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# Matrix Modulation of the Bioactivation of Estragole by Constituents of Different Alkenylbenzene-containing Herbs and Spices and Physiologically Based Biokinetic Modeling of Possible *In Vivo* Effects

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

***Key Words:*** **estragole; bioactivation; sulfotransferase inhibitor; flavonoid; matrix; physiologically based biokinetic (PBBK) modeling.**

Herbs and spices have a long history of use as food, flavoring and coloring agents throughout the world (Dearlove *et al.*, 2008; Smith *et al.*, 2002). In culinary usage, herbs are most commonly seasonings derived from leaves of plants. Spices, in contrast, are obtained from seeds, berries, fruits, barks, or roots (Dearlove *et al.*, 2008). Alkenylbenzenes such as estragole (1-allyl-4-methoxybenzene), present in the volatile oils, contribute to the characteristic taste and fragrance of many herbs and spices including nutmeg, basil, anise, mace, tarragon, fennel, and pimento (Smith *et al.*, 2002). Oral intake of estragole results primarily from consumption of foods, mainly herbs and spices, and of their essential oils. Based on rodent studies, estragole was found to be genotoxic and carcinogenic (Drinkwater *et al.*, 1976; Miller, 1983). Based on these findings, the use of estragole as a pure substance in foodstuffs has been prohibited since September 2008 within the European Union (Regulation [EC] No 1334/2008 of the European Parliament and of the Council of 16 December 2008). Realistic daily intake levels of estragole have been estimated to range from 0.01 mg/kg bw/day (Smith *et al.*, 2002) to 0.07 mg/kg bw/day (SCF, 2001). Given that at present addition of estragole as a pure compound to individual food categories is no longer allowed, the value of 0.01 mg/kg bw/day resulting mainly from herbs and spices (Smith *et al.*, 2002) is taken in the present study as the value for current levels of dietary human intake.

Figure 1 presents an overview of the bioactivation and detoxification pathways of estragole. Based on *in vivo* studies, at low doses (<10 mg/kg bw), O-demethylation appears to be more favored in humans and in rodents and yields phenolic derivatives which are to a large extent excreted as the sulfate or glucuronic acid conjugate (Anthony *et al.*, 1987; Sangster *et al.*, 1987). Bioactivation of estragole proceeds by 1′-hydroxylation to 1′-hydroxyestragole and subsequent sulfonation to 1′-sulfooxyestragole. The sulfate conjugate of 1′-hydroxyestragole is unstable and hydrolyzes to form a

