methyleugenol + 10 μM estragole). The parameters used are k_{cat} = 0.40 nmol min^{-1} nmol P450 $^{-1}$ and K_m = 2.4 × 10^{-3} mM for methyleugenol (17) and k_{cat} = 0.65 nmol min^{-1} nmol P450 $^{-1}$ and K_m = 11 × 10^{-3} mM for estragole.

4.6B as well. These reflect the theoretical total formation of the two 1′-hydroxymetabolites and their theoretical ratio of formation in the absence of any competitive interaction at the active site of P450 1A2.

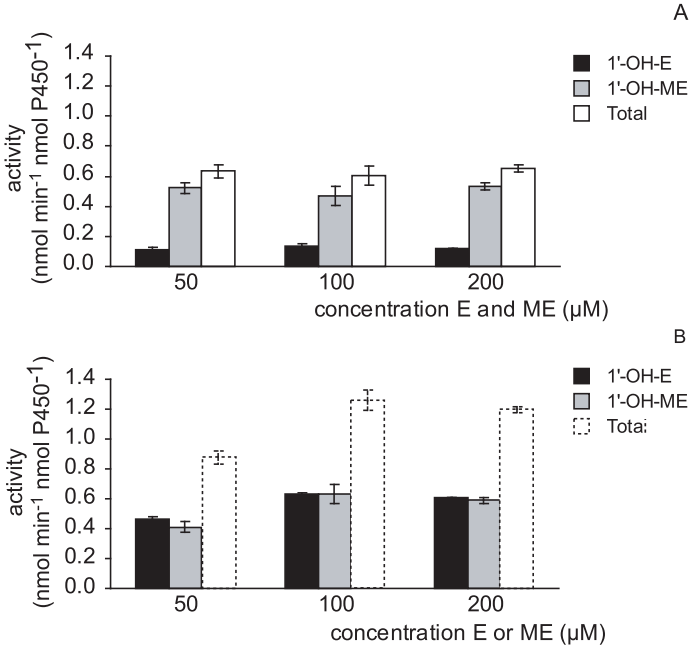
When equimolar concentrations of estragole and methyleugenol are present and competitive interaction is taken into account (Fig. 4.6A), total 1′-hydroxylation of the two alkenylbenzenes would amount to only 45% of the total activity predicted when the interaction is not taken into account (Fig. 4.6B). In the presence of equimolar concentrations of estragole and safrole, the total 1′-hydroxylation of the two alkenylbenzenes by P450 2A6 would amount to only 52% of the total activity that would be predicted when not taking the interaction into account (data not shown). Furthermore, taking the competitive interaction into account, the ratio between the formation of 1′-hydroxymethyleugenol and the formation of 1′-hydroxyestragole is constant and amounts to 2.82, whereas this ratio would decrease from 2.82 to 0.62 when no interaction is assumed. When taking the competitive interaction into account, the ratio between the formation of 1′-hydroxysafrole and 1′-hydroxyestragole is constant and amounts to 0.48 and this ratio would increase from 0.48 to 0.70 when no interaction is assumed.

To reveal whether the competitive interaction model reflects 1′-hydroxylation in case of co-exposure, Gentest microsomes expressing P450 1A2 were incubated with estragole or methyleugenol alone, and with different combinations of estragole and methyleugenol. Fig. 4.7A shows the 1′-hydroxylation of estragole, the 1′-hydroxylation of methyleugenol, and the total 1′-hydroxylation of both compounds in coexposure incubations at different concentrations. Fig. 4.7B shows the 1′-hydroxylation of estragole and the 1′-hydroxylation of methyleugenol measured in incubations with single compounds and the theoretical sum of those two conversions. When comparing the two figures, it is clear that especially estragole 1′-hydroxylation is inhibited in the presence of methyleugenol. Total 1′-hydroxylation measured in the coexposure incubations (Fig. 4.7A) is on average 58% of the theoretical sum of the 1′-hydroxylation of both compounds measured in individual incubations. This indicates that competitive interaction is taking place and that the 1′-hydroxylation in case of co-exposure can be predicted using the model assuming competitive interaction between two alkenylbenzenes. The predicted percentage assuming competitive interaction was 45%, and the difference between predicted and observed total amount can be explained by experimental variation, due to the use of another batch of P450 1A2 microsomes and the use of another HPLC system than used previously for obtaining the kinetic data for 1′-hydroxylation of methyleugenol (*17*).

Discussion

The aim of the present study was to characterize the human hepatic P450 enzymes that catalyze the 1′-hydroxylation of estragole and to determine the kinetics for the P450 enzymes involved. To allow comparison to the 1′-hydroxylation efficiencies for the enzymes involved in the bioactivation of the related compounds methyleugenol and safrole, we also

Figure 4.7. Estragole and methyleugenol 1′-hydroxylation and calculated total 1′-hydroxylation of the two alkenylbenzenes at three different concentrations A) in incubations with equimolar concentrations of both compounds and B) in incubations with single compounds. The bars in graph B for the calculated total 1′-hydroxylation are fictive amounts because due to competitive interactions, these amounts will not be reached in incubations in which the two alkenylbenzenes are simultaneously present.



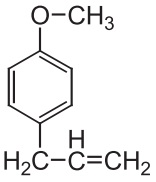
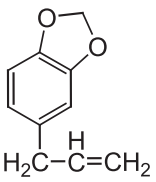
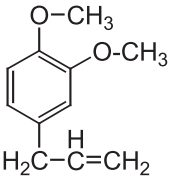
determined the kinetics for the P450 enzymes previously identified to be important in safrole 1′-hydroxylation. For methyleugenol these data are already described (*17*). In addition, we investigated the conversion of combinations of alkenylbenzenes that are bioactivated by the same P450 enzymes to demonstrate the consequences of conversion by a similar P450 for the overall 1′-hydroxylation of the alkenylbenzenes.

Several *in vitro* experiments were performed to elucidate the enzymes catalyzing the 1′-hydroxylation of estragole in the human liver. A pivotal role for P450 1A2 in estragole 1′-hydroxylation was elucidated by the significant correlation ($r = 0.71$, $P < 0.01$) between 7-ethoxyresorufin *O*-dealkylation activities and estragole 1′-hydroxylation activities in the correlation study and the significant 39% inhibition ($P < 0.01$) of estragole 1′-hydroxylation in incubations with human liver microsomes in the presence of the P450 1A2 inhibitor α-naphthoflavone. This was confirmed by the kinetic parameters obtained for P450 1A2 (enzyme efficiency k_{cat}/K_m, 59 min^{-1} mM^{-1}). Because the correlation coefficient for P450 1A2 does not approach 1 and estragole 1′-hydroxylation is only partly inhibited by α-naphthoflavone, this indicates that more enzymes contribute to this bioactivation step. However, no other significant

correlations between estragole 1′-hydroxylation and enzyme activities were observed in the correlation study with human liver microsomes and in addition no significant inhibition in estragole 1′-hydroxylation was observed with any other enzyme specific inhibitor/antibody. Most likely, also P450 2A6 is involved in estragole 1′-hydroxylation since for P450 2A6 the highest enzyme efficiency (k_{cat}/K_m, 341 min^{-1} mM^{-1}) was obtained. In addition, for P450 2A6 the *P*-value for the correlation ($r = 0.41$) between the coumarin-7-hydroxylation activities and the estragole 1′-hydroxylation activities was 0.1. Although this correlation is not significant, it is likely that P450 2A6 contributes to estragole 1′-hydroxylation in the human liver. Broad overlap of substrate specificity among P450 enzymes and their relative abundances in the human liver may reduce the reliability of correlation analyses (*31*). This could be an explanation for the absence of a significant correlation for P450 2A6 in the current study. P450 2A6 has a low abundance (6% of the total immunoquantified amount of P450) in the human liver (*32*) and P450 2A6 is not the only enzyme that is important in the bioactivation of estragole. According to the studies with recombinant enzymes, P450 2D6, 2C19, and 2E1 might also be involved; therefore kinetic studies were performed with these enzymes as well. However, the enzyme efficiencies of P450 2C19, 2D6, and 2E1 appeared to be an order of magnitude lower (k_{cat}/K_m, respectively, 4, 3, and 8 min^{-1} mM^{-1}) than those of P450 1A2 and 2A6 (k_{cat}/K_m, respectively 59 and 341 min^{-1} mM^{-1}). Moreover, their relative abundances in the liver [based on total immunoquantified (*32*) or spectroscopically (*33*) quantified P450 levels] are 2% for P450 2D6, 9% for P450 2E1 (*32*) and 4% for P450 2C19 (*33*) and are lower than or comparable to the abundances of P450 1A2 (18%) and P450 2A6 (6%) (*32*). This indicates that in the human liver P450 1A2 and P450 2A6 are the most important enzymes in estragole 1′-hydroxylation at physiologically relevant concentrations of estragole and only at relatively higher estragole concentrations, P450 2C19, 2D6, and 2E1 might contribute to some extent.

For the related alkenylbenzenes safrole and methyleugenol, we recently reported on the P450 enzyme specificities (*17,18*). P450 2C9, 2A6, 2D6, and 2E1 were shown to be the most important enzymes for safrole 1′-hydroxylation, based on data from experiments with human liver microsomes and recombinant P450 enzymes (*17*). According to the results obtained with recombinant P450 enzymes, also P450 2C19 may play a role in safrole 1′-hydroxylation (*17*). Enzyme efficiencies for safrole 1′-hydroxylation by these P450s were reported in the present study (Table 4.3). P450 2A6 was shown to have by far the highest enzyme efficiency for safrole 1′-hydroxylation (160 min^{-1} mM^{-1}), whereas the enzyme efficiencies for the other enzymes were ≤ 7 min^{-1} mM^{-1}. Although a significant correlation between P450 2C9 activity and safrole 1′-hydroxylation was found previously (*18*), kinetic analysis revealed that at lower substrate concentrations than the 500 μM used in the correlation study, this enzyme is not important in safrole 1′-hydroxylation. At low safrole concentrations P450 2A6 appears to be the most important enzyme involved in safrole 1′-hydroxylation. For methyleugenol, P450 1A2 was previously identified as the most important enzyme in the human liver at physiologically relevant concentrations, whereas P450 2C9 and P450 2C19 might contribute as well at higher substrate concentrations (*17*). Table 4.4 gives an overview of the catalytic efficiencies of the P450 enzymes now shown to be involved in 1′-hydroxylation of the three

Table 4.4 Comparison of enzyme efficiencies of P450 enzymes for different alkenylbenzenes.

	Estragole	**Safrole**	**Methyleugenol**
			
Enzyme	Enzyme efficiency k_{cat}/K_m (min^{-1} mM^{-1})	Enzyme efficiency k_{cat}/K_m (min^{-1} mM^{-1})	Enzyme efficiency k_{cat}/K_m (min^{-1} mM^{-1})
P450 1A2	59	nd	167
P450 2A6	341	160	nd
P450 2C9	nd*	9×10^{-2}	5
P450 2C19	4	7	3
P450 2D6	3	1	<3
P450 2E1	8	2	nd

** nd indicates that enzyme efficiencies are not determined because of lack of activity in the respective Gentest microsomes.*

related alkenylbenzenes. The most striking difference is the important role of P450 1A2 in the 1′-hydroxylation of both methyleugenol (enzyme efficiency, 167 min^{-1} mM^{-1}) and estragole (enzyme efficiency, 59 min^{-1} mM^{-1}) but not of safrole and the important role of P450 2A6 in the 1′-hydroxylation of both safrole (enzyme efficiency, 160 min^{-1} mM^{-1}) and estragole (enzyme efficiency, 341 min^{-1} mM^{-1}) but not of methyleugenol. These results illustrate that it is possible that compounds that are structurally similar differ in the pattern of P450 enzymes that convert them and indicate the need for characterization of the P450 enzymes involved in a certain metabolic conversion for each compound of interest.

Because P450 1A2 is an important enzyme in methyleugenol and estragole 1′-hydroxylation and P450 2A6 is an important enzyme in estragole and safrole 1′-hydroxylation, competitive interactions may occur for the active sites of the enzymes involved in the 1′-hydroxylation of these compounds when combinations of these alkenylbenzenes are present in the liver. This is especially relevant for herb-based exposure to these alkenylbenzenes, since in herbs such as anise, basil, and nutmeg, all three alkenylbenzenes are present (*1,34,35*). Coincubations of equimolar combinations of alkenylbenzenes (Figure 4.7) showed that such competitive interactions indeed occur. Modeling revealed that total 1′-hydroxylation of estragole and methyleugenol by P450 1A2 (Figure 4.6A) and 1′-hydroxylation of estragole and safrole by P450 2A6 amounted to only 45 and 52%, respectively, of the total 1′-hydroxylation calculated without taking the competitive interaction of the two substrates at the active site of P450 into account (Figure 4.6B).

For a better risk assessment for the alkenylbenzenes, identification of groups of people that might be at increased or decreased risk for the bioactivation of alkenylbenzenes is important. The activities of the two main enzymes in the bioactivation of the herb-based alkenylbenzenes, P450 1A2 and P450 2A6, may vary in the human population due to genotype- and lifestyle-based influences; therefore, interindividual differences in 1′-hydroxymetabolite formation may occur. People bearing polymorphisms in P450 2A6 that lead to poor metabolizer phenotypes or bearing whole deletion genotypes (*36*) might be at lower risk for adverse effects following 1′-hydroxylation of estragole and safrole. For P450 1A2, three mutations have been described that are associated with decreases in enzyme activity and one mutation has been described that is associated with enhanced inducibility. So far, no allelic variant that is associated with increased enzyme activity has been identified (http://www.cypalleles.ki.se/). Jiang *et al*. (*37*) concluded that P450 1A2 genotype cannot be unequivocally linked to a metabolic phenotype and this indicates that for interindividual differences in P450 1A2 activity, lifestyle factors are more important than genetic differences. Cigarette smoking (*38*) and the consumption of charbroiled food and cruciferous vegetables can increase the activity of P450 1A2 (reviewed in ref *39*) and might increase the chances on bioactivation of both estragole and methyleugenol.

The use of methyleugenol and estragole as an additive will be restricted in Europe due to the conclusions drawn by the Scientific Committee on Food that these compounds are carcinogenic and genotoxic and that restrictions in their use are necessary (*1,34,35*). Safrole is already banned from use as a flavor and fragrance substance (Federal Register of December 3, 1960, 25 FR 12412). The question remains whether the use of herbs that contain these alkenylbenzenes, herbal supplements, and foodstuffs in which these herbs are used should be restricted. For a risk assessment for herb-based exposure to alkenylbenzenes, possible interactions at the level of the P450-catalyzed bioactivation with other compounds present in the herbs should be taken into account. This includes not only the competitive interactions between the different alkenylbenzenes, shown to be relevant in the present study, but also the possible presence of P450 1A2 and/or P450 2A6 inhibitors in herbs, which might decrease the bioactivation and thus the genotoxicity of the alkenylbenzenes. Such P450 1A2 and/or P450 2A6 inhibitors might decrease the chances on 1′-hydroxylation-mediated adverse effects of the alkenylbenzenes and might thus act as anticarcinogens. Recently we showed that in basil, a herb that contains both estragole (*1*) and methyleugenol (*34*), several inhibitors of P450 1A2 are present (*40*). The P450 enzymes that catalyze *O*-demethylation and epoxidation of the alkenylbenzenes are not yet elucidated, but for these metabolic routes, the same issues raised above for P450-catalyzed 1′-hydroxylation are relevant. The same holds for the second step of the bioactivation pathway, the sulfation of the 1′-hydroxymetabolites by sulfotransferases (SULT). Also for the SULT enzymes involved interindividual differences in bioactivation, competitive interactions between the different 1′-hydroxyalkenylbenzenes, and possible interactions with other herbal compounds are to be expected. So far, no data are available on the individual human SULT enzymes involved in the bioactivation of these alkenylbenzenes. Recently, a method to quantify the sulfation of 1′-hydroxyestragole

indirectly, based on trapping of the reactive carbocations derived from 1′-sulfooxyestragole with 2′-deoxyguanosine and quantifying the major adduct formed, N^2-(*trans*-isoestragole-3′-yl)-2′-deoxyguanosine, using LC-ESI-MS/MS analysis was developed (*41*). This method is presently used to reveal the sulfotransferases involved in the bioactivation of these alkenylbenzenes.

Altogether, the data in the present paper show that P450 1A2 and P450 2A6 are the main enzymes involved in the bioactivation of herb-based alkenylbenzenes and that competitive interactions between the alkenylbenzenes may occur at the active site of the P450 enzymes involved in their 1′-hydroxylation. The knowledge on the P450 enzymes involved in 1′-hydroxylation of the alkenylbenzenes can be used to study the presence of possible anti-carcinogens in herbs acting through inhibition of P450 1A2 or 2A6 and to implement the possible consequences of genetic and phenotype polymorphisms in the risk assessment for these alkenylbenzenes.

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Development of an on-line high performance liquid chromatography detection system for human cytochrome P450 1A2 inhibitors in extracts of natural products

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Abstract

An on-line HPLC screening method for detection of inhibitors of human cytochrome P450 1A2 in extracts was developed. HPLC separation of extracts is connected to a continuous methoxyresorufin-*O*-demethylation (MROD) assay in which recombinant human P450 1A2 converts methoxyresorufin to its fluorescent metabolite resorufin. The system was tested with three P450 1A2 inhibitors, for which minimum detectable amounts (MDA) ranging from 0.7 to 9.5 ng were obtained. Analysis of a kava kava and a basil extract showed that the on-line system is applicable to complex mixtures, since in both extracts, peaks with P450 1A2 inhibiting activity were observed.

Introduction

Human cytochrome P450 enzymes (P450) are involved in the metabolism of drugs and other xenobiotics and play an important role in both detoxification and bioactivation of carcinogens (*1*). Many procarcinogens from the human diet are bioactivated to carcinogenic electrophilic metabolites by P450 enzymes. For instance, P450 1A2 bioactivates heterocyclic amines (e.g. 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PhIP) (*2,3*) and polycyclic aromatic hydrocarbons (e.g. benzo[*a*]pyrene) (*3*) present in heat-processed meat to carcinogens. P450 1A2 also bioactivates the herb-based alkenylbenzenes methyleugenol (*4*) and estragole (*5, 6*) to their proximate carcinogenic 1′-hydroxymetabolites. Consequently, compounds present in the diet that inhibit this enzyme might act as anticarcinogens by reducing or preventing the conversion of procarcinogens into (proximate) carcinogens. We are particularly interested in P450 1A2 inhibitors present in herbs containing methyleugenol and estragole such as basil (*7,8*) since these may reduce or even eliminate the possible risks associated with herb-based exposure to these alkenylbenzenes. Therefore, a method to screen herbal products for the presence of individual components that might inhibit P450 1A2 would be useful to get insight in the presence of possible anticarcinogens.