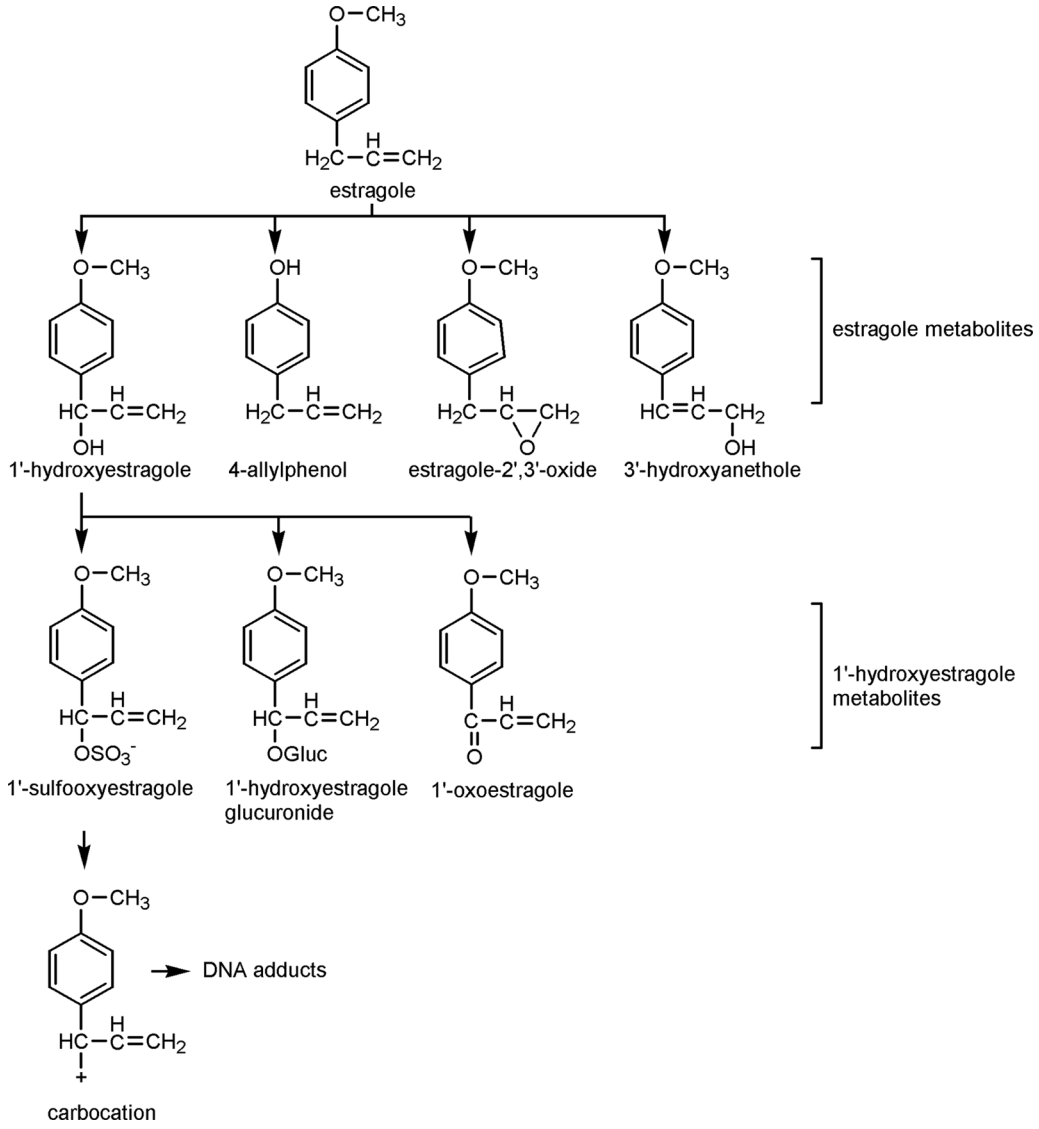


**FIG. 1.** Bioactivation and detoxification pathways of estragole.

reactive electrophilic intermediate (carbocation ion) which has been linked to glutathione (GSH) depletion, oxidative stress, protein-, DNA-, and GSH-adduct formation, probably at or near the site of formation (Phillips *et al.*, 1981; Smith *et al.*, 2002). Therefore, the sulfate conjugate of 1′-hydroxyestragole is considered to be the ultimate hepatotoxic and hepatocarcinogenic agent in rodents (Boberg *et al.*, 1983). This conclusion was corroborated by co-administration of the specific sulfotransferase (SULT) inhibitor pentachlorophenol (PCP), which resulted in a potent inhibition of hepatic tumor induction by long-term dietary administration of the closely related alkenylbenzenes safrole or 1′-hydroxysafrole and a significant decrease of SULT activity and hepatic DNA and RNA adduct formation by 1′-hydroxysafrole in mice (Boberg *et al.*, 1983).

A significant difficulty in evaluating the metabolic, biochemical, and toxicological data for estragole as well as other alkenylbenzenes is that human exposure to these substances results from exposure to a complex mixture of food, spice, and spice oil constituents which may significantly impact the biochemical fate and toxicological risk of the alkenylbenzenes. In this respect, we have previously demonstrated that a methanolic extract from basil, which contained estragole, also contained the flavone nevadensin (5,7-dihydroxy-6,8,4′-trimethoxyflavone) which was able to inhibit DNA-adduct formation in rat hepatocytes exposed to the proximate carcinogen 1′-hydroxyestragole (Alhusainy *et al.*, 2010; Jeurissen *et al.*, 2008). This inhibition by nevadensin was shown to occur at the level of SULT-mediated bioactivation of 1′-hydroxyestragole (Alhusainy *et al.*, 2010; Jeurissen *et al.*,

2008). Altogether, the results of our previous research point at a reduction of DNA-adduct formation and consequently a potential reduction of cancer risk when estragole exposure occurs within a food matrix containing SULT inhibitors compared with what is observed upon exposure to pure estragole. Given these previous results, the objectives of the present study were (1) to identify the presence and nature of possible SULT inhibitors able to interfere with estragole bioactivation in other alkenylbenzene-containing botanical preparations and (2) to predict the possible effects of combined exposure to a mixture of SULT inhibitors on formation levels of the ultimate carcinogen 1′-sulfooxyestragole at physiologically relevant concentrations by incorporating the kinetics for SULT inhibition by the compounds in the mixture into our recently developed physiologically based biokinetic (PBBK) models for estragole in the liver of rat (Punt *et al.*, 2008) and human (Punt *et al.*, 2009).

## MATERIALS AND METHODS

***Materials and chemicals.*** 1′-Hydroxyestragole and 1′-acetoxyestragole are genotoxic and carcinogenic compounds and caution should be taken when handling them. Twelve dried herbs and spices were purchased from local supermarkets: nutmeg and mace (*Myristica fragrans* Houtt.), star anise (*Illicium verum* Hook. f.), parsley (*Petroselinum crispum* (Mill.) Nyman ex A.W. Hill), pimento/allspice (*Pimenta dioica* (L.) Merr.), cinnamon (*Cinnamomum zeylanicum* J. Presl.), dill (*Anethum graveolens* L.), laurel (*Laurus nobilis* L.), tarragon (*Artemisia dracunculus* L.), fennel (*Foeniculum vulgare* Mill.), anise (*Pimpinella anisum* L.), and basil (*Ocimum basilicum* L.). 7-Hydroxycoumarin (7HC; 99%), 7-hydroxycoumarin sulfate (7HCS; 99%), quercetin, kaempferol, myricetin, apigenin, luteolin, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), GSH, uridine 5′-diphosphoglucuronic acid (UDPGA), and fetal bovine serum were obtained from Sigma Aldrich (Steinheim, Germany). Nicotinamide adenine dinucleotide (NAD+) was obtained from Roche Diagnostics (Mannheim, Germany).

Nevadensin was purchased from Apin Chemicals (Milton, U.K.). Trifluoroacetic acid was obtained from Mallinckrodt Baker B.V. (The Netherlands). Methanol (Ultra Liquid Chromatography/Mass Spectrometry, ULC/MS) and acetonitrile (ULC/MS) were obtained from Biosolve (Valkenswaard, The Netherlands), and pro-analysis grade acetic acid was purchased from VWR (Darmstadt, Germany). Hanks Balanced Salt Solution (HBSS), PBS, and gentamicin were purchased from Gibco (Paisley, U.K.). DMEM-F12 L-glutamine medium, tris-hydroxymethylaminomethane (tris), and trypsin were obtained from Invitrogen (Breda, The Netherlands). Dimethyl sulfoxide (DMSO), spectroscopic grade (99.9%) was supplied by Acros Organics (Geel, Belgium). Pooled male rat (Sprague Dawley), pooled human liver S9, and microsomal homogenates were obtained from BD Gentest (Woburn, MA), and PCP (98%) was obtained from Sigma-Riedel de Haen (Seelze, Germany). Nanopure water was obtained from a Barnstead Nanopure Type I ultrapure water system. 1′-Hydroxyestragole was synthesized as described previously by Drinkwater *et al*. (1976) based on a method described for the synthesis of 1′-hydroxysafrole (Borchert *et al.*, 1973). Structural confirmation by Gas Chromatography-Mass Spectrometry (GC-MS) was carried out as described previously (Punt *et al.*, 2007).

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

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

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

***Kinetics for inhibition of SULT-catalyzed conversion of 7HC into 7HCS and of oxidation and glucuronidation of 1′-hydroxyestragole into 1′-oxoestragole and 1′-hydroxyestragole glucuronide by selected flavonoids.*** The $K_i$ for SULT inhibition was determined by performing incubations for SULT activity with pooled male rat liver S9 fractions in the presence of increasing concentrations of 7HC (0–100 µM) in the absence or presence of 0.75 µM quercetin, 0.3 µM kaempferol, 5 µM myricetin, or 0.35 µM apigenin. These flavonoid concentrations were selected because they were close to the identified inhibitory concentration 50% ($IC_{50}$) values determined with pooled male rat liver S9 fractions (data not shown). Incubations were performed as described previously (Alhusainy *et al.*, 2010). The possible effects of the identified SULT inhibitors on the detoxification of 1′-hydroxyestragole by glucuronidation and oxidation (Figure 1) were quantified *in vitro* as well. The $K_i$ for the inhibition of 1′-hydroxyestragole oxidation was determined by performing incubations with pooled human liver microsomes in the presence of increasing concentrations of 1′-hydroxyestragole (0–1000 µM) in the absence or presence of 5 µ M quercetin, 5 µM kaempferol, 10 µM myricetin, and 10 µM apigenin. These flavonoid concentrations were selected because they were close to the identified $IC_{50}$ values determined with pooled human liver microsomal fractions (data not shown). Incubations were performed as previously described (Alhusainy *et al.*, 2010). Subsequently, the maximum velocity ($V_{max}$), the Michaelis–Menten constant ($K_m$), and the inhibition constant ($K_i$) for the formation of 7HCS and 1′-oxoestragole were determined as described below (Data Analysis). Incubations for testing the effect of the identified SULT inhibitors on 1′-hydroxyestragole glucuronidation were also performed. In short, the effect of the identified botanical constituents quercetin, kaempferol, myricetin, and apigenin on 1′-hydroxyestragole glucuronidation was examined with pooled human liver microsomes. The incubations were performed as described previously (Alhusainy *et al.*, 2010; Punt *et al.*, 2009) in the absence or presence of each respective flavonoid up to a concentration of 80 µM and at 1000 µM of 1′-hydroxyestragole (a concentration close to the $K_m$ value determined by Punt *et al*. (2009) for the formation of 1′-hydroxyestragole glucuronide by pooled human liver microsomes), both added from 200 times concentrated stock solutions in DMSO.