At present, many assays to study P450 inhibition are described in literature. Well plate assays to determine inhibition of P450 1A activity based on the conversion of alkoxyresorufins (*9-11*) or 3-cyano-7-ethoxycoumarin (*12*) into their fluorescent metabolites have been developed. These assays are suitable for high throughput screening of single compounds on their inhibition of P450 1A activity. For complex mixtures, such fluorescence-based assays are less suitable. Measurements may be disturbed by intrinsic fluorescence or quenching of fluorescence by compounds present in the mixture (*13*). Furthermore, assays that screen single compounds or mixtures for the inhibiting effect on different enzymes simultaneously, using cocktails of enzyme-selective substrates in combination with LC-MS analyses, have been reported (*14,15*). However, bioassay guided fractionation remains necessary to identify individual active compounds in a complex mixture, and this remains difficult and time-consuming.

Systems in which a separation technique is coupled on-line with a biochemical detection method, also called high resolution screening (*16*), detect bioactive compounds in a faster way. By including diode array detection (DAD), evaporative light scattering detection (ELSD), and/or mass spectrometry (MS) together with an on-line assessment of their activity in a bioassay, identification and quantification of bioactive compounds can be done simultaneously. The coupling of HPLC separation of herbal extracts with post-column assays for radical scavenging activity has been developed and applied successfully (*17,18*) and hyphenated systems are also described for among others acetylcholine esterase inhibitors (*19*), estrogen receptor α and β ligands (*20*), and phosphodiesterase inhibitors (*21*). Recently, Kool *et al.* published the first on-line system for the detection of cytochrome P450 inhibitors based on the conversion of ethoxyresorufin by β-naphthoflavone induced rat liver microsomes (*22*). Their system has not yet been applied to natural extracts. The aim of the present study is

to set-up an on-line system to detect specifically inhibitors of human cytochrome P450 1A2 based on the coupling of HPLC analysis to a continuous enzyme assay for human cytochrome P450 1A2 activity and to apply this system to natural products extracts.

Materials and methods

Chemicals

Acetic acid was obtained from Boom (Meppel, Netherlands). Coumarin (>99%), fluvoxamine maleate (>98%), methoxyresorufin (resorufin methyl ether, >98%), naringenin (4',5,7-trihydroxyflavanone, >98%), and tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride, 93%) were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). Dimethyl sulfoxide (DMSO) (spectophotometric grade), dipotassium hydrogen phosphate trihydrate (p.a.) and potassium dihydrogen phosphate (p.a.) were purchased from Merck (Darmstadt, Germany). Furafylline (>99%) was obtained from Ultrafine Chemicals (Manchester, UK). Kava kava capsules were a kind gift from Dr. Wouter Vaes from TNO Quality of Life (Zeist, Netherlands) (*23*). Methanol was high performance liquid chromatography (HPLC) grade from Lab-Scan, Analytical Sciences (Dublin, Ireland). NADPH (>98%) was obtained from Boehringer (Mannheim, Germany). DL-propranolol hydrochloride and resorufin were from Janssen Chimica (Beerse, Belgium). Supersomes, prepared from baculovirus infected insect cells expressing the human individual P450 1A2 enzyme, containing 1.0 nmol P450 1A2/mL, were from BD Gentest (Woburn, USA). Dried basil (Silvo) was obtained from the local supermarket.

Equipment

A Picoplus syringe pump was obtained from Harvard Apparatus (Holliston, USA) and 500-µL glass syringes were purchased from Hamilton (Bonaduz, Switzerland). A HyperShear™ ternary tee stainless steel static mixer (internal volume 10 µL) was obtained from Alltech (Breda, Netherlands). A manual Rheodyne 7125 injector was from Rheodyne Europe GmbH (Bensheim, Germany). A Gilson HPLC pump model 302 was purchased from Gilson International (Den Haag, Netherlands). A Heidolph EKT 3001 thermostat and Heidolph MR3001 K heating plate were purchased from Heidolph Instruments (Schwabach, Germany). A fluorescence plate reader (Varian Cary Eclipse) was obtained from Varian (Melbourne, Australia) and an FL2000 SpectraSYSTEM fluorescence detector was from Thermo Separation Products (Breda, Netherlands). Two recorders were obtained from Kipp & Zonen (Delft, Netherlands). A Mass Rate Attenuator was purchased from Shimadzu (Den Bosch, Netherlands). Evaporation of organic solvents was performed *in vacuo* by a rotavapor apparatus RE111 from Büchi Laboratoriumstechnik (Flawil, Switzerland). The HPLC system used consisted of a Waters 600E liquid chromatography system, equipped with a Waters 717 plus autosampler and a Waters 996 photodiode array detector (Waters, Breda, Netherlands).

Determination of optimal conditions for the continuous MROD assay in 96-well plates.
K_m and k_{cat} determination

The apparent K_m and k_{cat} of P450 1A2 for the substrate 7-methoxyresorufin (MR) were determined in a 96-well plate assay. Substrate concentrations of MR, ranging from 0 to 10 µM, were tested in triplicate. To each well, 50 µL of 0.1 M potassium phosphate pH 7.4 (KPi), 25 µL of 0 – 25 µM MR in KPi (final concentrations (fc) in well 0 – 5 µM), prepared from 80x concentrated stock solutions in DMSO, and 25 µL from a stock solution of 25 µL Supersomes/mL KPi (fc 5 nM P450) were added. The assay was started by adding 25 µL of 0.5 mM NADPH (fc 0.1 mM) and the initial fluorescence signal (λ_{ex} = 530 nm, λ_{em} = 590 nm) was measured on a fluorescence detector. Resorufin production was measured after 4 min of incubation at room temperature. A calibration curve was made with resorufin (0 – 200 nM, steps of 20 nM). Enzyme activities were calculated in pmol resorufin min^{-1} pmol $P450^{-1}$. The experiments were performed in duplicate. The data were fitted to the standard Michaelis−Menten equation $v = k_{cat}/(1+(K_m/[S]))$, with [S] = substrate concentration, using the Life Science Workbench (LSW) Data Analysis Toolbox (version 1.1.1, MDL Information Systems, San Leandro, CA).

Optimization NADPH concentration

To determine the optimal NADPH concentration, the same assay as described above was performed using an MR concentration (5 µM) at which k_{cat} is reached. The reaction mixture consisted of (fc) 5 µM MR, 5 nM P450 1A2 (Supersomes), and 0.01, 0.1, or 1 mM NADPH in a final volume of 125 µL KPi. Three replicates of each NADPH concentration were measured per experiment and the experiment was performed twice.

Effect of methanol on P450 1A2 activity

Since organic solvents inhibit P450s (*24*), the effect of methanol on P450 1A2 activity was tested in the same assay as described above. To each well, 25 µL of 2 µM MR in KPi (fc 0.4 µM), 50 µL KPi containing 0 – 100% methanol (fc 0 – 40%), and 25 µL from a stock solution of 25 µL Supersomes/mL KPi (fc 5 nM P450) were added. The assay was started by adding 25 µL of 0.5 mM NADPH (fc 0.1 mM). Each methanol concentration was tested in triplicate per experiment, and the experiment was performed twice. Furthermore, the effect of methanol on the inhibiting potential of two P450 1A2 inhibitors was tested. To this aim, to each well 25 µL KPi containing either propranolol (0 – 1500 µM stock solution = 0 – 300 µM fc) or fluvoxamine (0 – 500 µM stock solution = 0 – 100 µM fc) were added. Furthermore, to each well, 25 µL of 1 µM MR in KPi (fc 0.2 µM) and 25 µL from a stock solution of 5 µL Supersomes/mL KPi (fc 1 nM P450) were added. Each inhibitor concentration was tested in triplicate at four different methanol concentrations: 0-5-10-15% (v/v) (added as 25 µL of 0 – 25 – 50 – 75% (v/v) methanol stock solutions in KPi). The assay was started by adding 25 µL of 0.5 mM NADPH (fc 0.1 mM). IC_{50} values (concentration at which 50% inhibition is obtained) were calculated using the LSW Data Analysis Toolbox (version 1.1.1, MDL Information Systems, San Leandro, CA).

Continuous MROD assay

Experimental set-up

Based on the experimental data from the off-line experiments in 96-well plates, 0.1 mM NADPH, 0.2 µM MR, and an enzyme concentration of 1.5 nM recombinant P450 1A2 were used in the continuous MROD assay. In Fig. 5.1A, the set-up for this continuous MROD assay is shown. A syringe pump equipped with two 500-µL glass syringes was used. Syringe 1 was filled with enzyme (12 µL Supersomes/mL KPi = 12 nM P450 1A2) and syringe 2 was filled with NADPH (0.64 mg/mL KPi) and substrate (MR, 1.6 µL of an 1 mM stock solution prepared in DMSO/mL KPi). The two syringes were connected to a static mixer and infused at a flow rate of 7.5 µL/min each. A Gilson 302 HPLC pump (carrier pump) was connected to the mixer as well and operated at a flow rate of 45 µL KPi/min. A manual injector equipped with a 2-µL injection loop, made from polyether ether ketone (PEEK) tubing, was placed between the carrier pump and the mixing device. A 0.13-mm internal diameter (I.D.) reaction coil made of 15 m PEEK tubing (internal volume 0.20 mL) was connected to the mixing device. The total flow-rate was 60 µL/min, resulting in a residence time in the reaction coil of 3.3 min. The reaction coil was placed in a water bath of which the temperature was set at 45 °C with a thermostat and a heating plate. The coil was connected to a fluorescence detector that contained a 2-mm single-sided transmission (SST) flow cell with an illuminated volume of 3 µL and operated at λ_{ex} = 530 nm and λ_{em} = 590 nm.

Determination of IC_{50}-values and detection limits for P450 1A2 inhibitors

The continuous MROD assay was optimized and tested with the P450 1A2 substrates and/or inhibitors furafylline (*25*), propranolol (*26*), tacrine (*27*), fluvoxamine (*28*), and naringenin (*29*) (Fig. 5.2). Stock solutions (25 mM) of these inhibitors were prepared in DMSO and diluted in KPi to the desired concentrations (0 – 500 µM, maximum DMSO concentration 2%). Aliquots (2 µL) were injected into the flow system and detection limits in terms of concentration (limit of detection, LOD) and amount (minimum detectable amount, MDA) and IC_{50} values (inhibitory concentration at which 50% inhibition is obtained) were determined for all compounds in three separate runs. Intrinsic fluorescence of each compound was tested by injecting the highest concentration (500 µM) of each inhibitor while the system was flushed with KPi only, whereas quenching was tested by injecting the highest concentration while the system was flushed with 40 nM resorufin, the anticipated resorufin concentration in the assay.

Continuous MROD assay coupled to HPLC analysis

Experimental setup

Fig. 5.1B shows the set-up of the on-line MROD assay coupled to HPLC analysis. The mobile phase consisted of methanol and 20 mM potassium phosphate (pH 4.5) in ultra pure water. A photodiode array detector measured the absorbance (200–700 nm). After the diode array detector, a Mass Rate Attenuator was placed. The Mass Rate Attenuator directs ± 4 µL/min of the HPLC outlet into the stream of the Gilson 302 HPLC pump, which infused buffer

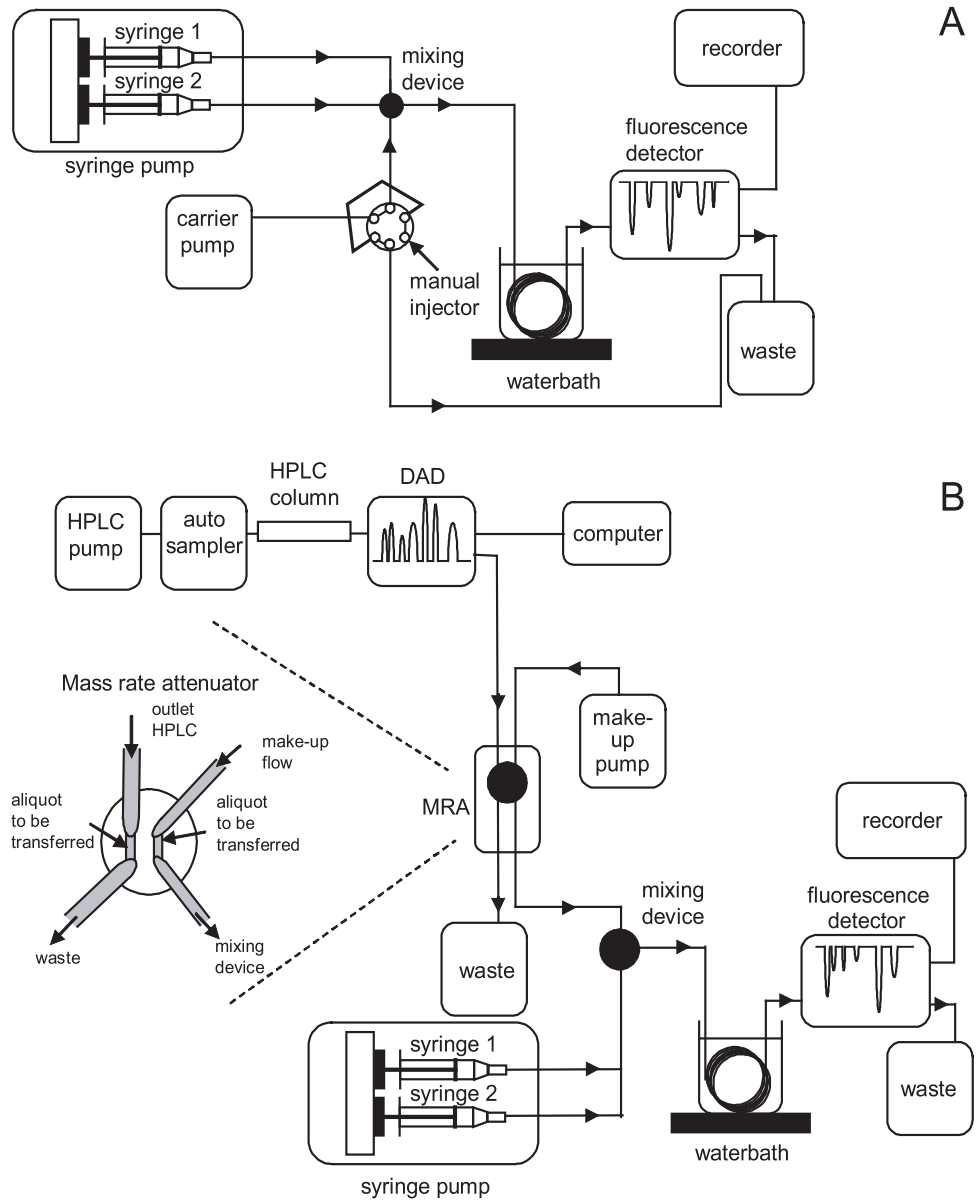


Figure 5.1. Schematic set-up of (A) the continuous MROD assay and (B) the on-line HPLC MROD assay for detection of P450 1A2 inhibitors.

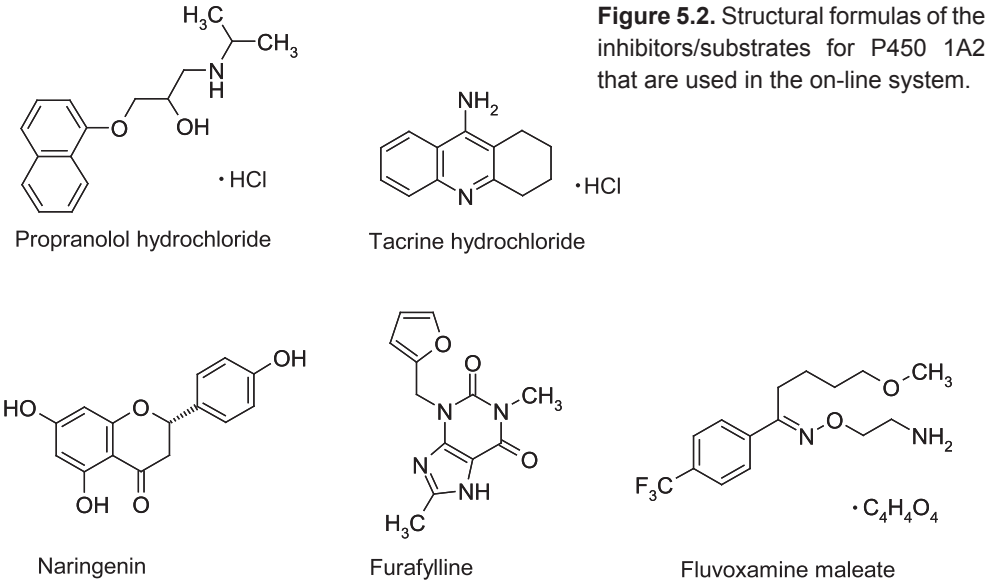


Figure 5.2. Structural formulas of the inhibitors/substrates for P450 1A2 that are used in the on-line system.

(KPi) at a rate of 45 µL/min. The outlet of the MRA device is connected to the static mixer. The two syringes (contents of the syringes are similar as in the continuous enzyme assay), operated by a syringe pump at an infusion rate of 7.5 µL/min, were connected to the mixer. The mixing device was connected to the reaction coil. The total flow rate was 60 µL/min and the reaction coil was put into a water bath of 37 °C.

Determination of detection limits for P450 1A2 inhibitors

Stock solutions (25 mM) of propranolol, tacrine, and fluvoxamine were prepared in DMSO and diluted in KPi to the desired concentrations (0 – 1000 µM, maximum DMSO concentration 4%). Aliquots (40 µL) were analyzed on an Alltima C8 5 µm particle size column, 150 x 3.2 mm (Alltech, Breda, Netherlands) using the HPLC in isocratic mode (57% methanol, 43% 20 mM potassium dihydrogen phosphate in ultra pure water) at a flow rate of 500 µL/min and detection limits for P450 inhibition in the on-line assay were determined for all compounds in two separate runs.

Analysis of kava kava and basil extract

Sample preparation

A kava kava extract was prepared, according to Unger *et al.* (*30*), by extracting 250 mg of the contents of a kava kava capsule with 2.5 mL methanol in an ultrasonic bath at ± 40 °C for 30 min. The resulting extract was centrifuged (Sigma 202M, 10 min, 10,000 rpm). To 1 mL of the supernatant of the methanol extract 1 mL of ultra pure water was added and this solution was passed through a 500 mg Varian Bond Elut RP18 SPE cartridge preconditioned

with 5 mL methanol and 5 mL of 50% methanol in ultra pure water. After application of the extract, the column was washed with 2 mL of 50% methanol, dried, and consecutively eluted with 4 mL methanol. The eluate was evaporated *in vacuo* and stored at −20 °C until further use. The extraction yield was about 10% using this method.