***Effect of flavonoid mixtures on SULT activity and on oxidation of 1′-hydroxyestragole.*** Incubations for testing the SULT inhibition by a mixture of quercetin, kaempferol, apigenin, and nevadensin (each flavonoid added from a 400 times concentrated stock solution in DMSO) were performed using

pooled male rat liver S9 fractions at concentrations equal to $0K_i$ (DMSO control), $0.2K_i$, $0.5K_i$, $1K_i$, $2K_i$, $5K_i$, or $10K_i$ of each flavonoid together with 25 µM of the substrate 7HC with incubation conditions similar to what was described previously (Alhusainy *et al.*, 2010). Incubations for testing inhibition of conversion of 1′-hydroxyestragole to 1′-oxoestragole by a mixture of quercetin, kaempferol, myricetin, and apigenin were performed using pooled human liver microsomes. These flavonoids were added to the incubation mixtures from 400 times concentrated stock solutions in DMSO at final concentrations equal to $0K_i$ (DMSO control), $0.1K_i$, $0.2K_i$, $0.5K_i$, $1K_i$, or $2K_i$ of each flavonoid together with 400 µM of the substrate (a concentration close to the $K_m$ value determined by Punt *et al*. (2009) for the conversion of 1′-hydroxyestragole to 1′-oxoestragole by pooled human liver microsomes) using incubation conditions similar to what was described previously (Alhusainy *et al.*, 2010).

***Cultivation and treatments of HepG2 cells.*** A total of approximately $1 \times 10^6$ HepG2 cells/well were seeded in a six-well plate with each well containing DMEM/F12 medium containing GlutaMax, 10% fetal bovine serum, and 50 µg/ml gentamycin and incubated in a humidified incubator, 5% $CO_2$ and 95% air at 37°C until confluent monolayers were obtained. Then, cells were washed with HBSS. Cytotoxicity of the test compounds was evaluated using the MTT test (Hussain *et al.*, 1993). For testing detoxification and bioactivation of 1′-hydroxyestragole in response to different flavonoid treatments, cells were exposed to 50 µM 1′-hydroxyestragole, added from a 1000 times concentrated stock solution in DMSO, in the absence or presence of the flavonoid mixtures. All flavonoids when tested individually or in a mixture (Table 4) were added individually from 800 times concentrated stock solutions in DMSO to DMEM/F12 containing GlutaMax and 50 µg/ml gentamicin and incubations were performed for 22 h at 37°C in a humidified atmosphere. To measure the effect of the different flavonoids or their mixtures on the formation of the major DNA adduct $N^2$-(*trans*-isoestragole-3′-y1)-2′-deoxyguanosine (E-3′-N2-dGuo), cells were scraped in PBS and the cells of six wells were pooled in an Eppendorf vial and centrifuged at 6000 rpm for 5 min (Eppendorf centrifuge, type 5415C, Hamburg, Germany) to obtain pellets. The pellets were stored at −20°C until DNA isolation using a Dojindo Get pure DNA Kit Cell, Tissue kit (Dojindo, Amsterdam, The Netherlands), according to the accompanying protocol. Extraction, digestion, and quantification of E-3′-N2-dGuo were performed as previously described (Paini *et al.*, 2010).

***Identification, analysis, and quantification of metabolites.*** Identification and analysis of 7HCS, 1′-hydroxyestragole glucuronide, and 1′-oxoestragole by HPLC–UV and their quantification by calibration curves using available standards was performed as described in our previous work (Alhusainy *et al.*, 2010; Punt *et al.*, 2008, 2009). Detection and quantification of the major DNA adduct $N^2$-(*trans*-isoestragole-3′-y1)-2′-deoxyguanosine (E-3′-N2-dGuo) by Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS/MS) was performed as previously described (Paini *et al.*, 2010).