A basil extract was prepared by stirring 5 g of dried basil twice for 2 h, each time with 50 mL of a mixture of methanol, ultra pure water, and acetic acid (ratio 80:19:1), at room temperature. Subsequently, the solvent was evaporated *in vacuo* after pooling the filtrates and the extract was stored under nitrogen at −20 °C until use. The extraction yield was about 10% using this method. To 300 mg of the extract, 2 mL of ultra pure water were added, and the solution was successively extracted with four 1 mL portions of dibutyl ether. The four dibutyl ether layers were combined and the solvent was removed *in vacuo*. The resulting 42 mg of dibutyl ether extract were dissolved in 500 µL of 80% methanol in ultra pure water (v/v).

Kava kava and basil in the MROD assay in 96-well plates

To determine adequate extract concentrations for analysis in the on-line HPLC MROD assay, the activity of both extracts was estimated using the off-line 96-well plate MROD assay described under 'effect of methanol on P450 1A2 activity' (enzyme concentration 1 nM). For kava kava extract, concentrations ranging from 0 to 50 µg extract/mL were tested and for the methanol extract of basil, concentrations ranging from 0 to 200 µg extract/mL were tested.

Kava kava and basil in the on-line MROD assay coupled to HPLC analysis

For the kava kava extract, aliquots (40 µL, 8.3 mg extract/mL in methanol/water 1:1, filtered through a 0.45 µm PTFE filter, Alltech, Breda, Netherlands) were analyzed on an Alltima C18 5 µm particle size column, 250 × 2.1 mm (Alltech, Breda, Netherlands) using the HPLC in isocratic mode (60% methanol, 40% 20 mM potassium dihydrogen phosphate in ultra pure water, flow rate 0.2 mL/min). The run time was 50 min. Absorbance was measured from 200 to 700 nm using diode array detection.

For the basil extract, initially methanol extract aliquots (40 µL, 150 mg extract/mL in methanol/water 1:1, filtered through a 0.45 µm PTFE filter, Alltech, Breda, Netherlands) were analyzed on the same column using various gradients made using methanol and 20 mM potassium dihydrogen phosphate in ultra pure water as solvents. The flow rate was 0.2 mL/min. Absorbance was measured from 200 to 700 nm using diode array detection. Thereafter, the methanol extract was extracted with dibutyl ether as described previsouly. Aliquots (40 µL, 84 mg extract/mL in methanol/water 4:1 before filtration) were analyzed on the same column. A linear gradient from 31 to 69% methanol in 20 mM potassium dihydrogen phosphate in ultra pure water was applied during 35 min and 69% methanol was maintained for another 40 min. Due to relatively long delay time caused by system dead volume and the low flow rate used, aliquots were injected 20 min after the gradient was started. Run time (after injection) was 55 min. Three separate runs were performed with kava kava extract and the dibutyl ether extract of basil. During the first run, the eluate was mixed only with KPi

in the reaction coil, to investigate which components showed intrinsic fluorescence. During the second run, the eluate was mixed with the metabolite resorufin (final concentration in fluorimeter 40 nM) to detect compounds that quenched the signal of resorufin. Finally, the extracts were analyzed in the on-line MROD assay in triplicate.

Results and discussion

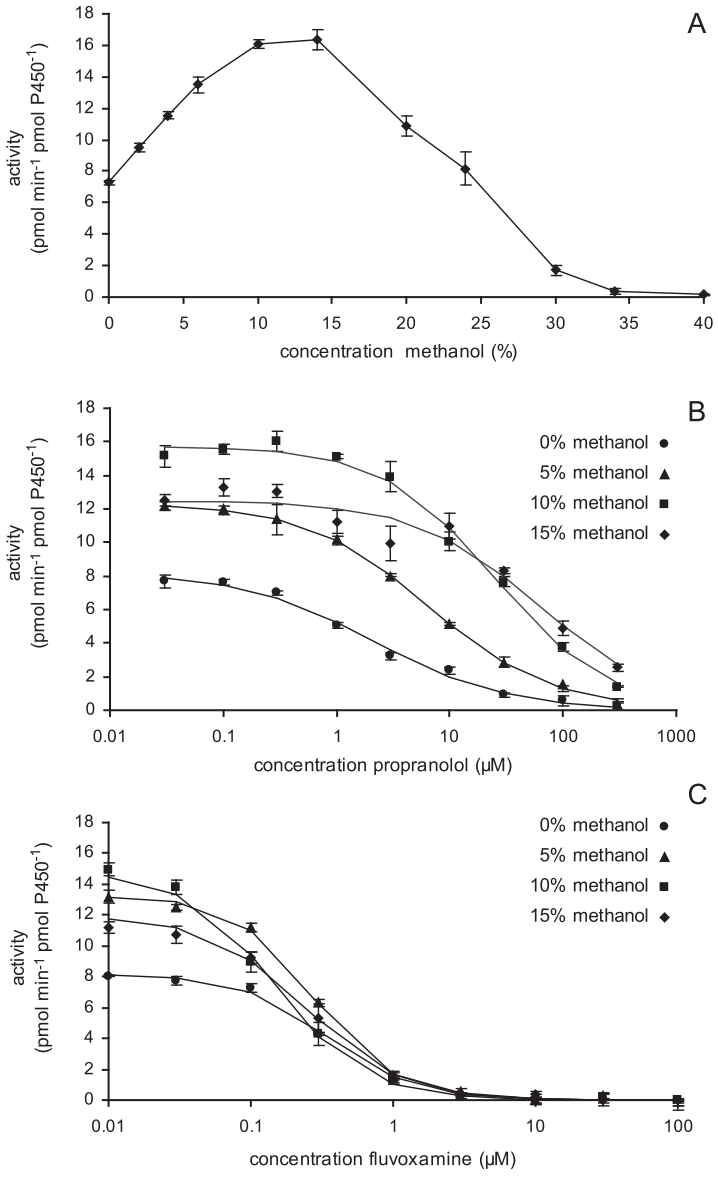
Off-line optimization of the assay

The aim of the work presented here was to develop an on-line HPLC detection system for detection of human cytochrome P450 1A2 inhibitors in complex mixtures based on the methoxyresorufin-*O*-demethylase (MROD) assay (*9-11*). In this assay, the P450 1A2 selective substrate methoxyresorufin (MR) is converted into its fluorescent metabolite resorufin by commercially available human recombinant P450 1A2.

Initial tests in 96-well plates were performed to optimize the parameters for the MROD assay. A reaction time of 4 min was used for all tests; during this period resorufin production was linear with time (data not shown). For the continuous MROD inhibition assay, the concentration of the substrate MR should be around the K_m for MR in order to measure proper inhibition, and for economical reasons low consumption of both NAPDH and enzyme is preferable. In two individual experiments, an average K_m value of 0.3 μM MR was obtained for human P450 1A2 and based on this K_m value an MR concentration of 0.2 μM was used in all further experiments. In the system developed by Kool *et al* (*22*), a different but comparable substrate was used in a similar concentration (0.15 μM ethoxyresorufin versus 0.2 μM MR in the present system). NADPH concentrations of 0.01, 0.1 and 1 mM were tested, and 0.1 mM turned out to be a saturating NADPH concentration when an enzyme concentration < 5 nM P450 1A2 was used. For the continuous enzyme assay, the enzyme concentration was lowered to 1.5 nM P450 1A2 since this concentration already gave sufficient resorufin production to detect inhibition.

Furthermore, in the off-line 96-well plate assay the effects of organic solvents on the MROD assay were investigated. Preliminary experiments (data not shown) revealed that methanol was more compatible with the MROD assay than acetonitrile, tetrahydrofuran, and *tert*-butyl methyl ether. In Fig. 5.3A, the effect of increasing concentrations of methanol on the resorufin production is shown. At high concentrations (≥ 15%, v/v) the resorufin production was inhibited, probably due to enzyme denaturation and/or inhibition, and at lower concentrations (1–15%, v/v) resorufin production is increased. A small part (< 10%) of this increase in fluorescence can be explained by a direct effect of methanol on the fluorescence of resorufin, but the major part of this increase is an increase in resorufin production and could be due to higher solubility of the substrate methoxyresorufin, conformational changes of the enzyme, modified membrane incorporation of the enzyme, or altered affinity/binding of NADPH in the presence of a higher methanol concentration. To test the influence of methanol on the sensitivity of the assay, inhibition curves were made for propranolol and fluvoxamine at methanol concentrations of 0–5–10–15% (concentrations higher than 15% methanol are

Figure 5.3. The effect of various concentrations methanol on (A) P450 1A2 activity (B) P450 1A2 inhibition by propranolol and (C) P450 1A2 inhibition by fluvoxamine. Data points represent mean ± SEM of triplicate measurements.



not suitable for the on-line system because of enzyme inhibition at these concentrations). The results are shown in Fig. 5.3B and 5.3C. A substrate dependent loss of sensitivity is observed in the assay, since with increasing methanol concentrations, the inhibition curve for propranolol shifts to the right (IC_{50} value at 15% methanol ± 30 times higher than at 0% methanol), whereas for fluvoxamine, no shift in IC_{50} is visible. For the continuous MROD assay coupled to HPLC analysis methanol concentrations around 5% will be optimal. No inhibition was observed at this concentration (see Fig. 5.3A). Although the detection limits for some substrates will increase at 5% methanol as compared to lower methanol concentrations (see Fig. 5.3B), less dilution of the HPLC eluent will be necessary when allowing methanol concentrations up to 5%, so higher concentrations of potential inhibitors can enter the continuous enzyme assay, which leads to an increase in sensitivity for the on-line system. Kool *et al.* also found that methanol concentrations from 3 – 6% were optimal (*22*).

Continuous MROD assay

With the parameters selected above (0.2 µM MR, 0.1 mM NADPH, 1.5 nM P450 1A2) the continuous MROD assay was set up. By the combination of length and diameter of the reaction coil and the total flow rate of 60 µL/min, the reaction time in the coil was 3.3 min. The set-up was tested with the P450 1A2 substrates/inhibitors furafylline, propranolol, tacrine, fluvoxamine, and naringenin (Fig. 5.2). None of the compounds showed quenching of fluorescence or intrinsic fluorescence at the highest concentration tested (500 µM). In Table 5.1 the IC_{50} values and also the limits of detection/minimum detectable amounts (LOD/MDA, defined as the concentration/amount that gives a peak ≥ 3 SD of the baseline noise) are given for all inhibitors tested. From the small standard error of the mean (SEM) values, it can be concluded that inter-run and inter-day reproducibility is high, since for all three compounds the runs were performed on at least 2 different days. The MDAs ranged from 0.4 to 5.4 ng.

Table 5.1. IC_{50} values, limits of detection (LOD), and minimum detectable amounts (MDA) obtained for five inhibitors in the continuous enzyme assay.

Compound	IC_{50} (µM)[a]	IC_{50} (ng)	LOD (µM)[a]	MDA (ng)
Fluvoxamine	1 ± 0	12 ± 1	0.5	0.4
Tacrine	10 ± 1	47 ± 5	2	0.9
Furafylline	16 ± 2	83 ± 10	5	2.6
Propranolol	21 ± 3	124 ± 18	1	0.6
Naringenin	88 ± 6	479 ± 33	10	5.4

[a] Concentrations shown are concentrations in the injection loop. Injection volume in the continuous enzyme assay is 2 µL.

On-line HPLC MROD assay

Next, the continuous assay was coupled on-line to an HPLC system. For the coupling of the HPLC system to the MROD assay, a Mass Rate Attenuator (MRA) was used. The MRA is a flow-control device that periodically gates a small aliquot from one liquid stream into another (*31*). In the expansion in Fig. 5.1B, the device is shown in detail. This device directs ± 4 µL/min of the HPLC outlet into the stream of the HPLC pump which infuses buffer into the continuous enzyme assay. The total flow in the enzyme assay is 60 µL/min so in this way, the HPLC outlet is diluted 15 times and the methanol percentage in the enzyme assay may vary between 0 and 7% in an HPLC run, depending on the gradient used.

The continuous MROD assay was initially tested with three known P450 1A2 inhibitors and/or substrates, using the HPLC in isocratic mode. In Fig. 5.4, the results for one typical run with a concentration range of tacrine are shown and in Table 5.2, the LODs and MDAs of two separate runs for all three inhibitors are summarized and compared to those of the continuous enzyme assay without coupling to HPLC. In Table 5.2, a correction is made for the fact that the MRA directs only 4 µL/min of the HPLC outlet (total flow 500 µL/min) to the enzyme assay, so less than 1% of the amount injected enters the reaction coil. When comparing the MDAs of the continuous enzyme assay to the MDAs of the on-line HPLC MROD system, for fluvoxamine and tacrine, a loss of sensitivity of about two-fold is observed when HPLC analysis is included in the system. For propranolol, however, the loss of sensitivity is much larger since the MDA value for the on-line HPLC MROD system is about 16-fold higher. This is in line with the results shown in Fig. 5.3, where propranolol showed a much greater loss of sensitivity in the presence of methanol than fluvoxamine. The increases in MDAs can be explained by the presence of methanol and the additional peak broadening that is caused by the HPLC separation. During the set-up of the system, it was tested which components of the system contribute most to the peak broadening (HPLC separation, splitter, mixer, reaction coil, detector) and it appeared that the reaction coil had the most important contribution to the peak broadening, followed by HPLC separation.

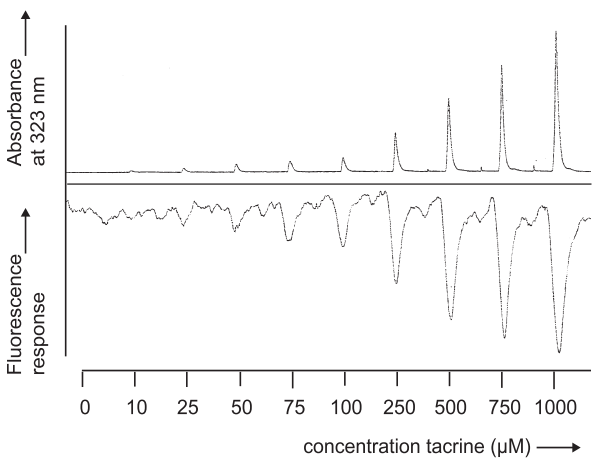


Figure 5.4. UV and fluorescence profiles of a concentration range of tacrine (0-1000 µM injected concentration) in the on-line HPLC MROD assay. HPLC analysis was performed on a C8 column (150 × 3.2 mm) using HPLC in isocratic mode (57% methanol, 43% 20 mM potassium dihydrogen phosphate in ultra pure water; flow rate 0.5 mL/min). The upper part presents the absorbance at 323 nm and the lower part shows the fluorescence response.

Table 5.2. Comparison between limits of detection (LOD) and minimum detectable amounts (MDA) in the continuous enzyme assay with and without coupling to HPLC analysis.

Compound	Continuous enzyme assay		Continuous enzyme assay coupled to HPLC (uncorrected)		Continuous enzyme assay coupled to HPLC (corrected)[a]
	LOD (μM)[b]	MDA (ng)	LOD (μM)[c]	MDA (μg)	MDA (ng)
Fluvoxamine	0.5	0.4	5	0.09	0.7
Tacrine	2	0.9	25	0.23	1.9
Propranolol	1	0.6	100	1.18	9.5

[a] The LOD values are corrected for the dilution caused by the Mass Rate Attenuator. Since the MRA directs only 4 μL/min from the HPLC outlet (total flow 500 μL/min) to the enzyme assay, the amount injected is reduced 125 times before entering the enzyme assay.
[b] Concentrations shown are concentrations in injection loop. Injection volume in the continuous enzyme assay is 2 μL.
[c] Concentrations shown are concentrations in injection loop. Injection volume in the continuous enzyme assay coupled to HPLC analysis is 40 μL.

Since the corrected MDAs for the on-line HPLC MROD system are only 2 – 16 fold higher than the MDAs for the continuous MROD assay, the on-line HPLC-MROD assay is working properly. Kool *et al.* found MDAs of 10 pmol injected for their highest affinity ligands (ellipticine and 9-hydroxyellipticine) in their on-line HPLC system (*22*). In combination with their split ratio (1:9), the MDAs in their enzyme assay are 1 pmol and this is in line with our corrected MDA for fluvoxamine which is 0.7 ng (= 2 pmol) (Table 5.2). Since ellipticine has higher affinity for P450 1A2 than fluvoxamine (*22*), according to the data of Kool *et al.*, the sensitivity of our system is at least comparable to their set-up. The drawback of our set-up is that less than 1% of the amount injected on the HPLC enters the enzyme assay in order to keep the percentage of methanol compatible with the enzyme assay. Therefore, large amounts of inhibitors have to be injected in the on-line HPLC system, and especially for complex mixtures that have many components, the maximum load of the HPLC column might be a limitation. In the on-line HPLC system, a flow of only 60 μL/min is used in the enzyme assay. If higher flows would be used, a larger amount of HPLC effluent could be directed to the enzyme assay, and thus smaller amounts of extract could be injected. This would lead to less peak broadening and to a higher sensitivity of the system. However, when increasing the flow in the enzyme system, more recombinant enzyme and NADPH would be necessary.

We have tested the applicability of our on-line HPLC system with two extracts, a kava kava extract of which it is known that it contains P450 1A2 inhibitors (*29*) and a basil extract for which we are interested in the possible presence of P450 1A2 inhibitors because of the concomitant presence in basil of procarcinogens that are bioactivated by P450 1A2 *(4-6)*. In the off-line MROD assay, kava kava extract and the methanolic extract of basil showed IC_{50} values of, respectively, 1.4 μg/mL and 11 μg/mL. For kava kava, an HPLC system was developed, using a C18 250 x 2.1 mm column with a flow rate of 200 μL/min, and 40 μL

aliquots of extract (concentration kava kava extract 8.3 mg/mL before filtration). For the basil extract, a sample with a concentration of 150 mg/mL (before filtration) was analyzed in the on-line HPLC MROD system on the same column. No constituents caused quenching of the fluorescence of resorufin. In Fig. 5.5A, a typical HPLC on-line MROD run for kava kava extract is shown. One component of the extract is fluorescent itself (retention time = 16 min) at the excitation and emission wavelengths used. Five main peaks are visible at 355 nm, and these components all show P450 1A2 inhibition to some extent. Both in Fig. 5.5A and Fig. 5.5B it is observed that the fluorescent signal during the second half of the chromatogram

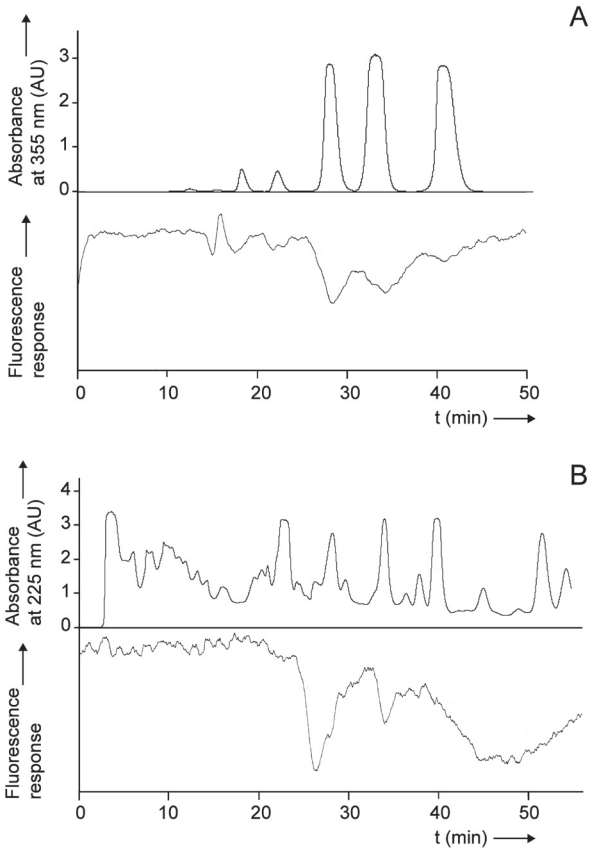


Figure 5.5. UV and fluorescence profiles of (A) 40 µL of 8.3 mg/mL kava kava extract (filtered) and (B) 40 µL of 84 mg/ml dibutyl ether extract of basil (filtered) in the on-line HPLC MROD assay. Both HPLC analyses were performed on a C18 column (250 × 2.1 mm). Kava kava was analyzed using HPLC in isocratic mode (60% methanol, 40% 20 mM potassium dihydrogen phosphate in ultra pure water; flow rate 0.2 mL/min) and basil using a linear gradient (31 – 69 % methanol in 20 mM potassium dihydrogen phosphate in ultra pure water in 35 min; flow rate 0.2 mL/min). The upper part of (A) presents the absorbance at 355 nm and the lower part of (A) shows the fluorescence response and the upper part of (B) presents the absorbance at 225 nm and the lower part of (B) shows the fluorescence response.

(from ± 25 min onwards) does not return to baseline anymore between two fluorescence dips. This is not due to baseline drift, since this effect is not observed in blank runs (to check for intrinsic fluorescence and quenching of the fluorescence) of both extracts, and is most likely due to peak broadening.

For the basil extract, initial tests with the crude methanol extract indicated that especially the less polar components of the basil extract cause the inhibition (data not shown). Therefore, the experiments were continued using a dibutyl ether extract. In Fig. 5.5B, the UV absorbance at 225 nm and the corresponding on-line fluorescence response are shown for this extract (40 µL aliquot, 84 mg/mL in methanol/water 4:1 before filtration). No intrinsic fluorescence was observed for any of the components. From these chromatograms it can be concluded that the compounds eluting until 25 min after injection do not have detectable P450 1A2 inhibitory capacities. Three main dips in the fluorescence were observed (t = 26, 34, and 48 min) indicating that P450 1A2 inhibitors are present in the less polar part of the dibutyl ether extract of basil. It is not possible to link this inhibitory activity unequivocally to a peak present in the UV-based chromatogram. When comparing the UV chromatograms at different wavelengths (absorbance was measured between 200 and 700 nm), it was obvious that multiple components elute at the time at which inhibition of P450 1A2 activity is observed. To identify the basil components that are causing this inhibition, further fractionation and optimization of the HPLC separation is necessary, possibly in combination with MS. This was, however, beyond the scope of this study.

The off-line MROD assay and the results of the analysis of basil in the on-line HPLC MROD assay showed that basil contains P450 1A2 inhibitors. This indicates that the chances on bioactivation of methyleugenol and estragole by P450 1A2 might be less in a matrix of other herbal ingredients than expected on the basis of experiments using methyleugenol and/or estragole as single compounds.

Conclusions

An on-line HPLC MROD system for the detection of human P450 1A2 inhibitors was developed and tested with typical P450 1A2 inhibitors and two natural products extracts. Our system was developed independently of a comparable on-line system that was published recently by Kool *et al.* (*22*) and when comparing the two systems there are great similarities but also clear differences. Many technical parameters such as type and concentration of substrate, temperature, and concentration of organic solvent are very similar and are apparently intrinsic to the type of assay. An important advantage of the present system is the use of recombinant P450 enzymes instead of β-naphthoflavone induced rat liver microsomes (*22*). Because of the relatively low enzyme concentration (1.5 nM) in our system, it is possible to use commercially available enzyme preparations (Supersomes) expressing individual human P450 1A2, and this makes the assay more specific for human P450 1A2 inhibitors. Furthermore, by switching to Supersomes expressing other P450 enzymes, such as P450 1A1 and P450 1B1, this system can be applied to screen specifically for inhibitors for these

enzymes as well since these enzymes also catalyze the *O*-demethylation of MR (*32*).

Another important difference is the way in which the two systems deal with HPLC gradients. Kool *et al.* used a counter gradient and a fixed split in their system to keep the percentage of methanol stable during HPLC gradients (*22*). We used a mass rate attenuator which directs ± 4 µL/min of the HPLC eluate to the reaction coil, and in this way, the HPLC eluent is diluted 15 times with buffer. As no counter gradient is applied, this system is considerably simpler. A result of this approach is that the methanol concentration varies during gradient runs from 0 to 7 %, which moderately affects the P450 activity and the detection limits but not to an extent that it hampers detection of P450 inhibitors.

The applicability of the on-line HPLC MROD system presented here to plant extracts was proven by analyzing a kava kava extract and a basil extract. For both extracts, inhibitory activity was observed in the on-line HPLC MROD system (Fig. 5.5A and B). This indicates that the system is in principle capable of detecting inhibitors in natural products extracts. The main drawbacks of the current system are the dilution of potential inhibitors present in the HPLC eluate before entering the enzyme assay and the peak broadening in the reaction coil. The necessary injection of high concentrations of extract into the HPLC causes the separation to be less than optimal and the sensitivity of our system should be increased before high resolution screening of herbal extracts becomes feasible. The sensitivity of the system might be improved by replacement of the current fluorescence detector by a capillary laser induced fluorescence detector. This would lead to both significantly higher signal-to-noise ratios in the enzyme assay and less band broadening in the detector cell. Both effects will lower LODs. Additionally, the use of such an on-line capillary detector would open the door for a miniaturization of the flow scheme on a chip in combination with nano-LC. The low flow rates on such a chip would allow the use of higher enzyme concentrations without a concomitant increase in the amount of enzyme used per assay. An alternative approach is the use of mass spectrometry instead of fluorescence detection. This allows very selective monitoring of substrate and product and more freedom in the choice of substrate (*14, 15*). Both approaches to improve the current assay will be pursued by us.

In order to estimate the chances on bioactivation of food-derived procarcinogens, knowledge on the presence of both procarcinogens and potential inhibitors of the bioactivation enzymes involved in their metabolism in the food matrix is essential, and this on-line system can be valuable for gaining this knowledge. The data presented here on analysis of the basil extract illustrate that the system is sensitive enough to detect several P450 1A2 inhibitors in this extract. These data indicate that the chances on bioactivation of methyleugenol and estragole by P450 1A2 might be less in a matrix of other herbal ingredients than expected on the basis of experiments using methyleugenol and/or estragole as single compounds.

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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.

Based on: Jeurissen SMF, Punt A, Delatour T, and Rietjens IMCM
Submitted

Abstract

The effects of a basil extract on the sulfation and concomitant DNA adduct formation of the proximate carcinogen 1′-hydroxyestragole were studied using rat and human liver S9 homogenates and the human hepatoma cell line HepG2. Basil was chosen since it contains the procarcinogen estragole that can be metabolized to 1′-hydroxyestragole by cytochrome P450 enzymes. Basil extract addition to incubations of rat and human liver S9 homogenates with 1′-hydroxyestragole, the sulfotransferase cofactor PAPS, and 2′-deoxyguanosine resulted in a dose-dependent inhibition of N^2-(*trans*-isoestragole-3′-yl)-2′-deoxyguanosine formation. Because the inhibition resembled the inhibition by the sulfotransferase inhibitor pentachlorophenol and since the inhibition was not observed in incubations with the direct electrophile 1′-acetoxyestragole it is concluded that the inhibition occurs at the level of the sulfotransferase mediated bioactivation step. Additional experiments in HepG2 cells revealed the same protective effect of basil extract in intact cells, demonstrating that the inhibitors are able to enter the cells. The results of this study suggest that bioactivation and subsequent adverse effects of 1′-hydroxyestragole might be lower in a matrix of other basil ingredients than what would be expected on the basis of experiments using 1′-hydroxyestragole as a single compound.

Introduction

Estragole (4-allyl-1-methoxybenzene) is a natural constituent of herbs such as tarragon, basil, fennel, and anise (*1*). The Scientific Committee on Food (SCF) of the European Union concluded that estragole is genotoxic and carcinogenic and this will lead to restrictions in the use of the compound as such as a food additive in the European Union (*1,2*). The question remains what this means for the use of herbs that contain estragole, foodstuffs in which these herbs are used, and herbal supplements. The carcinogenicity of estragole is demonstrated in animal experiments using high doses of the single compound (*3-5*), and its carcinogenicity depends on the formation of genotoxic electrophilic metabolites generated through a two step bioactivation pathway catalyzed by cytochrome P450 enzymes (P450) and sulfotransferases (SULT) (*3*).

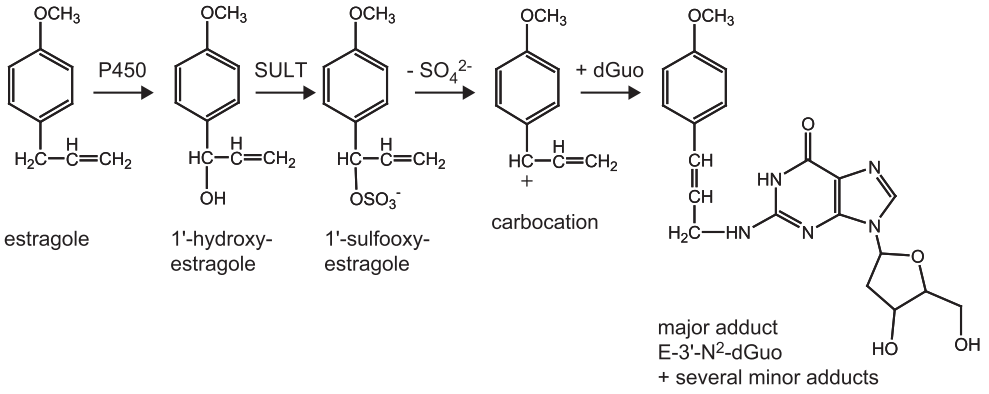
The bioactivation pathway of estragole (Fig. 6.1) starts with the conversion of estragole into its proximate carcinogen 1′-hydroxyestragole by P450 enzymes (*3,6*), in particular by P450 1A2 and P450 2A6 (*7*). The 1′-hydroxymetabolite is sulfated by sulfotransferases to the ultimate carcinogenic species 1′-sulfooxyestragole. 1′-Sulfooxyestragole is unstable in an aqueous environment and gives rise to a reactive carbocation upon loss of the sulfate group. This carbocation may bind to DNA and proteins which ultimately might lead to the formation of liver tumors (*8*).

Based on the involvement of P450 enzymes and sulfotransferases in the bioactivation of estragole to its ultimate carcinogen, a risk assessment for herb-based exposure to low doses of estragole should take into account the potential modulating effects of other bioactive ingredients present in herbs at the level of the P450 and SULT catalyzed bioactivation steps. Compounds present in herbs that inhibit the P450 enzymes or sulfotransferases involved in the bioactivation of estragole could decrease the possible adverse effects of estragole present in the same herbs and might thus act as anticarcinogens.

Previously, we have shown that a basil extract contains several inhibitors of P450 1A2 (*9*), the major P450 enzyme known to be involved in the bioactivation of methyleugenol (*10*) and one of the major P450 enzymes in the bioactivation of estragole (*7*). Basil was chosen as the model herb of interest because it contains significant levels of the alkenylbenzenes estragole (*11*) and methyleugenol (*12,13*). The objective of the present work was to study the potential basil extract-mediated inhibition of the SULT catalyzed second step of the bioactivation of estragole and the concomitant formation of DNA adducts. This requires the quantification of the ultimate carcinogen and/or the DNA adducts formed. Different adducts with the nucleobases guanine and adenine were found in mice after exposure to the proximate carcinogenic metabolite 1′-hydroxyestragole (*14,15*). The major adduct found in mice, characterized as N^2-(*trans*-isoestragole-3′-yl)-2′-deoxyguanosine (E-3′-N^2-dGuo, Fig. 6.1), can arise after an allylic shift of the carbocation prior to the reaction with the exocyclic amino group of 2′-deoxyguanosine (*14,15*).

Since 1′-sulfooxyestragole is unstable in an aqueous environment, direct quantification of this metabolite cannot be carried out. Therefore, we recently developed an indirect

Figure 6.1 Main bioactivation pathway of estragole. P450 = cytochrome P450 enzymes; SULT = sulfotransferases; dGuo = deoxyguanosine; E-3′-N^2-dGuo = N^2-(trans-isoestragol-3′-yl)-2′-deoxyguanosine.



method for the measurement of the sulfation of 1′-hydroxyestragole (*16*). This method is based on trapping of the reactive carbocation derived from 1′-sulfooxyestragole with 2′-deoxyguanosine, followed by the quantification of the major adduct, E-3′-N^2-dGuo, using an LC-ESI-MS/MS technique. In the current work, the possible inhibition of the SULT-catalyzed bioactivation of 1′-hydroxyestragole by basil extracts was studied by quantifying E-3′-N^2-dGuo in incubations of 1′-hydroxyestragole, PAPS, and 2′-deoxyguanosine with rat and human S9 liver homogenates in the absence and presence of increasing concentrations of basil extract. To investigate whether effects observed in these sub-cellular model systems would also be observed in intact cells, the formation of E-3′-N^2-dGuo was also quantified in the human hepatoma HepG2 cell line exposed to 1′-hydroxyestragole in the absence and presence of basil extract. HepG2 cells contain enzymes relevant in the phase II metabolism of 1′-hydroxyestragole such as SULT and UDP-glucuronosyltransferases (*17-19*). By measuring adduct formation in intact cells, various processes are taken into account, including uptake of 1′-hydroxyestragole, the effect of possible SULT inhibitors present in basil, the actions of other biotransformation routes, and DNA repair mechanisms. The results are discussed with regard to their possible implications for risk assessment on herb-based exposure to estragole.

Material and Methods

Materials

1′-Hydroxyestragole was synthesized by Dr. Yiannis Fiamegos (*7*) and 1′-acetoxyestragole and E-3′-N^2-dGuo were synthesized as described previously (*16*). Pooled male rat liver S9 (Sprague Dawley) and pooled human liver S9 were purchased from Gentest (Woburn, MA). Acetonitrile (chromatography grade), dimethyl sulfoxide (DMSO), dipotassium hydrogen

phosphate trihydrate, formic acid, sodium acetate, potassium dihydrogen phosphate, and zinc sulfate heptahydrate were purchased from Merck (Darmstadt, Germany). 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) and triethylammonium acetate buffer were obtained from Fluka (Buchs, Switzerland). 2′-Deoxyguanosine, ethylene-diamine-tetra-acetic acid (EDTA), nuclease P1 from *Penicillium citrinum* (EC 3.1.30.1), pentachlorophenol, phosphodiesterase I type VI from *Crotalus adamanteus venom* (EC 3.1.4.1), phosphodiesterase II from calf spleen (EC 3.1.16.1), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Steinheim, Germany). Alkaline phosphatase from calf intestine (EC 3.1.3.1) and hydrochloric acid (37%) were purchased from Roche (Mannheim, Germany). Acetic acid was obtained from Boom (Meppel, Netherlands). Tri s(hydroxymethylaminomethane) was from Invitrogen Life Technologies (Paisley, United Kingdom). Methanol was high performance liquid chromatography (HPLC) grade from Lab-Scan, Analytical Sciences (Dublin, Ireland). Dried basil (Silvo) was obtained from the local supermarket. The human hepatoma cell line HepG2 was purchased from the American Type Culture Collection (Manassas, VA). Fetal calf serum (FCS), DMEM/F12 with glutamax, Hanks Balanced Salt Solution (HBSS), phosphate buffered saline (PBS), and gentamicin were obtained from Gibco (Paisley, UK).

Preparation of a basil extract

A basil extract was prepared by stirring 5 g of dried basil twice for 2 h, each time with 50 mL of a mixture of methanol, ultra pure water, and acetic acid (ratio 80:19:1), at room temperature. Subsequently, the solvent was removed *in vacuo* by a rotavapor apparatus RE111 (Büchi Laboratoriumstechnik, Flawil, Switzerland) after pooling the filtrates. The extract was stored under nitrogen at −20 °C until use. The extraction yield was about 10% using this method.

In vitro incubations of 1′-hydroxyestragole with rat and human S9 homogenates

Incubations with rat and human S9 were performed in a 150 µL incubation mixture, containing (final concentrations) 0.1 M Tris-HCl (pH 7.4), 2 mM 2′-deoxyguanosine, 1 mg/mL S9 protein and 1′-hydroxyestragole (added from 200 times concentrated stock solutions in DMSO) at final concentrations of 50, 100, or 200 µM (for rat S9 incubations because the apparent K_m for sulfation of 1′-hydroxyestragole was previously defined to be 63 µM (*16*)) or 200, 500, or 1000 µM (for human S9 incubations because the apparent K_m for sulfation of 1′-hydroxyestragole was previously determined to be 296 µM (*16*)). 1′-Hydroxyestragole is a chiral molecule and previously it was shown that the synthesized 1′-hydroxyestragole used in the present incubations is a racemic mixture and that male rat and pooled human S9 homogenates form both enantiomers (in a ratio of about 3:1) (*16*). In the present study it is therefore assumed that a racemic mixture of 1′-hydroxyestragole can be used as substrate to determine the sulfotransferase dependent formation of 1′-hydroxyestragole DNA adducts. To these incubations, different concentrations of basil extract (0 – 100 µg/mL, added from 200 times concentrated stock solutions in methanol) were added. The reaction was started by

addition of the cofactor PAPS (2 mM final concentration). Incubations were performed for 30 min and under these circumstances sulfation was linear with time and protein concentration for both species (*16*). Control experiments without the cofactor PAPS were performed and in these blank incubations low amounts of E-3′-N^2-dGuo were formed, indicating that 1′-hydroxyestragole can also react with 2′-deoxyguanosine to some extent without its sulfation. Therefore all incubations were corrected for the amount of E-3′-N^2-dGuo formed in the blank incubations. The reactions were terminated by addition of 37.5 µL of ice-cold acetonitrile. The samples were centrifuged for 5 min at 16.000 g (14.000 rpm, Eppendorf Centrifuge, type 5415C, Hamburg, Germany) and the supernatant was stored at −20 °C until LC-ESI-MS/MS analysis. To investigate whether the inhibition of E-3′-N^2-dGuo formation can be mediated by sulfotransferase inhibition, additional incubations in the presence of 25 µM of the sulfotransferase inhibitor pentachlorophenol (PCP, added from a 5 mM stock solution in DMSO) were performed. Finally, also the effects of basil extract (100 µg/mL) on the E-3′-N^2-dGuo formation in incubations of 1′-acetoxyestragole with deoxyguanosine were studied in the absence of S9 and PAPS to test if the adduct formation of this direct electrophile would also be hampered by the basil extract, since this would point at a mechanism of inhibition of the DNA adduct formation independent from sulfotransferase inhibition and due to indirect scavenging effects.