***Estragole PBBK model.*** To obtain insight in the quantitative dose- and species-dependent effects of the flavonoid inhibitors on the formation of the ultimate carcinogenic metabolite 1′-sulfooxyestragole, in the target organ, the liver, we modified our previously developed PBBK models to take the inhibition into account. Supplementary figure 1 presents the basic concept of these models, which were originally developed to simulate the bioactivation, and detoxification of estragole in both rat (Punt *et al.*, 2008) and human (Punt *et al.*, 2009). The original models were described in detail previously (Punt *et al*., 2008, 2009) and include separate compartments for liver, lung, and kidney, representing organs which were found to be involved in the metabolism of estragole. A separate compartment for fat tissue was included in order to take into account the relatively higher partition coefficient of estragole in fat tissue. All other tissues were lumped into either a rapidly perfused or slowly perfused tissue group (Ramsey and Andersen, 1984). The physiological parameters were obtained from literature (Brown *et al.*, 1997; Supplementary table 1). The partition coefficients were estimated from the log Kow based on a published method (DeJongh *et al.*, 1997). Log Kow values were estimated using the software package ClogP version 4.0 (Biobyte, Claremont, CA) and amounted to 3.1 for estragole and 1.6 for 1′-hydroxyestragole (Supplementary

**TABLE 1**
**Flavonoids Present in Some Alkenylbenzene-containing Herbs and Spices Based on Literature**

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

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

**TABLE 2**
**Kinetic Parameters of SULT-dependent Formation of 7HCS by Pooled Male Rat Liver S9 in the Absence or Presence of Different Flavonoids**

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

[a]Protein from pooled male rat liver S9.
[b]Calculated using the Michaelis–Menten equation for noncompetitive inhibition ($V^{app}_{max} = V_{max}/(1+([I]/K_i))$, where $[I]$ is the flavonoid concentration (µM). An asterisk (*) indicates a significant inhibition compared with the incubation without inhibitor ($p < 0.001$).

*Note*. The control (no inhibitor) values were: $V_{max} = 2.7 \pm 0.2$ nmol/min/mg S9 protein, and $K_m = 4.0 \pm 1.0$ µM.

table 2). The apparent $V_{max}$ values for the different phase I and II reactions, expressed as nmol/min (mg microsomal or S9 protein)$^{-1}$ were determined *in vitro* and were scaled to the liver using a microsomal protein yield of 32 mg/g liver (Barter *et al.*, 2007) or using an S9 protein yield of 143 mg/g liver (Medinsky *et al.*, 1994; Supplementary table 2). The apparent $K_m$ values were also determined *in vitro* and were assumed to correspond to the apparent *in vivo* $K_m$ values. The absorption rate constant (Ka) was set to 1.0/h, resulting in a rapid absorption of estragole from the gastrointestinal tract with an absorption half-life of 0.7 h (Anthony *et al.*, 1987; Supplementary table 2). Mass balance equations were applied to describe the Absorption, Distribution, Metabolism, Excretion (ADME) of estragole in the different tissue compartments, and the kinetic parameters for the different bioactivation and detoxification reactions of estragole were based on *in vitro* kinetic data obtained using relevant rat and human tissue fractions. A full description of the mass balance equations can be found in our previous papers (Punt *et al*., 2008, 2009). Model equations were coded and numerically integrated in Berkeley Madonna 8.0.1 (Macey and Oster, UC Berkeley, CA, USA) using the rosenbrock's algorithm for stiff systems. The predictions of the PBBK model for male rat (Punt *et al.*, 2008) for the formation of 4-allylphenol and 1′-hydroxyestragole glucuronide were in good agreement with observations in the literature in female Wistar rats *in vivo* (Anthony *et al.*, 1987), and the PBBK model for human (Punt *et al.*, 2009) could predict levels of the same metabolites within the same order of magnitude compared with the reported levels *in vivo* in two human volunteers (Sangster *et al*., 1987).

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

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

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

and

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

where $dAM_{HES}/dt$ is the rate of 1′-sulfooxyestragole formation in μmol/h) and $dAM_{HEO}/dt$ is the rate of 1′-oxoestragole formed μmol/h), $V_{max,L\text{-}HES}$ is the maximum rate of formation of 1′-sulfooxyestragole, $V_{max,L\text{-}HEO}$ is the maximum rate of formation of 1′-oxoestragole, $[I]$ is the concentration of quercetin (second scenario, Table 5) or nevadensin (third scenario, Table 5) in the liver μmol/l). Correspondingly, $K_i$ is the inhibition constant for the inhibition by the respective flavonoids μmol/l). $K_{m,L\text{-}HES}$ is the Michaelis–Menten constant for the formation of 1′-sulfooxyestragole μmol/l), $K_{m,L\text{-}HEO}$ is the Michaelis–Menten constant for the formation of 1′-oxoestragole μmol/l), $CL_{HE}$ is the concentration of 1′-hydroxyestragole in the liver μmol/l), $PL_{HE}$ is the liver/blood partition coefficient.