Cytotoxicity tests

Cytotoxicity of the test compounds was evaluated using the MTT test (*21,22*). HepG2 cells were plated at a density of 2×10^4 cells per well in a 96-well plate 24 h prior to exposure. The medium was removed and cells were washed with 100 µL HBSS. Cells were exposed to the test compounds in exposure medium (DMEM/F12 + glutamax + 50 µg/mL gentamicin). The DMSO concentration was 0.5%. After 4 h of exposure in a humidified atmosphere, 5 µL of a 5 mg/mL MTT solution in PBS were added and the cells were incubated for another hour. Then, the medium was removed and 100 µL of DMSO were 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 of basil extract, 1′-hydroxyestragole, or pentachlorophenol treated versus untreated cells.

Formation of E-3′-N^2-dGuo in HepG2 cells

HepG2 cells were grown in culture medium (DMEM/F12 with glutamax containing 10% foetal bovine serum and 50 µg/mL gentamycine) in 6-well plates until confluent monolayers were obtained. Then, cells were washed with HBSS and exposed (3 wells per sample) to 50 µM 1′-hydroxyestragole (added from 400 times concentrated stock solutions in DMSO) in exposure medium (DMEM/F12 + glutamax + 50 µg/mL gentamicin). In addition, basil extract (0, 2.5, 12.5, or 50 µg/mL added from 400 times concentrated stock solutions in methanol) or pentachlorophenol (12.5 µM, added from a 400 times concentrated stock solution in DMSO) were added. The cells were exposed for 4 h at 37 °C in a humidified atmosphere. The compounds were not cytotoxic to the HepG2 cells under these conditions according to

the MTT assay described above. After exposure, cells were scraped in PBS and the cells of three wells were pooled in an Eppendorf vial and centrifuged for 5 min at ± 480 g (1500 rpm, Eppendorf Centrifuge, type 5415C, Hamburg, Germany). The pellets were stored at −20 °C until DNA isolation. DNA was isolated from the cells using a Dojindo Get *pure* DNA Kit-Cell, Tissue kit (Dojindo, Amsterdam, Netherlands), according to the accompanying protocol. DNA pellets were dissolved in 80 µL ultra pure water and the DNA was stored at 4 °C until DNA digestion. To 80 µL of the DNA solution, 15 µL of a 0.4 mU/µL phosphodiesterase II solution (6 mU), 5 µL of an 1 U/µL nuclease P1 solution (5 U), and 10 µL buffer (300 mM sodium acetate, 1 mM zinc sulphate, pH 5.3) were added. The samples were incubated for 4 h at 37 °C. Thereafter, 1.3 µL of an 1 U/µL alkaline phosphatase solution (1.3 U), 5 µL of a 0.26 mU/µL phosphodiesterase I solution (1.3 mU) and 10 µL buffer (500 mM Tris, 1 mM EDTA, pH 8) were added. The samples were incubated for another 2 h at 37 °C. After the enzymatic digestion, 500 µL of ice-cold ethanol were added and the samples were centrifuged at 16.000 g (14.000 rpm, Eppendorf Centrifuge, type 5415C, Hamburg, Germany) for 5 min and the supernatant was transferred to a new 1.5 mL tube and evaporated to dryness using a Speedvac Concentrator (New Brunswick Scientific). Samples were reconstituted in 180 µL water and analyzed using the LC-ESI-MS/MS method described below for the quantification of the amount of E-3′-N^2-dGuo, followed by HPLC analysis for quantification of the amount of 2′-deoxyguanosine as described below in order to express DNA adduct formation as pmol E-3′-N^2-dGuo formed/1000 pmol dG.

To investigate whether the digestion was complete, an oligonucleotide adduct was synthesized by incubating equimolar concentrations (approximately 140 µM) of oligonucleotide (5′-TTGTT-3′) and 1′-acetoxyestragole for 48 h at 37 °C in 0.2 g/L ammonium bicarbonate (pH 7.6). Thereafter, the sample was extracted three times with 150 µL of diethyl ether in order to remove remaining 1′-acetoxyestragole and the aqueous layer was freeze-dried. The sample was reconstituted in 500 µL ultra pure water and the adduct was purified and collected using HPLC-UV on an HP1050 liquid chromatography system (Hewlett-Packard, Geneva, Switzerland) equipped with a Supelcosil LC-18-DB, 25 cm x 4.6 mm, 5 µm column (Supelco, Buchs, Switzerland). The gradient was made with 10 mM triethylammonium acetate buffer (pH 7) and acetonitrile. The flow rate was 1 mL/min. A linear gradient was applied from 0% to 25% acetonitrile over 20 min after which the percentage of acetonitrile was increased to 100% over 2 min, kept at 100% for 3 min, lowered to 0% in 1 min and equilibrated at these initial conditions for 14 min. Detection was carried out at 260 nm. The adduct was collected at a retention time of approximately 18 min. The HPLC eluent of several runs was pooled and freeze-dried and adduct was reconstituted in 1 mL ultra pure water. One part of the sample was stored at 4°C and the other part of the sample was digested using the method described above. Both samples were again analyzed using HPLC analysis as described above. The results indicated that the digestion was complete, since no oligonucleotide adduct was measured after digestion.

Sample analysis

LC-ESI-MS/MS analysis of E-3′-N^2-dGuo

The LC-ESI-MS/MS method was adapted from (16). The analysis was performed on a Thermo Finnigan HPLC system coupled to an LCQ mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Aliquots of 20 µL (injected volume) were separated on a Phenomenex Gemini column, 2 × 150 mm (Phenomenex, Torrance, CA). The gradient was made with ultra pure water containing 0.1% (v/v) formic acid and acetonitrile and the flowrate was set to 0.1 mL/min. A linear gradient was applied from 20% to 30% acetonitrile in 30 min after which the percentage acetonitrile was increased to 100% in 2 min, kept at 100% for 1 min, lowered to 20% in 2 min and re-equilibrated at the initial conditions for 15 min. Under such conditions, the typical retention time of E-3′-N^2-dGuo was 24 min. An automatic switching device was used to prevent the electrospray source from excessive pollution. Mass spectrometrical analysis was carried out in the positive ion mode. The electospray capillary voltage was set at 4 kV and the capillary temperature at 275 ºC. Nitrogen was used as sheath gas (60 arbitrary units ~ 0.9 L/min). Sample analysis was performed in the selected reaction monitoring (SRM) mode, using transition *m/z* 414 → 298 for E-3′-N^2-dGuo with a collision energy set at –36 eV. The ion at *m/z* 414 corresponds to the protonated molecule $[M+H]^+$ and the major product ion at *m/z* = 298 is formed by loss of the 2′-deoxyribose moiety (ΔM= -116 Da) and corresponds to the adducted base (Fig. 6.2) (*16*). For each sample set, a calibration curve was prepared using synthesized E-3′-N^2-dGuo as standard (typical range 0.25 – 4 pmol). The quantification limit was estimated at 0.25 pmol on column and the signal was estimated to be linear up to ~ 8 pmol on column. To test whether the basil extract influenced the ionization of the adduct, a standard curve was measured in the presence of 100 µg/mL basil extract which revealed that the presence of basil extract did not have an effect on ionization of the adduct (data not shown).

Quantification of 2′-deoxyguanosine using HPLC

The quantitative determination of 2′-deoxyguanosine was carried out with 20 µL-sample aliquots on a Waters C18 column, 4.6 × 150 mm using a Waters 600 Controller HPLC equipped with a Waters 717plus autosampler coupled to a Waters 2996 photodiode array detector (detection wavelength at 260 nm) (Waters Corp., Etten-Leur, The Netherlands). A linear gradient was applied from 0% to 7% acetonitrile in 30 min after which the percentage acetonitrile was increased to 100% in 2 min, kept at 100% for 2 min, lowered to 100% in 2 min and re-equilibrated at the initial conditions for 15 min. The gradient was prepared with ultra pure water containing 0.1% (v/v) trifluoroacetic acid and acetonitrile. The flow rate was 1 mL/min and the retention time of 2′-deoxyguanosine under these conditions was 22 min. Quantification of 2′-deoxyguanosine was achieved by external calibration using standard solutions of 2′-deoxyguanosine.

Statistical analysis

To test whether the inhibition of the adduct formation in the presence of basil extract or pentachlorophenol was significant, two sample *t*-tests (one-sided) were performed, after F tests for equal variances were done, using Excel (Microsoft Office 2000).

Results

Inhibition of E-3′-N²-dGuo formation in rat and human liver S9 homogenates

Fig. 6.2 shows the ESI-MS/MS chromatogram for the 414 → 298 transition of an incubation of rat liver S9 homogenates with 200 μM 1′-hydroxyestragole, 1 mM PAPS, and 2 mM 2′-deoxyguanosine. The major adduct with a retention time of approximately 24 min corresponds to the synthesized E-3′-N²-dGuo and in addition to this major adduct two minor dGuo adducts are visible. With liver homogenates, a 1′-hydroxyestragole dose dependent increase in E-3′-N²-dGuo formation is observed for both rat and human models (Fig. 6.3). To investigate whether the observed adduct formation was related to SULT activity, incubations were performed in the presence of pentachlorophenol (PCP, 25 μM), a known SULT inhibitor (23). The formation of E-3′-N²-dGuo was significantly decreased (ca. 80-100%) in the presence of 25 μM pentachlorophenol.

To study possible inhibition of DNA adduct formation by a basil extract, similar incubations were performed in the absence and presence of increasing concentrations of basil extract. With basil extract, a dose dependent inhibition is observed and 25 and 100 μg/mL basil extract almost completely block E-3′-N²-dGuo formation in incubations with both rat and human liver S9. Interestingly, 100 μg basil extract/mL did not inhibit E-3′-N²-dGuo formation in

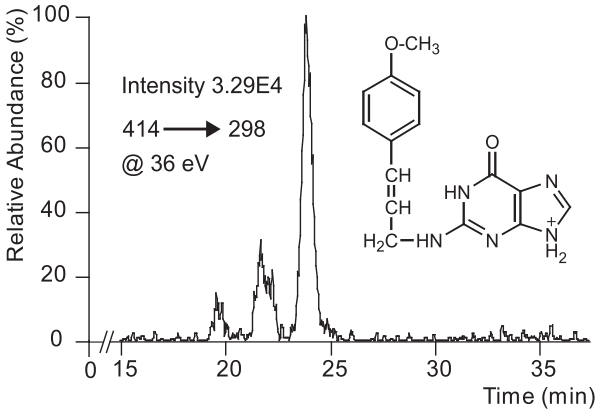
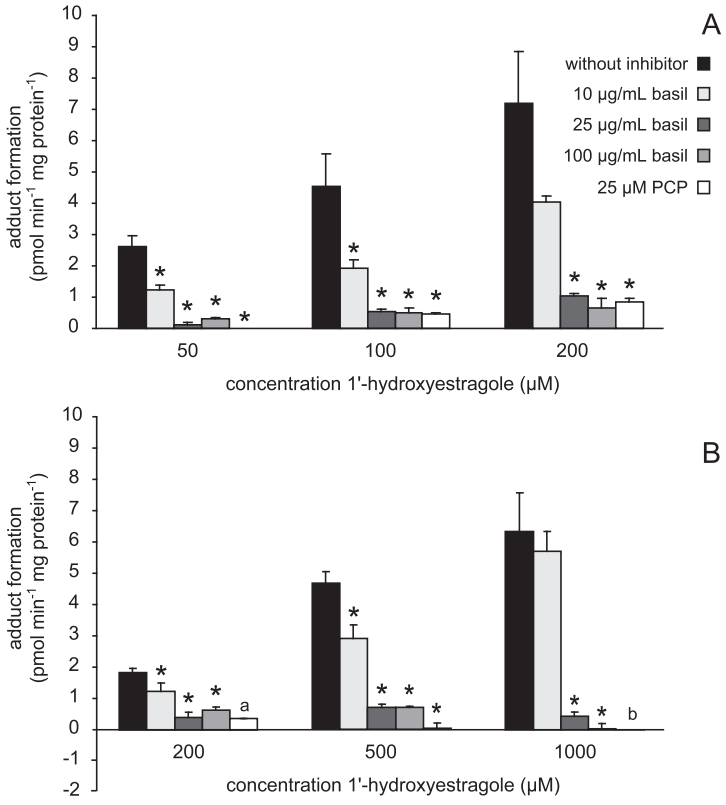


Figure 6.2. LC-ESI-MS/MS analysis of an incubation with rat liver S9 homogenate, 200 μM 1′-hydroxyestragole, 1 mM PAPS, and 2 mM 2′-deoxyguanosine. The chromatogram corresponds to the recorded transition from *m/z* 414 → *m/z* 298, and the proposed structure of the fragment ion with *m/z* 298 (*16*) is shown. The peak at approximately 24 min corresponds with E-3′-N²-dGuo.

Figure 6.3. E-3′-N^2-dGuo formation in A) rat liver S9 homogenates and B) human liver S9 homogenates incubated with different concentrations of 1′-hydroxyestragole in the absence or presence of increasing concentrations of basil extract or 25 µM of the sulfotransferase inhibitor pentachlorophenol (PCP). Data points represent mean (+ SEM) of triplicate measurements (for the rat liver S9 incubations without inhibitor n=5, for the human liver S9 incubations without inhibitor n=4).
An asterisk (*) indicates a significant inhibition compared to the incubation without inhibitor ($P < 0.05$). [a] Data point represents a single measurement, [b] Data point below quantification limit.

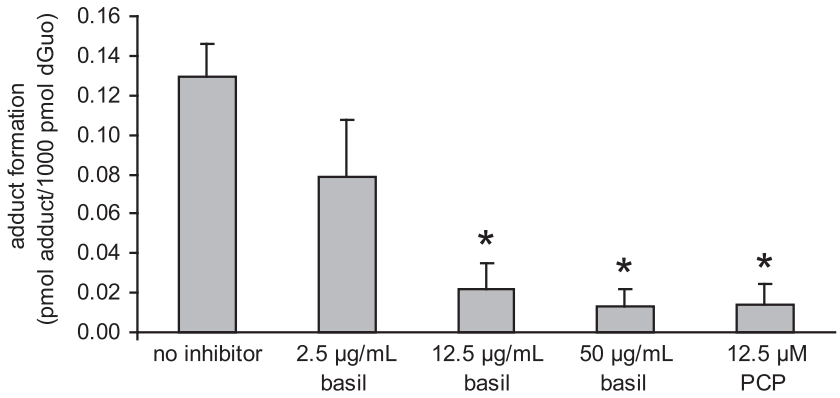


incubations with the direct electrophile 1′-acetoxyestragole (instead of 1′-hydroxyestragole) that generates E-3′-N^2-dGuo without the need for sulfotransferase activity (data not shown). Together our data indicate that the observed inhibition of E-3′-N^2-dGuo formation by S9 homogenates by the basil extract can be ascribed to modulation of SULT activity.

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

Fig. 6.4 presents the E-3′-N^2-dGuo formation detected in HepG2 cells exposed for 4 h to 50 µM 1′-hydroxyestragole in the absence and presence of increasing concentrations of

Figure 6.4. E-3′-N^2-dGuo formation in HepG2 cells exposed for 4 h to 50 µM 1′-hydroxyestragole in the absence or presence of increasing concentrations of basil extract or 12.5 µM of the sulfotransferase inhibitor pentachlorophenol (PCP). Data points represent mean (+ SEM) of data points obtained during three independent experiments (controls n=8, 2.5 µg/mL basil n=6, 12.5 µg/mL basil n=9, 50 µg/mL basil n=9, 12.5 µM PCP n=8). An asterix (*) indicates a significant inhibition compared to the incubation without inhibitor ($P < 0.05$).



basil extract or 12.5 µM pentachlorophenol. The results presented reveal that E-3′-N^2-dGuo formation in HepG2 cells exposed to 1′-hydroxyestragole is almost completely blocked by pentachlorophenol. For basil, a dose dependent inhibition is observed with 12.5 and 50 µg/mL extract almost completely inhibiting E-3′-N^2-dGuo formation in 1′-hydroxyestragole exposed HepG2 cells.

Discussion

The objective of the present study was to investigate whether a basil extract inhibits the SULT catalyzed second step of estragole bioactivation and the subsequent formation of DNA adducts with 2′-deoxyguanosine. Basil contains significant amounts of estragole (*11*) and we showed previously that basil extract inhibits P450 1A2 (*9*), one of the major P450 enzymes involved in the first step of the bioactivation of estragole (*7*). The effect of a basil extract on the SULT-mediated DNA adduct formation (estimated on the basis of E-3′-N^2-dGuo measurement) by 1′-hydroxyestragole was investigated with human and rat liver S9 homogenates and in the human hepatoma cell line HepG2. The quantification of E-3′-N^2-dGuo was adapted from a recently developed method encompassing the LC-ESI-MS/MS technique (*16*). In the current work, the mass spectrometer was of an ion trap type while a triple quadrupole was used in the method reported by Punt *et al.* (*16*), and our quantification was based on external calibration. Our results were comparable with the data provided by Punt *et al.* (*16*).

Incubations with both rat and human liver S9 homogenates (Fig. 6.3) revealed a dose

dependent inhibition of SULT-catalyzed E-3′-N^2-dGuo formation by basil extracts at concentrations ranging from 10 to 100 µg extract/mL. At 100 µg basil extract /mL the E-3′-N^2-dGuo formation was almost completely inhibited. The inhibited E-3′-N^2-dGuo formation studied was completely dependent on the presence of PAPS and SULT containing S9 homogenates, since a correction was made for E-3′-N^2-dGuo that was formed from 1′-hydroxyestragole without prior sulfation. Furthermore, since E-3′-N^2-dGuo formation was significantly inhibited by the SULT inhibitor pentachlorophenol it is concluded that the inhibition of E-3′-N^2-dGuo formation by basil extract occurs at the level of the sulfotransferase mediated bioactivation step. This conclusion is supported by the fact that the formation of E-3′-N^2-dGuo was not inhibited with a basil extract at 100 µg /mL when incubations were performed with the direct electrophile 1′-acetoxyestragole (instead of 1′-hydroxyestragole) which generates E-3′-N^2-dGuo without the need for sulfation. This result indicates that the basil extract-induced inhibition is not caused by either direct scavenging of the reactive carbocation or its precursor (Fig. 6.1), 2′-deoxyguanosine trapping by basil extract constituents, or inhibition of the chemical reaction underlying the E-3′-N^2-dGuo formation.