The Michaelis–Menten equations representing the sulfonation or oxidation of 1′-hydroxyestragole in the presence of a mixture of noncompetitive inhibitors were derived assuming an additive effect (Results) and that each enzyme molecule can combine with no more than one of the inhibitors at a time and were as follows:

$$dAM_{HES}/dt = V_{max,L\text{-}HES}/(1+([I_1]/K_{i1}) +([I_2]/K_{i2})+([I_3]/K_{i3})+([I_4]/K_{i4}))*CL_{HE}/PL_{HE}/(K_{m,L\text{-}HES}+(CL_{HE}/PL_{HE}))$$

and

$$dAM_{HEO}/dt = V_{max,L\text{-}HEO}/(1+([I_1]/K_{i1}) +([I_2]/K_{i2})+([I_3]/K_{i3})+([I_4]/K_{i4}))*CL_{HE}/PL_{HE}/(K_{m,L\text{-}HEO}+(CL_{HE}/PL_{HE}))$$

where $[I_1]$, $[I_2]$, $[I_3]$, and $[I_4]$ are the concentrations of the inhibitors: quercetin, kaempferol, myricetin, and apigenin in the liver μmol/l), respectively. Correspondingly, $K_{i1,}$ $K_{i2,}$ $K_{i3,}$ and $K_{i4,}$ are the inhibition constants for the inhibition by the respective inhibitors (μmol/l), which were assumed to be equal in liver of human and rat.

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

$$V=V_{max}/(1+([I_1]/K_{i1}) +([I_2]/K_{i2})\ldots +([I_n]/K_{in}))*[S]/(K_m+[S])$$

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

## RESULTS

### *Inhibition of SULT Activity by Methanolic Herb and Spice Extracts*

Figure 2 presents the effect of increasing concentrations of a series of methanolic herb and spice extracts on SULT activity. With the exception of fennel, increasing concentrations of all methanolic herb and spice extracts inhibited SULT enzyme activity in a dose-dependent manner. The extract from basil

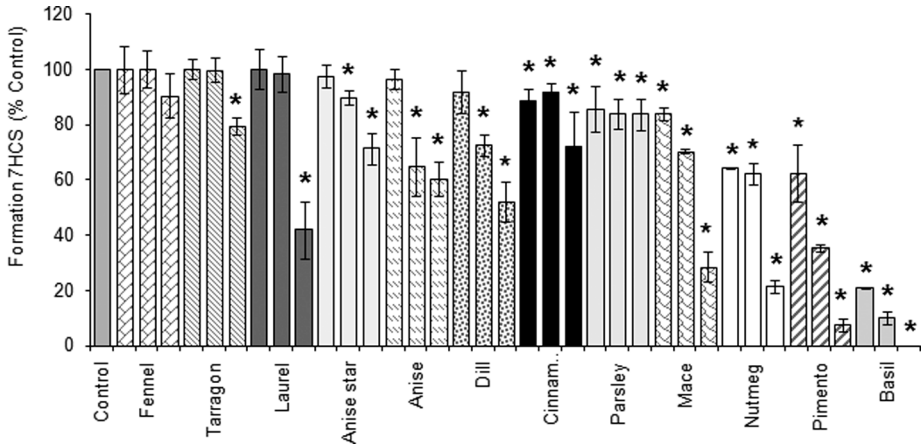


**FIG. 2.** Inhibition of SULT-catalyzed conversion of 7HC into 7HCS by pooled male rat liver S9 fractions in the absence (control) or presence of increasing concentrations (10, 25, and 100 µg/ml) of different methanolic herb and spice extracts. 100% 7HCS formation is equal to 1.3 ± 0.1 nmol/min/mg S9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

was the most potent followed by the extracts from pimento/allspice, nutmeg, and mace. Extracts from parsley, star anise, and cinnamon displayed only moderate SULT inhibition and the extracts from tarragon and laurel significantly inhibited SULT activity only at the highest concentration tested (100 µg extract/ml). Blank incubations, which lacked the cofactor PAPS or which lacked the pooled male rat liver S9 fraction, did not show any formation of 7HCS (data not shown). This indicates that the inhibition of 7HCS formation by the herb and spice extracts was fully dependent on the presence of PAPS and the S9 fraction containing SULT. Incubations with the SULT-inhibitor PCP inhibited SULT activity completely at 25 µM (data not shown).