To investigate whether the inhibitors of the E-3′-N^2-dGuo formation present in basil extract were also able to exert a protective effect in intact cell systems, the adduct formation was investigated in HepG2 cells exposed to 1′-hydroxyestragole in the absence and presence of basil extract or PCP. HepG2 cells contain different enzymes involved in the phase II metabolism of 1′-hydroxyestragole such as SULT and UDP-glucuronosyltransferases (*17-19*). The level of formation of the DNA adduct E-3′-N^2-dGuo in these cells is the result of the simultaneous contribution of various processes such as the effects of sulfation, potential other biotransformation (detoxification) routes, and DNA repair mechanisms. Especially glucuronidation provides a detoxification route for 1′-hydroxyestragole (*3, 24-26*). The results of the present study revealed that E-3′-N^2-dGuo is formed in HepG2 cells after exposure to 1′-hydroxyestragole and our data further suggest that its formation is sulfotransferase dependent. Indeed, the formation of E-3′-N^2-dGuo was almost completely inhibited when the incubations were performed in the presence of PCP at 12.5 µM as SULT inhibitor. Basil extract was able to inhibit E-3′-N^2-dGuo formation (Fig. 6.4) in HepG2 cells exposed to 50 µM 1′-hydroxyestragole in a dose dependent manner with 12.5 and 50 µg/mL basil extract almost completely inhibiting adduct formation, demonstrating that the SULT-inhibiting compounds present in the basil extract are able to pass the cell membrane and exert effects in the cytosol.

The inhibition of P450 1A2 and SULT mediated bioactivation of estragole and 1′-hydroxyestragole by basil ingredients suggests that the chances on bioactivation and subsequent adverse effects of both estragole and 1′-hydroxyestragole are decreased in a matrix of other basil ingredients than what would be expected on the basis of experiments using estragole and 1′-hydroxyestragole as single compounds. Of course, this may also depend on the alternative metabolic routes of estragole and 1′-hydroxyestragole that will be increased when their 1′-hydroxylation and/or sulfation is inhibited by other herbal ingredients,

and the effects of herbal ingredients on these alternative routes. For 1′-hydroxyestragole, alternative biotransformation routes include glucuronidation, epoxidation, and isomerization to 3′-hydroxyestragole. Glucuronidation of 1′-hydroxyestragole yields a metabolite that can be excreted in urine (*25,26*). P450 catalyzed epoxidation of 1′-hydroxyestragole leads to a metabolite that is genotoxic *in vitro* (*3-5*), but *in vivo* this epoxide has been demonstrated to be efficiently detoxified by epoxide hydrolases and glutathione S-transferases and is therefore unlikely to contribute to the genotoxic effects of estragole (*14,27,28*). Isomerization of 1′-hydroxyestragole leads to the formation of the more stable 3′-hydroxyestragole, of which the hydroxyl group serves as a primary alcohol and can be oxidized to yield a cinnamic acid derivative (*25,26*). This cinnamic acid derivative may undergo β-oxidation and cleavage to yield a benzoic acid derivative which may be excreted as the glycine conjugate (*25,26*). All three alternative biotransformation pathways thus lead to a reduction in the toxicity of 1′-hydroxyestragole as compared to the SULT mediated bioactivation. For estragole, the two main alternative routes are *O*-demethylation and epoxidation, which both also reduce the toxicity of estragole as compared to the 1′-hydroxylation and SULT mediated pathway that leads to DNA adduct formation (*26,29*).

In the present study, basil extract employed at 50 µg/mL almost completely inhibited the DNA adduct formation with 50 µM (= 8.2 µg/mL) 1′-hydroxyestragole in HepG2 cells. The amount of essential oil in *Ocimum basilicum* corresponds to ~ 0.5% (*12,29*) and the percentage of estragole in the essential oil is described to range from 20 up to 88% (*1,11*). Assuming that this is on a w/w basis, and using the worst case scenario that the essential oil of basil contains 88% of estragole, this would mean that 5 g of basil contains 22 mg estragole (= 5 g × 0.5% × 88%). The basil extract used in the present study was obtained with a yield of approximately 10%, so ± 500 mg of extract was obtained from 5 g of basil. The ratio between basil extract and 1′-hydroxyestragole which leads to an almost complete inhibition of DNA adduct formation in the present experiments in HepG2 cells (50 µg/mL inhibitor versus 8.2 µg/mL substrate) is 6.1 (w/w). The ratio that is roughly to be expected between 'basil extract' and estragole in basil (500 mg inhibitor versus 22 mg substrate) is 22.7 (w/w/). When comparing these ratios, it is obvious that the ratio inhibitor/substrate is higher in basil than in our present experiments. The actual *in vivo* ratio between the concentrations of the SULT inhibitors and 1′-hydroxyestragole upon consumption of basil is difficult to predict, since it depends on the absorption, metabolism and distribution of estragole and the SULT inhibitors in the body. Therefore, the present results should be confirmed in an *in vivo* animal study before accurate extrapolation to the human situation could be done. However, since only a small amount of estragole will be bioactivated into 1′-hydroxyestragole, the *in vivo* ratio between potential SULT inhibitors and 1′-hydroxyestragole is probably even more favorable, and our results suggest that the SULT-mediated bioactivation step will be reduced when estragole consumption occurs via basil consumption.

In basil oil, Müller *et al.* showed that the genotoxic potential of estragole is not masked by other ingredients of the oil (*30*). The genotoxic potentials of basil oil and estragole were compared in the unscheduled DNA synthesis (UDS) test, using basil oil with an estragole

content of 88%, and it was concluded that basil oil induced UDS in the same concentration range as estragole (*30*). This lack of protective effect in basil oil can be explained by the high ratio between estragole (88%) and other herbal oil ingredients (12%) in the essential oil fraction of basil compared to the ratio between estragole and other herbal ingredient in the total herb.

In conclusion, we have shown in the present study that a basil extract is able to efficiently inhibit the SULT-mediated DNA adduct formation of 1′-hydroxyestragole with rat and human liver S9 homogenates. Furthermore, our data show that the compounds in basil that are responsible for the inhibitory effect in this subcellular model system are able to enter cells and exert the same protective effect in the human hepatoma cell line HepG2. In basil, the ratio between compounds with SULT inhibiting effects and estragole is more favorable than the ratios that lead to almost full inhibition of DNA adduct formation in our present study, and only a small part of estragole will be bioactivated into 1′-hydroxyestragole upon consumption of basil. Therefore, our results suggest that the bioactivation and subsequent adverse effects of estragole and 1′-hydroxyestragole are probably lower when consumed in a matrix of other basil ingredients than what would be expected on the basis of experiments using estragole and 1′-hydroxyestragole as single compounds. In future experiments this conclusion should be confirmed with an *in vivo* animal experiment in which formation of E-3′-N^2-dGuo in the liver could be quantified.

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7

Summary and general discussion

Summary of the results

The alkenylbenzenes safrole, estragole, and methyleugenol are genotoxic carcinogens and combinations of these compounds are present in among others basil, cinnamon, fennel, mace, nutmeg, star anise, and tarragon. *In vitro* and *in vivo* studies have unraveled that the genotoxicity of these alkenylbenzenes proceeds via electrophilic metabolites generated by cytochrome P450 enzymes (P450) and sulfotransferases (SULT). Their carcinogenicity has been demonstrated in animal experiments in which rats and mice were exposed to high doses of single compounds. Human intake of these alkenylbenzenes is low in comparison to the exposure in animal experiments and occurs via consumption of herbs, but also via use of essential oils from herbs and via food products to which estragole and methyleugenol have been added. The Scientific Committee on Food of the European Union (EU-SCF) concluded that estragole and methyleugenol are carcinogenic and genotoxic and that reductions in exposure and restrictions in use are indicated (*1,2*). As a result the use of the pure compounds will be restricted within the EU and limitations to the maximum amounts of estragole and methyleugenol in certain food products may be set (*3*). For safrole, such measures have already been implemented in the legislation in Europe (since 1988) and in the United States (since 1960) [Federal Register of December 3, 1960, 25 FR 12412, (*4*)]. Contrary to the EU-SCF, the Food and Extract Manufacturers Associations (FEMA) of the United States concluded that present exposure to methyleugenol and estragole resulting from food, mainly spices and added as such, does not pose a significant cancer risk. The Joint WHO/FAO Expert Committee on Food Additives (JECFA) has scheduled estragole for a re-evaluation in June 2007.

The differences in expert opinions on the safety of these compounds result in part from the absence of adequate data to enable unequivocal extrapolation from animal experimental data to the human situation. Additional knowledge of interspecies and interindividual differences in bioactivation and genotoxicity of the alkenylbenzenes, dose-responses at low doses, human exposure to these alkenylbenzenes, and interactions of alkenylbenzenes with other (herbal) food components is necessary to facilitate the risk assessment on these compounds. In this thesis, several studies were undertaken to provide additional insight in the human bioactivation and genotoxicity of these three alkenylbenzenes and in the interactions of these compounds with other herbal ingredients.

The first aim of this thesis was to identify the human P450 enzymes that bioactivate safrole, methyleugenol, and estragole to their proximate carcinogenic 1′-hydroxymetabolites. This knowledge enables prediction of interindividual differences in bioactivation and subsequent adverse effects. For identifying the enzymes responsible for a certain metabolic step, it is recommended to use an approach in which several *in vitro* assays with recombinant P450 enzymes and human liver microsomes are used (*5-7*). In Chapters 2, 3, and 4, the human P450 enzymes responsible for the 1′-hydroxylation of, respectively, safrole, methyleugenol, and estragole were elucidated. The contributions of P450 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, which are the enzymes that are most abundant in the human liver and

most often involved in drug and xenobiotic metabolism, were investigated using the above mentioned approach of various *in vitro* methods. Incubations with Supersomes, prepared from baculovirus-infected insect cells that express individual human P450 enzymes at high levels, were performed to identify which enzymes are intrinsically able to 1′-hydroxylate the three alkenylbenzenes. Also, incubations with Gentest microsomes, prepared from lymphoblastoid cells that express individual human P450 enzymes to roughly average liver levels, were performed. These incubations indicate which enzymes might be involved at enzyme concentrations present in the human liver. In addition, two approaches using human liver microsomes were used in this thesis. Correlations between the 1′-hydroxylation of the alkenylbenzenes and individual P450 activities in a batch of fifteen human liver microsomal samples were determined. Furthermore, also inhibition of the 1′-hydroxylation of the alkenylbenzenes by enzyme specific chemical inhibitors and antibodies was measured in pooled human liver microsomes.

In Chapter 2, an important role in safrole 1′-hydroxylation was ascribed to P450 2A6, 2C9, 2D6, and 2E1 based on results obtained with recombinant enzymes, in a correlation study, and in an inhibition experiment with P450 2A6. Furthermore, according to the results obtained in studies using recombinant enzymes, also P450 2C19 may play a role in safrole 1′-hydroxylation. In Chapter 4, additional kinetic experiments were performed to determine the k_{cat}, K_m, and enzyme efficiency (k_{cat}/K_m) for safrole 1′-hydroxylation in Gentest microsomes containing these P450 enzymes. These additional studies demonstrated that at low substrate concentrations, P450 2A6 is the main enzyme for safrole 1′-hydroxylation and that at higher concentrations, P450 2C19, 2D6, and 2E1 might contribute to some extent. Although a significant correlation between P450 2C9 activity and safrole 1′-hydroxylation was found in Chapter 2, kinetic analysis revealed that at lower substrate concentrations than the 500 μM used in the correlation study and used in incubations with recombinant enzymes in Chapter 2, this enzyme is not important in safrole 1′-hydroxylation. This demonstrates the importance of the determination of kinetic parameters when identifying enzymes involved in a certain metabolic step.

A comparable study by Ueng *et al.* (*8*) showed that all bacterial expressed human P450 enzymes tested (1A1, 1A2, 1B1, 2A6, 2C9, 2D6, 2E1, and 3A4) were capable of catalyzing safrole 1′-hydroxylation to some extent. Based on results from a correlation study (using 2 mM safrole) and an inhibition study (using 200 μM safrole; no P450 2A6 inhibitor included), P450 2C9 and 2E1 were selected for kinetic analyses and it was concluded that those two enzymes are the main P450 enzymes involved in human hepatic safrole 1′-hydroxylation. However, the role of P450 2C19 was not studied in these experiments, and P450 2A6 was not included in the inhibition study and in the kinetic analysis. Therefore, the results of this study can not be fully compared to the results obtained in this thesis, and do not exclude P450 2A6 from being involved at low substrate concentrations of safrole.

For methyleugenol (Chapter 3) and estragole (Chapter 4), kinetic analyses using Gentest microsomes and a full inhibitor study for all P450 enzymes were also included in the P450 characterization. For methyleugenol, all studies pointed towards P450 1A2 and 2C9 as the

two important enzymes in the bioactivation of methyleugenol, except that the K_m of P450 1A2 was 100-fold lower than the K_m of P450 2C9, and the resulting enzyme efficiency (k_{cat}/K_m) was much higher for P450 1A2 than for P450 2C9 (167 min^{-1} mM^{-1} versus 5 min^{-1} mM^{-1}). This indicates that P450 1A2 is the main enzyme responsible for methyleugenol 1′-hydroxylation at low, physiologically relevant, substrate concentrations. At higher substrate concentrations, P450 2C9 but also P450 2C19 might be involved to some extent. For estragole, the importance of P450 1A2 was demonstrated in all assays. Kinetic studies revealed that, at low and physiologically probably more relevant concentrations of estragole, besides P450 1A2 (k_{cat}/K_m 59 min^{-1} mM^{-1}) also P450 2A6 (k_{cat}/K_m 341 min^{-1} mM^{-1}) is involved in the bioactivation of estragole in the human liver. The difference in enzyme efficiencies is mainly caused by the higher k_{cat} value of P450 2A6 compared to P450 1A2. At relatively high estragole concentrations, P450 2C19, 2D6, and 2E1 might contribute to some extent.

Altogether it can be concluded that the main players in the 1′-hydroxylation of the alkenylbenzenes are P450 1A2 for methyleugenol and estragole, and P450 2A6 for safrole and estragole (for an overview of the catalytic efficiencies of all P450 enzymes involved see Table 4.4 in Chapter 4). Although the three alkenylbenzenes are structurally very similar, the P450 enzyme specificities for the three compounds are different. A possible explanation for the differences in the involvement of P450 1A2 could be that safrole acts as an inhibitor of P450 1A2 rather than as a substrate for 1′-hydroxylation. Inhibition of microsomal activities by safrole, but also methyleugenol and estragole, has been reported before (*9,10*) and recently it has been published that safrole is a potent inhibitor of P450 1A2 (*11*). The authors indicated that inhibition of P450 1A2 catalyzed 7-ethoxyresorufin *O*-deethylation by safrole (K_i = 1.5 μM) was competitive, which indicates that safrole in able to bind to the active site of P450 1A2 and could therefore, in theory, also act as a substrate. Safrole was also reported to inhibit P450 2A6 (K_i = 31 μM) and P450 2E1 (K_i = 2.8 μM) noncompetitively (*11*). Characterizing safrole as a noncompetitive inhibitor of P450 would imply that it binds to a site distinct from the active site as well as to the active site and this could still be in line with our results that show that especially P450 2A6 has a low K_m value (12 μM) and a high enzyme efficiency (160 min^{-1} mM^{-1}) for safrole with regard to its 1′-hydroxylation.

The activities of the two main enzymes involved in the bioactivation of the alkenylbenzenes, P450 1A2 and 2A6, vary in the human population due to genotype- and lifestyle-based influences. In the human liver samples used for the correlation studies in Chapters 2-4, interindividual differences in 1′-hydroxylation of alkenylbenzenes are not that large and range from 2.5-fold (safrole) to 5-fold (methyleugenol). At lower concentrations than the 200 μM (for methyleugenol) and 500 μM (for safrole and estragole) used in the correlation study, larger differences may occur, since at low concentrations, 1′-hydroxylation will be highly dependent on the activity of only one or two enzymes that have the highest affinity for these alkenylbenzenes.

Differences in P450 1A2 activities in the human population result mainly from lifestyle factors (*12*) such as cigarette smoking and the consumption of charbroiled food and cruciferous vegetables that increase the activity of P450 1A2 (*13,14*). In the human

liver samples used in the correlation study, a 15-fold difference in P450 1A2 activity and an about 1000-fold difference in P450 2A6 activity was observed (Chapters 2-4). For P450 2A6, genetic polymorphisms are the main cause of the large interindividual variation in the human population (*15,16*). Many mutant alleles are described, and gene duplications, gene deletions, and decreased enzyme activities are reported. Also one mutation possibly associated with increased activity is reported (http://www.cypalleles.ki.se). These lifestyle factors and genetic polymorphisms that influence the activities of P450 2A6 and P450 1A2 may lead to interindividual differences in the chances on 1′-hydroxylation and subsequent adverse effects of the alkenylbenzenes.

The second aim of this thesis was to study the influence of other herbal ingredients on the bioactivation and the genotoxicity of herb-based alkenylbenzenes. Compounds in herbs that inhibit the P450 or SULT catalyzed bioactivation of alkenylbenzenes might decrease or even eliminate the possible adverse effects of alkenylbenzenes present in the same herbs. Since combinations of safrole, estragole, and methyleugenol are present in various herbs, competitive interactions between methyleugenol and estragole for the active site of P450 1A2 and between safrole and estragole for the active site of P450 2A6 were investigated in Chapter 4. Co-incubations of combinations of alkenylbenzenes showed that such competitive interactions indeed occur. Modeling revealed that total 1′-hydroxylation at equimolar concentrations of estragole and methyleugenol by P450 1A2 and of estragole and safrole by P450 2A6 amounted to only 45% and 52%, respectively, of the total 1′-hydroxylation calculated without taking the competitive interaction of the two substrates at the active site of P450 into account.

Besides the competitive interactions between alkenylbenzenes, the presence of inhibitors of the P450 enzymes involved in the 1′-hydroxylation of the alkenylbenzenes in herbs was studied. The search for bioactive compounds in natural products is shifting nowadays from bioassay guided fractionation towards screening using hyphenated systems. In such systems, a separation technique is directly coupled to a biochemical detection method. In addition, diode array detection, evaporative light scattering detection, and/or mass spectrometry are often included in these systems as well. This enables simultaneous detection and (to some extent) identification and quantification of bioactive compounds (also called high resolution screening). In Chapter 5, an on-line system was developed for the detection of P450 1A2 inhibitors in natural extracts. This system was based on HPLC analysis coupled to diode array detection and a continuous methoxyresorufin *O*-demethylation (MROD) assay in which recombinant human P450 1A2 converts methoxyresorufin to its fluorescent metabolite resorufin. P450 1A2 inhibitors will inhibit the MROD activity, leading to less resorufin production and a concomitant decrease in fluorescence signal.