### *Identification of SULT Inhibitors in the Methanolic Herb and Spice Extracts*

Based on our previous work (Alhusainy *et al.*, 2010), the flavonoid nevadensin was identified as the major SULT-inhibiting constituent in the methanolic extract from basil. In the present work, based on comparison of HPLC–UV and retention time characteristics to HPLC–UV and retention time characteristics of commercially available reference compounds, apigenin was identified as the second-most potent constituent in the same methanolic extract. Among the other most potent methanolic herb and spice extracts, the extract from nutmeg was fractionated and the compounds in the most active fractions were identified as (+) catechin and (−) epicatechin, whereas in the methanolic extract from pimento/allspice, eugenol was identified as the major SULT inhibitor. Based on the observation that in potent extracts flavonoids appeared to be the major SULT inhibitors identified, a literature review was made to screen for major flavonoids present in alkenylbenzene-containing herbs and spices focusing on flavonoids which have been reported to be SULT inhibitors (Eaton *et al.*, 1996; Morimitsu *et al.*, 2004). Table 1 presents an overview of the flavonoids reported to be present in alkenylbenzene-containing herbs and spices as derived from literature and also reported to be SULT inhibitors. The overview presented reveals that in a series of 15 selected alkenylbenzene-containing herbs and spices, quercetin, kaempferol, apigenin, and luteolin are among the most abundant flavonoids, and of these flavonoids, quercetin and kaempferol are the most widespread in alkenylbenzene-containing herbs and spices (Justesen and Knuthsen, 2001).

### *Selection of SULT-inhibiting Flavonoids to be Tested in Subsequent Studies*

Given the identification of flavonoids as an important category of food-borne SULT inhibitors, and the fact that these flavonoids will not only be present in the herbs and spices analyzed in this study, the selection of the flavonoids to be used in the subsequent studies was based on their abundance in the diet as a whole. Generally, estimation of the daily dietary flavonoid intake is based on the intake of three quantitatively important dietary flavonols (quercetin, kaempferol, and myricetin) and two quantitatively important dietary flavones (apigenin and luteolin) (Hertog *et al.*, 1993a,b). These flavonoids are all present in alkenylbenzene-containing herbs and spices, as well as throughout the diet, they are all reported to have SULT inhibiting potency; the total estimated daily intake (EDI) levels of these flavonoids as recorded by different studies varies between 18.3 ± 3.4 and 25.9 ± 14.5 mg/day (Hertog *et al.*, 1993a, b; Lugasi *et al.*, 2003; Mullie *et al.*, 2008). Given these observations, the flavonoids quercetin, kaempferol, myricetin, apigenin, luteolin, and nevadensin, the latter being the major flavone constituent in basil (Grayer *et al.*, 2004), were used in our subsequent experiments to examine the influence of flavonoids in mixtures or individually on the bioactivation of 1′-hydroxyestragole by SULT and on the detoxification of 1′-hydroxyestragole via

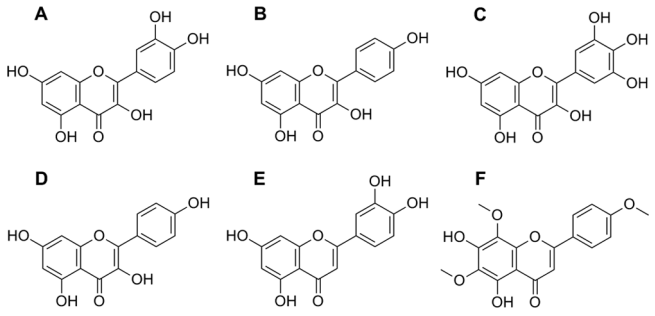


**FIG. 3.** Structural formulas of (A) quercetin, (B) kaempferol, (C) myricetin, (D) apigenin, (E) luteolin, and (F) nevadensin, the major SULT inhibitors in alkenylbenzene-containing herbs and spices and/or throughout the diet.

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

*Inhibition of SULT-catalyzed Conversion of 7HC Into 7HCS and of Oxidation and Glucuronidation of 1′-Hydroxyestragole Into 1′-Oxoestragole and 1′-Hydroxyestragole Glucuronide by the Selected Flavonoids*