First, the performance of this on-line system was proven with typical P450 1A2 substrates/inhibitors for which minimum detectable amounts (MDA) ranged from 0.7 to 9.5 ng. Thereafter, a kava kava extract (known to contain P450 1A2 inhibitors) was analyzed for the presence of P450 1A2 inhibitors using the newly developed on-line HPLC MROD system (Chapter 5, Fig. 5.5). The results obtained indicated that the system is capable of detecting

inhibitors in natural products extracts, and that the system can be used to gain information on the presence of P450 1A2 inhibitors in food matrixes. Before high resolution screening of herbal or other food matrixes becomes feasible, the sensitivity of the present system should be further improved, for example by using a laser induced fluorescence detector. A reason for the limited sensitivity of the assay is the incompatibility of P450 enzymes with organic solvents that are needed for HPLC analysis. Consequently, dilution of the HPLC eluate is necessary to keep the organic solvent concentration compatible with the enzyme assay (< 7% methanol in the present system).

Besides the system described in this thesis, a comparable system for P450 1A inhibitors was developed by Kool *et al.* (*17*), and recently a new on-line system has been published in which microsomes from β-naphthoflavone (P450 1A), phenobarbital (P450 2B), and dexamethansone (P450 3A) induced rats were used to determine inhibition of three P450 classes simultaneously (*18*). Especially when recombinant P450 enzymes would be used to improve the specificity of the enzyme assays, the latter system can be valuable in giving insight in the presence of P450 inhibitors in food matrixes. However, both systems have not yet been tested with natural product extracts, so the applicability of these on-line systems for such purposes is not yet clear.

In Chapter 5, the on-line HPLC system was used to gain insight in the presence of P450 1A2 inhibitors in a basil extract. Initially, the activity of the extract was measured in an off-line well-plate assay and an IC_{50}-value for P450 1A2 inhibition of 11 µg extract/mL was determined. Analysis in the on-line system revealed that this inhibition was caused by several constituents of the extract. Structural elucidation of these inhibitors was not undertaken in the present study. Nevertheless these data indicate that the chances on bioactivation of methyleugenol and estragole by P450 1A2 might be less in a matrix of other basil ingredients than expected on the basis of experiments using methyleugenol and/or estragole as single compounds, because of the presence of P450 1A2 inhibitors in basil.

Also the possible inhibitory effects of basil extract on the sulfotransferase catalyzed second step in the bioactivation and the subsequent DNA adduct formation of alkenylbenzenes were investigated. The sulfate metabolites of the alkenylbenzenes are unstable in an aqueous environment and therefore it is not possible to quantify these metabolites directly. Recently, an indirect method to measure sulfation of 1′-hydroxyestragole was developed based on trapping of the reactive carbocation derived from 1′-sulfooxyestragole with 2′-deoxyguanosine, followed by quantification of the major adduct, N^2-(*trans*-isoestragole-3′-yl)-2′-deoxyguanosine (E-3′-N2-dGuo), using an LC-ESI-MS/MS technique (*19*). In Chapter 6, the influence of a basil extract on the SULT mediated DNA adduct formation of 1′-hydroxyestragole was investigated. Basil extract was able to efficiently inhibit the SULT-mediated DNA adduct formation of 1′-hydroxyestragole by rat and human liver S9 homogenates. Furthermore, the compounds in basil that are responsible for the inhibition of SULT catalyzed DNA adduct formation by 1′-hydroxyestragole in S9 incubations were able to enter cells and exert the same protective effect in the human hepatoma cell line HepG2 at levels that did not cause cytotoxicity.

Basil extract employed at 50 µg/mL almost completely inhibited the DNA adduct formation with 50 µM (= 8.2 µg/mL) 1′-hydroxyestragole in HepG2 cells. The amount of essential oil in *Ocimum basilicum* corresponds to ~ 0.5% (*20,21*) and the percentage of estragole in the essential oil is described to range from 20 up to 88% (*2,22*). In the worst case scenario, this would mean that 5 g of basil contains 22 mg estragole. The basil extract used in Chapter 6 was obtained in a yield of approximately 10%, so ± 500 mg of extract was obtained from 5 g of basil. When comparing the ratio between basil extract and 1′-hydroxyestragole which leads to an almost complete inhibition of DNA adduct formation in the experiments in HepG2 cells (50 µg/mL inhibitor versus 8.2 µg/mL substrate, the ratio (w/w) being 6.1) to the ratio that is roughly to be expected between 'basil extract' and estragole in basil (500 mg inhibitor versus 22 mg substrate, leading to a ratio (w/w) of 22.7), it is obvious that the ratio inhibitor/substrate is higher in basil than in the experiments described in Chapter 6.

The actual *in vivo* ratio between the concentrations of the SULT inhibitors and 1′-hydroxyestragole resulting from consumption of basil is difficult to predict and depends on absorption, metabolism, and distribution of estragole and the SULT inhibitors in the body. However, since only a small amount of the estragole present in basil will be bioactivated into 1′-hydroxyestragole upon basil consumption, the *in vivo* ratio between potential SULT inhibitors and 1′-hydroxyestragole is likely to be even higher and thus more favorable than the ratio used in the experiments with HepG2 cells. These results suggest that the SULT-mediated bioactivation step will be reduced when estragole exposure occurs via basil consumption. For unequivocal extrapolation, this hypothesis should be confirmed *in vivo* by for example measuring DNA adduct formation and/or cancer incidence in livers of rats or mice exposed to estragole with and without basil extract.

Implications for risk assessment and future perspectives

The new results presented in this thesis on the bioactivation and genotoxicity of the alkenylbenzenes safrole, estragole, and methyleugenol and the influence of other herbal ingredients on these processes may contribute to a more refined extrapolation of animal carcinogenicity data on the alkenylbenzenes to the human situation. In this thesis, the P450 enzymes responsible for the 1′-hydroxylation of safrole, estragole, and methyleugenol are defined. Interindividual differences in formation of the proximate carcinogenic 1′-hydroxymetabolites may occur due to genotype- or lifestyle-based differences in P450 1A2 and 2A6 activities. The interindividual differences also depend on the activities of the enzymes involved in the two other main metabolic routes, *O*-demethylation and epoxidation. These enzymes have not yet been identified, but for estragole, these studies are underway in our laboratory. Eventually, the kinetic data on estragole 1′-hydroxylation will be implemented, together with kinetic data for *O*-demethylation and epoxidation, in a pharmacologically based pharmacokinetic (PBPK) model. Such a model enables estimation of liver concentrations of the proximate carcinogen 1′-hydroxyestragole resulting from exposure to estragole and gives insight in the effects of genetic polymorphisms and lifestyle factors on the toxicokinetics of the alkenylbenzenes.

Kinetic data on all three main phase I routes will give insight in the existence of the 'metabolic shift' in phase I metabolism that could explain the disproportional high increase in excretion of the glucuronidated metabolite of 1′-hydroxyestragole in the urine of rats and mice with increasing dose of estragole (Table 1.3, Chapter 1) (*27*). The disproportional increase was explained by a fall in *O*-demethylation with increasing dose of estragole and a concomitant increase in estragole 1′-hydroxylation (*21,27*). This was one of the important arguments for the FEMA to conclude that exposure to estragole and methyleugenol at the low levels does not pose a significant cancer risk for humans (*21*). In addition, also phase II metabolism of the 1′-hydroxymetabolites should be included in an evaluation of dose-dependency of the bioactivation of the alkenylbenzenes. In general, sulfation is regarded as a high affinity, low capacity conversion, whereas glucuronidation is regarded as a low affinity, high capacity reaction (*28*). Therefore, it might be possible that sulfation of the 1′-hydroxymetabolites takes place (and might even be saturated) before glucuronidated metabolites can be detected in urine. In such a situation the level of glucuronidated 1′-hydroxestragole detected in urine may not reflect the actual levels of 1′-hydroxyestragole and 1′-sulfooxyestragole formed in the liver. Therefore PBPK modeling may prove a better method to obtain insight in the actual formation of 1′-hydroxyestragole and 1′-sulfooxyestragole in liver tissue and the shape of the dose-response curve for bioactivation at low concentrations.

When extrapolating animal experimental data to the human situation, interspecies differences in bioactivation have to be taken into account. Gardner *et al.* determined methyleugenol 1′-hydroxylation in human liver microsomal samples and in rat liver microsomes, and found that the most active human sample out of 13 samples tested had a comparable activity to rat liver microsomes (*29*). Punt *et al.* recently estimated that the catalytic efficiency of human liver S9 homogenates for sulfation of 1′-hydroxyestragole is approximately 12 times lower than of rat S9 homogenate and 6 times higher than of mice samples (*19*). In line with this observation, Chapter 6 of the present thesis revealed that DNA adduct formation as a consequence of the sulfation of 1′-hydroxyestragole (at 200 μM) is approximately 4-fold higher using rat compared to human S9 homogenates. This indicates that when extrapolating data from rat studies with estragole, the risks for humans may be overestimated, whereas when using data from mice studies these risks may be underestimated, as far as sulfation is concerned (*19*).

Another important factor for extrapolation of rodent carcinogenicity data obtained in animal experiments using high concentrations of isolated alkenylbenzenes to the human situation with exposure to alkenylbenzenes in a complex food matrix is the interaction of alkenylbenzenes with other (herbal) components. From the *in vitro* experiments undertaken in this thesis, several indications for reduced risks posed by alkenylbenzenes in a matrix of other herbal ingredients as compared to risks posed by the individual compounds can be derived. First, basil contains inhibitors of P450 1A2, involved in bioactivation of methyleugenol and estragole. In addition to these P450 1A2 inhibitors, also the alkenylbenzenes themselves may act as inhibitors competing for the active site of P450 1A2 (estragole and methyleugenol) or P450 2A6 (estragole and safrole). Furthermore it was demonstrated that basil extract is able

to strongly inhibit sulfation and subsequent DNA adduct formation of 1′-hydroxyestragole in incubations with rat and human S9 homogenate and in the human hepatoma HepG2 cell line.

The *in vitro* findings on the protective effects of basil extract on both the P450 and the SULT catalyzed bioactivation of methyleugenol and/or estragole and subsequent DNA adduct formation have to be confirmed *in vivo*. This could be done by performing a carcinogenicity study in which rats or mice are exposed to estragole or methyleugenol in the absence or presence of basil extract. Comparison of the tumor incidences caused by the different treatments will reveal whether the carcinogenic effects of estragole and/or methyleugenol can be reduced (or even eliminated) by other ingredients from basil, and whether the results of the *in vitro* experiments can be extrapolated to the *in vivo* situation.

Risk assessment for natural compounds that are genotoxic carcinogens but are part of the normal diet, such as the alkenylbenzenes, is a difficult issue. For genotoxic compounds that can directly or via their metabolites react with DNA, such as the herbal alkenylbenzenes, a no threshold situation is assumed in which there is no dose level without a potential effect (*23*). Consequently, the advice of risk assessors is often to keep the levels of these compounds in food as low as reasonably achievable ('ALARA' approach). However, this approach neglects differences in potencies of genotoxic carcinogens and does not take into account the actual human exposure to the carcinogen of interest. Therefore, new approaches are being developed that can be used to set priorities in risk management of genotoxic carcinogens. Recently the Scientific Committee of the European Food Safety Authority (EFSA) recommended the use of the margin of exposure (MOE) approach (*24*). In this approach, a reference point of an animal study (for example the BMDL10, the lower confidence limit of the benchmark dose at 10% cancer incidence, or the T25, the dose corresponding to 25% incidences of tumors) is selected and compared to dietary intakes in humans (*24*). In general, using the BMDL10 or the T25 as starting point, a margin of exposure (BMDL10 or T25/dietary intake) of respectively 10000 (using BMDL10) or higher (using T25) would be of low concern from a public health point of view and might be considered as a low priority for risk management actions (*24*).

For estragole and methyleugenol, tumor incidences higher than 25% are found for example in rats and mice exposed to 75 mg methyleugenol/kg bw/day (*25*) and in mice exposed to 150-300 mg estragole/kg bw/day (*26*). Human daily intakes are estimated at 72 µg/kg bw/day (*2*) and < 10 µg/kg bw/day (*21*) for estragole and at 0.22 mg/kg bw/day (*1*) and < 10 µg/kg bw/day (*21*) for methyleugenol. These intake estimates may result in MOE values of less than 10000 indicating that more information is needed on actual human intake and on human carcinogenicity of these compounds at low doses of exposure taking into account possible matrix effects as observed in the present thesis. *In silico* models for toxicokinetics and toxicity presently developed for rat and man, incorporating, respectively, kinetics for the different phase I and phase II conversions and the rate of DNA adduct formation in hepatocytes, may provide better insight in low dose extrapolation, taking into account species differences in kinetics or even toxicodynamics of alkenylbenzenes.

Altogether, restrictions in the use of methyleugenol and estragole as pure compounds are foreseen in the European Union based on the opinions of the EU-SCF that these compounds are genotoxic and carcinogenic. Nevertheless, exposure to low doses of alkenylbenzenes will continue via consumption of herbs or via consumption of food products in which these herbs and/or their essential oils are used. Definition of the actual risk and/or a virtual safe dose for exposure to the various alkenylbenzenes awaits the detailed definition of species differences in their bioactivation and detoxification, the development of better models for low dose cancer risk extrapolation, more refined intake estimates, and also a better definition of modulation of the risks by matrix effects. The results of the present thesis reveal protective effects of constituents from basil extract on P450 and SULT catalyzed bioactivation and DNA adduct formation in *in vitro* models. Upon validation of these effects *in vivo*, it may turn out that rodent carcinogenicity data on estragole and methyleugenol considerably overestimate the risks posed when humans are exposed to these compounds via herbs.

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LIST OF ABBREVIATIONS

DAD	Diode array detection
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethylsulfoxide
EU-SCF	Scientific Committee on Food of the European Union
f.c.	final concentration
FEMA	Food and Extract Manufacturers' Assocation
GC	Gas chromatography
GC-MS	Gas chromatography - mass spectrometry
GSH	Gluthatione
HBSS	Hanks' Balanced Salt Solution
HPLC	High performance liquid chromatography
IC50	Concentration at which 50% inhibition is observed
KPi	Potassium phosphate buffer
LC-MS	Liquid chromatography mass spectrometry
LD50	Dose at which 50% lethality is observed
LOD	Limit of detection
MDA	Minimum detectable amount
MR	Methoxyresorufin
MRA	Mass rate attenuator
MROD	Methoxyresorufin-*O*-demethylation
MS	Mass spectrometry
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
P450	Cytochrome P450 enzyme
PAPS	3'-Phosphoadenosine-5'-phosphosulfate
PCP	Pentachlorophenol
PEEK	Polyether ether ketone
SD	Standard deviation
ST	Sulfotransferase
SEM	Standard error of the mean
UDP	Uridine-5'-phosphoglucuronic acid
UDS	Unscheduled DNA synthesis
UV	Ultra-violet

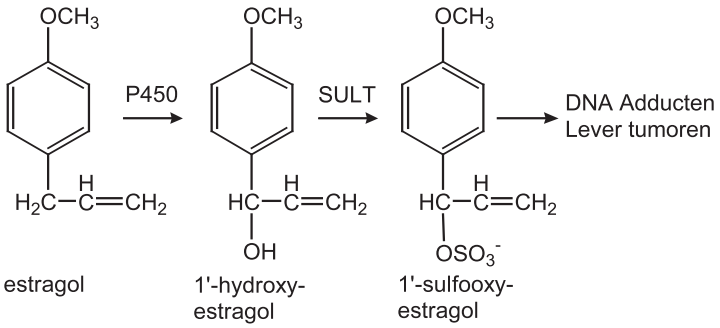
NEDERLANDSE SAMENVATTING

Inleiding

Kruiden en specerijen worden al vele duizenden jaren gebruikt voor culinaire en medische doeleinden. De markt voor kruidenproducten zoals kruidenthee, kruidenpreparaten en medicinale kruiden groeit nog steeds en componenten uit kruiden worden onder andere gebruikt in voedingssupplementen, als geur- en smaakstoffen en als bioactieve ingrediënten in zogenaamde 'functional foods'. Hoewel bij veel consumenten de gedachte leeft dat 'natuurlijk' gelijk is aan 'gezond', kunnen kruiden en kruidenextracten componenten bevatten die giftig en soms zelfs genotoxisch en kankerverwekkend zijn.

Dit proefschrift beschrijft onderzoek naar de stoffen safrol, methyleugenol en estragol die tot de groep van de alkenylbenzenen behoren. Combinaties van deze drie stoffen komen voor in verschillende kruiden zoals nootmuskaat, foelie, kaneel, dragon, basilicum, steranijs en venkel. Safrol, methyleugenol en estragol zijn genotoxische carcinogenen en kunnen dus veranderingen in het DNA veroorzaken waardoor kanker kan ontstaan. De effecten van deze stoffen treden met name op in de lever. *In vitro* studies (studies met bijvoorbeeld levercellen die buiten het lichaam gekweekt worden of met leverfracties waarin bepaalde enzymen zitten) en dierexperimenten hebben uitgewezen dat deze stoffen hun genotoxische werking uitoefenen via hun omzettingsproducten (metabolieten) die in het lichaam gevormd worden door enzymen (zie figuur 1). De carcinogeniteit van safrol, methyleugenol en estragol is aangetoond in dierstudies waarin ratten en muizen blootgesteld werden aan hoge doseringen van de individuele stoffen. De humane inname van deze stoffen is lager dan de doseringen die aan de proefdieren werden toegediend en treedt op via consumptie van kruiden die alkenylbenzenen bevatten, door gebruik van vluchtige oliën uit deze kruiden en via consumptie van levensmiddelen waaraan methyleugenol en estragol zijn toegevoegd. Het wetenschappelijke panel dat de Europese Commissie adviseert over voeding (voorheen Scientific Committee on Food, EU-SCF en tegenwoordig European Food Safety Authority, EFSA) heeft geconcludeerd dat safrol, estragol en methyleugenol carcinogeen en genotoxisch zijn en dat daarom een afname van de blootstelling aan deze stoffen en beperkingen van het gebruik van deze stoffen wenselijk zijn. Het gebruik van de pure stoffen estragol en methyleugenol zal daarom beperkt worden in de Europese Unie en er komen mogelijk maximum gehaltes voor estragol en methyleugenol in bepaalde producten. Voor safrol bestaat zulke wetgeving al in de Europese Unie en in de Verenigde Staten. In tegenstelling tot de EU-SCF heeft een expert panel van de Amerikaanse voedingsindustrie (Food and Extract Manufacturers Association, FEMA) geconcludeerd dat de huidige blootstelling aan methyleugenol en estragol via voedselinname geen significant risico met zich meebrengt. In juni 2007 zal estragol opnieuw geëvalueerd worden door een internationale commissie van deskundigen op het gebied van voedingsadditieven (Joint WHO/FAO Expert Committee on Food Additives, JECFA).

De verschillen in de opinies van deze commissies over de veiligheid van de alkenylbenzenen zijn deels te verklaren door een gebrek aan adequate gegevens die een ondubbelzinnige



Figuur 1. Bioactiveringsroute voor estragol (gelijk aan de bioactiveringsroutes voor safrol en methyleugenol). P450 = cytochroom P450 enzymen, SULT = sulfotransferases.

extrapolatie van de dierexperimentele naar de humane situatie mogelijk maken. Meer kennis over interspecies-verschillen (verschillen tussen verschillende soorten, bijvoorbeeld tussen rat en mens) en inter-individuele verschillen (verschillen binnen één soort, dus tussen verschillende individuen) in de bioactivering en de genotoxiciteit van de alkenylbenzenen is nodig om de risicoschattingen voor deze stoffen te verbeteren. Ook informatie over de relatie tussen de dosis en de toxicologische respons bij lage doseringen, een exactere bepaling van de humane blootstelling aan alkenylbenzenen en interacties van alkenylbenzenen met andere kruidenbestanddelen is wenselijk voor meer betrouwbare risicoschattingen voor safrol, estragol en methyleugenol. Dit proefschrift beschrijft verschillende studies die zijn uitgevoerd om meer inzicht te krijgen in de humane bioactivering en genotoxiciteit van de drie alkenylbenzenen en in de mogelijke effecten van andere kruidenbestanddelen op de genotoxiciteit van deze alkenylbenzenen.

Identificatie van P450 enzymen

Het eerste doel van dit proefschrift was het identificeren van de humane P450 enzymen die verantwoordelijk zijn voor de eerste stap in de bioactivering van de alkenylbenzenen: de omzetting van safrol, estragol en methyleugenol in hun respectievelijke 1′-hydroxymetabolieten. In de *hoofdstukken 2, 3* en *4* van dit proefschrift zijn verschillende *in vitro* studies beschreven waarmee de bijdrage van de enzymen P450 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 en 3A4 in de bioactivering van respectievelijk safrol, methyleugenol en estragol is onderzocht. Deze negen P450 enzymen zijn de enzymen die het meest voorkomen in de humane lever en die het vaakst betrokken zijn bij de omzetting van medicijnen en andere lichaamsvreemde stoffen. Allereerst is onderzocht welke van de negen enzymen intrinsiek in staat zijn om de betreffende 1′-hydroxymetaboliet te vormen. Hiervoor werden Supersomes gebruikt, enzympreparaten gemaakt van genetisch gemodificeerde insectencellen die de verschillende humane P450 enzymen in hoge concentraties bevatten. Uit de verschillende studies bleek dat bijna alle geteste P450 enzymen intrinsiek in staat zijn om één of meerdere alkenylbenzenen om te zetten in hun respectievelijke 1′-hydroxymetabolieten. De werkelijke bijdrage van de verschillende P450 enzymen aan de 1′-hydroxylering hangt echter niet alleen

af van de intrinsieke activiteit van een specifiek P450 enzym, maar ook van de hoeveelheid enzym die aanwezig is in de lever en van de affiniteit van het enzym voor het alkenylbenzeen. Hiertoe werden incubaties met recombinante enzymen (Gentest microsomen) uitgevoerd, werd een correlatiestudie uitgevoerd met een serie van vijftien humane levermicrosomen, en werd een remmingsstudie uitgevoerd met een pool van humane levermicrosomen en specifieke remmers voor de verschillende P450 enzymen. Voor de enzymen die op basis van de hiervoor genoemde experimenten een rol zouden kunnen spelen in de bioactivering van de alkenylbenzenen werd daarna een aantal belangrijke kinetische parameters bepaald. Dit waren k_{cat}, een maat voor de maximale omzettingssnelheid van het enzym, K_m ofwel de Michaelis-Menten constante, een maat voor de affiniteit van het enzym, en enzymefficiëntie (k_{cat}/K_m). Uit deze studies bleek dat P450 2A6 het belangrijkste enzym is in de 1′-hydroxylering van safrol bij lage-, en fysiologisch waarschijnlijk relevante, safrol concentraties en dat P450-2C19, -2D6 en -2E1 mogelijk bij kunnen dragen bij hogere substraatconcentraties. Voor methyleugenol bleek P450-1A2 de grootste bijdrage te leveren bij lage concentraties en kunnen 2C9 en 2C19 een rol spelen bij hogere substraatconcentraties. Voor estragol zijn P450-1A2 en P450-2A6 de meest belangrijke enzymen. De twee belangrijkste enzymen in de bioactivering van de alkenylbenzenen zijn dus P450-1A2 (voor methyleugenol en estragol) en P450-2A6 (voor estragol en safrol).

Op basis van deze kennis kan gekeken worden of inter-individuele verschillen in de 1′-hydroxylering van de drie alkenylbenzenen te verwachten zijn. De activiteiten van de enzymen P450 1A2 en P450 2A6 variëren in de humane populatie door genetische verschillen en door verschillen die veroorzaakt worden door leefstijlfactoren. In de vijftien humane leverpreparaten die gebruikt zijn in *hoofdstukken 2, 3* en *4* is een factor 15 verschil in P450 1A2 activiteit en ongeveer een factor 1000 verschil in P450 2A6 activiteit gemeten. Verschillen in P450 1A2 activiteit in de humane populatie worden voornamelijk veroorzaakt door leefstijlfactoren. Het roken van sigaretten, de consumptie van gebarbecued vlees en de consumptie van koolachtige groenten zoals broccoli en spruitjes kunnen het enzym P450 1A2 induceren. Voor P450 2A6 zijn genetische verschillen de voornaamste oorzaak van inter-individuele verschillen. Genetische verschillen kunnen leiden tot verminderde enzymactiviteit, een vermeerdering van het aantal P450 2A6 genen en een verwijdering van het P450 2A6 gen. Deze leefstijl factoren en genetische factoren kunnen leiden tot inter-individuele verschillen in de kansen op 1′-hydroxylering en de daarop volgende schadelijke effecten na blootstelling aan alkenylbenzenen. Dat maakt verschillende mensen verschillend gevoelig voor de genotoxische effecten van de alkenylbenzenen.

Interacties van alkenylbenzenen met andere ingrediënten uit kruiden

Het tweede doel van dit proefschrift was het bestuderen van de invloed van andere kruidenbestanddelen op de bioactivering en de genotoxiciteit van de alkenylbenzenen. Componenten uit kruiden die de P450 en de SULT gekatalyseerde bioactivering van de alkenylbenzenen remmen zouden de mogelijke schadelijke effecten van de alkenylbenzenen die aanwezig zijn in hetzelfde kruid kunnen remmen of teniet kunnen doen.

Aangezien combinaties van safrol, estragol en methyleugenol voorkomen in verschillende kruiden, zijn eerst de competitieve interacties tussen methyleugenol en estragol voor de actieve site van P450 1A2 en de competitieve interacties tussen estragol en safrol voor de actieve site van P450 2A6 onderzocht in *hoofdstuk 4*. Uit experimenten met combinaties van deze stoffen en het modelleren van de interacties tussen deze stoffen bleek dat zulke competitieve interacties inderdaad optreden. Bij gelijke concentraties van respectievelijk methyleugenol en estragol (P450 1A2) of safrol en estragol (P450 2A6) werd slechts ongeveer de helft van de totale hoeveelheid 1′-hydroxymetaboliet gevormd door deze enzymen in vergelijking met de hypothetische situatie waarin geen competitieve interacties tussen de alkenylbenzenen zouden optreden.

Naast deze competitieve interacties tussen alkenylbenzenen is de aanwezigheid bestudeerd van remmers van P450 1A2 in basilicum, een kruid dat estragol en methyleugenol bevat. Het opsporen van bioactieve stoffen (zoals P450 1A2 remmers) in natuurlijke producten gebeurt tegenwoordig steeds vaker met behulp van zogenaamde ‘on-line’ of ‘hyphenated systems’ (gekoppelde systemen) in plaats van met een combinatie van bioassays en het fractioneren van natuurlijke extracten. In zulke gekoppelde systemen wordt een scheidingstechniek direct gekoppeld aan een biochemische detectiemethode, waardoor scheiding, detectie en -tot op zekere hoogte- ook kwantificering van de actieve stoffen gelijktijdig plaats kunnen vinden. Dit wordt high-resolution screening genoemd. Soms worden ook nog extra detectoren aan het systeem gekoppeld om de structuur van de actieve stoffen (gedeeltelijk) op te kunnen helderen. In *hoofdstuk 5* is een online systeem opgezet voor de detectie van P450 1A2 remmers in natuurlijke extracten. Dit systeem is gebaseerd op de koppeling van een scheidingstechniek (high performance liquid chromatography, HPLC), een detectietechniek (diode array detection) en een enzym assay waarin recombinant P450 1A2 het substraat methoxyresorufine omzet in de fluorescerende metaboliet resorufine. P450 1A2 remmers zullen de vorming van de fluorescerende metaboliet remmen en worden zo gedetecteerd door een tijdelijke afname van het fluorescente signaal. Het systeem is uitgetest met individuele stoffen, waarvan bekend is dat ze P450 1A2 remmen, en met een kavakava-extract, een extract waarvan bekend is dat het P450 1A2 remmers bevat. Daarna is het online HPLC systeem gebruikt om de aanwezigheid van P450 1A2 remmers in basilicum, een kruid dat zowel methyleugenol als estragol bevat, te onderzoeken. Eerst werd de activiteit van een basilicumextract bepaald in een offline enzym assay en werd een IC_{50}-waarde van 11 µg extract/mL gemeten. Uit analyse in het online systeem bleek dat deze remming veroorzaakt werd door verschillende componenten uit het basilicumextract. Voor deze componenten is nog geen structuuropheldering uitgevoerd. Toch geven deze resultaten aan dat de kans op bioactivering van methyleugenol en estragol lager zou kunnen zijn in een matrix van andere basilicumbestanddelen dan wat op basis van experimenten met de pure stoffen te verwachten is, door de aanwezigheid van P450 1A2 remmers in basilicum.

In *hoofdstuk 6* is de mogelijk remmende werking onderzocht van een basilicumextract op de door sulfotransferases gekatalyseerde tweede stap in de bioactivering van de alkenylbenzenen (zie figuur 1), de omzetting van de 1′-hydroxymetabolieten in de 1′-sulfooxymetabolieten, en

de daarop volgende vorming van DNA adducten. De 1′-sulfooxymetabolieten zijn onstabiel in een waterig milieu. De sulfaatgroep kan afsplitsen waardoor een reactief carbokation ontstaat. Dit carbokation kan dan binden aan DNA en eiwitten, en is direct verantwoordelijk voor de genotoxische effecten van de alkenylbenzenen. Vanwege de instabiliteit van de 1′-sulfooxymetabolieten is het niet mogelijk deze metabolieten direct te kwantificeren. Recentelijk is een methode ontwikkeld om de sulfatering van 1′-hydroxyestragol indirect te meten door de reactieve carbokationen die ontstaan na het uiteenvallen van 1′-sulfooxyestragol te vangen met de DNA-base 2′-deoxyguanosine. Het DNA-adduct dat daarbij ontstaat [(N^2-*trans*-isoestragole-3′-yl)-2′-deoxyguanosine, E-3′-N2-dGuo] wordt gekwantificeerd door middel van LC-ESI-MS/MS. Deze methode is gebruikt in hoofdstuk 6. Basilicumextract was in staat om de SULT-afhankelijke DNA-adductvorming van 1′-hydroxyestragol door ratten- en humane lever S9-fracties te remmen. Bovendien bleken de stoffen uit het extract die verantwoordelijk zijn voor de remming van de SULT-afhankelijke DNA-adductvorming, ook te worden opgenomen in humane lever hepatoma(HepG2) cellen en in deze cellen dezelfde remmende werking te vertonen.

Hoewel moeilijk te voorspellen is wat de verhouding tussen de metaboliet-1′-hydroxyestragol en de SULT-remmers in het lichaam (*in vivo)* zal zijn na consumptie van basilicum, suggereren de resultaten van deze experimenten dat de SULT-afhankelijke bioactiveringsstap en de daarop volgende DNA-adduct vorming lager is wanneer blootstelling aan estragol plaatsvindt door basilicumconsumptie in vergelijking met blootstelling aan pure estragol. Deze hypothese moet nu vervolgens getoetst worden in de *in vivo* situatie.

Implicaties voor de risicobeoordeling van de alkenylbenzenen en toekomstperspectieven

In *hoofdstuk 7* zijn de implicaties van de nieuwe bevindingen van dit proefschrift voor de risicobeoordeling van de alkenylbenzenen beschreven en zijn enkele toekomstperspectieven voor het onderzoek aan deze stoffen uitgelicht. De resultaten van het eerste gedeelte van het proefschrift, de 1′-hydroxyleringsexperimenten, zullen in de toekomst, samen met vergelijkbare data voor de twee andere metabole routes voor de alkenylbenzenen, te weten de *O*-demethylering en epoxidering, geïmplementeerd worden in een pharmacokinetisch model (pharmacologically based pharmacokinetic model, PBBK) waarmee de leverconcentraties van de 1′-hydroxymetabolieten voorspeld kunnen worden. Met behulp van dit model kunnen de effecten van genetische polymorfismen en leefstijlfactoren op de toxicokinetiek van de alkenylbenzenen bepaald worden. Met behulp van PBBK-modellen kan ook meer inzicht verkregen worden in de vorm van de dosisresponscurve bij lage concentraties.

De resultaten beschreven in dit proefschrift geven meerdere aanwijzingen voor een gereduceerd risico op schadelijke effecten van alkenylbenzenen wanneer deze aanwezig zijn in een matrix van kruidenbestanddelen vergeleken met het risico op schadelijke effecten wanneer de alkenylbenzenen als individuele stof worden toegediend. Op de eerste plaats bevat basilicum remmers van het enzym P450- 1A2 dat betrokken is bij de bioactivering van methyleugenol en estragol. Naast deze P450 1A2 remmers in basilicum kunnen ook de alkenylbenzenen zelf als competitieve remmers optreden voor de actieve site van P450 1A2

(estragol en methyleugenol) of P450 2A6 (estragol en safrol). Verder is aangetoond dat een basilicumextract in staat is om de sulfatering en de daarop volgende DNA-adductvorming van 1′-hydroxyestragol door zowel leverfracties (S9 homogenaten) als in humane levercellen (HepG2 cellen) sterk te remmen.

Om te bepalen of de *in vitro* bevindingen geëxtrapoleerd kunnen worden naar de situatie *in vivo* is echter *in vivo* validatie nodig. Dit zou gedaan kunnen worden in een carcinogeniteitstudie waarin ratten of muizen blootgesteld worden aan estragol of methyleugenol in de aan- en afwezigheid van basilicumextract. Vergelijking van de tumorincidenties die de verschillende behandelingen veroorzaken zal uitwijzen of andere basilicumbestanddelen de carcinogene effecten van estragol en/of methyleugenol kunnen verminderen (of zelfs kunnen elimineren).

Tot slot, het gebruik van methyleugenol en estragol als pure stoffen zal beperkt worden in de Europese Unie op basis van de opinie van de EU-SCF dat deze stoffen genotoxische carcinogenen zijn. Desalniettemin zal blootstelling aan lage doses alkenylbenzenen voortduren door de consumptie van kruiden en van levensmiddelen waarin deze kruiden, en/of de vluchtige olie uit deze kruiden, gebruikt worden. Voor het bepalen van het daadwerkelijke risico van de blootstelling aan deze stoffen of de ‘virtual safe dose’ voor deze stoffen is een verdere gedetailleerde bepaling van speciesverschillen in bioactivering en detoxificatie nodig, evenals betere modellen voor het extrapoleren van kankerrisico’s naar lage doses en zouden een exactere innameschatting voor de alkenylbenzenen en een betere bepaling van matrixeffecten wenselijk zijn. De resultaten van dit proefschrift laten zien dat bestanddelen van basilicum beschermen tegen de P450- en SULT-gekatalyseerde bioactivering en de vorming van DNA-adducten in *in vitro* modellen. Validatie van deze resultaten *in vivo* zou kunnen uitwijzen dat de carcinogeniteitsgegevens voor estragol en methyleugenol, verkregen in dierexperimenten met ratten en muizen, de risico’s voor de mens die blootgesteld wordt aan deze stoffen via kruiden aanzienlijk overschatten.

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CURRICULUM VITAE

Suzanne Maria Francisca Jeurissen werd geboren op 21 mei 1980 te Vlodrop. In 1998 behaalde zij haar gymnasium diploma op het Bisschoppelijk College Schönden te Roermond. In datzelfde jaar begon ze met de studie Voeding en Gezondheid aan de Landbouwuniversiteit Wageningen (nu Wageningen Universiteit). Tijdens deze studie heeft ze een afstudeervak gedaan bij de Sectie Toxicologie van Wageningen Universiteit onder begeleiding van Laura de Haan, Dr. Ir. Jac Aarts en Prof. Dr. Ir. Ivonne Rietjens. Daarna heeft zij bij het Laboratory for Endocrinology van het Babraham Institute te Cambridge (UK) een tweede afstudeervak gedaan onder begeleiding van Dr. Jane Robinson. In januari 2003 behaalde ze haar doctoraal diploma met lof. In datzelfde jaar is ze begonnen met haar promotieonderzoek dat beschreven is in dit proefschrift. Dit promotieonderzoek is uitgevoerd bij de Sectie Toxicologie en het Laboratorium voor Organische Chemie van Wageningen Universiteit in samenwerking met TNO Kwaliteit van Leven (Zeist). Tijdens haar promotieonderzoek heeft ze tevens deelgenomen aan de Postdoctorale Opleiding Toxicologie.
Sinds 1 januari 2007 is ze werkzaam als beoordelaar humane blootstelling en toxicologie bij het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven, bij de afdeling Stoffen en Integrale Risicoschattingen (SIR).

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TRAINING AND SUPERVISION PLAN

Courses

VLAG PhD week	2003
Radiation expert 5B (Wageningen University)	2003
Organising and supervising MSc thesis projects (Wageningen University)	2004
Basic course ‘ Molecular sepctroscopy’ (ANAC)	2003
Food Toxicology (Postdoctoral Education in Toxicology, P.E.T)	2003
Ecotoxicology (P.E.T)	2003
Legal and Regulatory Toxicology (P.E.T)	2004
Immunotoxicology (P.E.T.)	2004
Scientific writing (Centa)	2004
Risk Assessment (P.E.T.)	2004
Medical and Forensic Toxicology (P.E.T.)	2005
NWO Talent Day ‘Career perspectives’	2006

Meetings

PhD study tour Laboratory for Organic Chemistry (Switzerland)	2003
PhD symposium (Netherlands Society of Toxicology)	2004
NWO meeting analytical chemistry	2004
Young Investigators Symposium (European Society of Phytochemistry, Italy)	2004
PhD symposium (Netherlands Society of Toxicology)	2005
PhD study tour Laboratory for Organic Chemistry (USA)	2005
NWO meeting analytical chemistry	2005
PhD symposium (Netherlands Society of Toxicology)	2006
Symposium European Environmental Mutagen Society (Czech-Republic)	2006

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