After identification of major SULT inhibitors present in the selected alkenylbenzene-containing herbs and spices (nevadensin, quercetin, kaempferol, myricetin, and apigenin), initial studies testing the effect of the selected flavonoids quercetin, kaempferol, myricetin and apigenin on SULT-mediated conversion of 7HC into 7HCS and on oxidation or glucuronidation of 1′-hydroxyestragole revealed that only SULT and oxidation activity appeared to be inhibited. Nevadensin was not included in these inhibition studies because its effect on sulfonation, glucuronidation, and oxidation was already determined in our previous work (Alhusainy *et al.*, 2010). Oxidation and glucuronidation of 1′-hydroxyestragole were not inhibited by nevadensin to any significant extent even at nevadensin concentrations up to 20 μM (Alhusainy *et al*., 2010). The effect of the other selected flavonoids quercetin, kaempferol, myricetin, and apigenin on glucuronidation of 1′-hydroxyestragole was examined by adding each flavonoid up to 80 μM together with 1000 μM of the substrate 1′-hydroxyestragole in incubations with pooled human liver microsomes and the cofactor for glucuronidation (UDPGA). None of the flavonoids tested resulted in any significant effect on the formation of 1′-hydroxyestragole glucuronide. Because the catalytic efficiency for the SULT-catalyzed conversion of 7HC into 7HCS and for 1′-hydroxyestragole into 1′-sulfooxyestragole is higher in the liver of rat compared with the liver of human (Alhusainy *et al.*, 2010; Wang *et al.*, 2006), *in vitro* incubations for inhibition of SULT activity by the selected flavonoids was performed using pooled male rat liver S9. Oxidation of 1′-hydroxyestragole to 1′-oxoestragole represents a minor metabolic route in the liver of male rat as compared with the liver of human suggesting that the inhibition of this pathway would hardly influence the overall bioactivation of estragole in the liver of male rat but may influence the overall metabolism in human. Therefore, *in vitro* incubations for inhibition of oxidation of 1′-hydroxyestragole by the selected flavonoids were performed in pooled human liver microsomes. In this way, the kinetics for inhibition of SULT activity and of oxidation of 1′-hydroxyestragole were determined, and the mode of inhibition and the respective $K_i$ values were defined. Tables 2 and 3 display the Michaelis–Menten parameters ($V_{max}$ and $K_m$) and inhibition constants ($K_i$) for 7HCS and 1′-oxoestragole formation by pooled male rat liver S9 and pooled human liver microsomal fractions, respectively, in the presence or absence of the flavonoids quercetin, kaempferol, myricetin, and apigenin. The apparent $V_{max}$ values for sulfonation were lowered significantly (minus 30–33%; $p < 0.001$ ) in the presence of each flavonoid, whereas the apparent $K_m$ values were not statistically significantly different from the $K_m$ value determined in the absence of each flavonoid. The apparent $V_{max}$ values for oxidation of 1′-hydroxyestragole were lowered significantly (minus 52–77%; $p < 0.001$ ) in the presence of each flavonoid, whereas the apparent $K_m$ values were not statistically significantly different from the $K_m$ value determined in the absence of each flavonoid. These results point at a noncompetitive type of inhibition of sulfonation, as well as oxidation by each individual flavonoid. Applying the Michaelis–Menten equation for noncompetitive inhibition, the inhibition constant ($K_i$) for SULT inhibition as calculated from the data amounted to, respectively, 1.5, 0.6, 11.9, and 0.7 μM for quercetin, kaempferol, myricetin, and apigenin. The inhibition constants ($K_i$) for inhibition of the conversion of 7HC to 7HCS by the different flavonoids are not expected to be significantly different when using 7HC instead of 1′-hydroxyestragole as a substrate. This is based on the type of inhibition that was shown to be noncompetitive meaning that only the apparent $V_{max}$ and not the apparent $K_m$ of the enzyme was found to be affected, which corroborates the assumption that flavonoids do not interfere with the substrate binding site and exert their inhibiting effect by interaction with another site on the enzyme than the site involved in substrate binding (Simmons, 1996). In our previous work (Alhusainy *et al.*, 2010), the type of SULT inhibition (noncompetitive) and the $K_i$ (4 nM) for this inhibition by the flavonoid nevadensin was similar in pooled male rat and pooled human liver S9 fractions. Based on this result, it was assumed that the $K_i$ and the type of SULT inhibition by the flavonoids quercetin, kaempferol, myricetin, and apigenin determined in pooled male rat liver S9 will also be similar in human liver S9, given that the type of inhibition by each of them was also noncompetitive.

The inhibition constant ($K_i$) for inhibition of oxidation of 1′-hydroxyestragole to 1′-oxoestragole by quercetin, kaempferol, myricetin, and apigenin amounted to 4.5, 1.5, 4.8, and 6.5 μM, respectively (Table 3).

**TABLE 3**
**Kinetic Parameters of 1′-Oxoestragole Formation by Pooled Human Liver Microsomes in the Absence or Presence of Different Flavonoids**

| Inhibitor | Apparent $V_{max}$ (nmol/min/mg protein)[a] | Apparent $K_m$ (μM) | $K_i$[b] (μM) |
|---|---|---|---|
| Quercetin (5 μM) | 4.8 ± 1.0* | 1046 ± 258 | 4.6 |
| Kaempferol (5 μM) | 2.3 ± 0.3* | 605 ± 69 | 1.5 |
| Myricetin (10 μM) | 3.5 ± 0.9* | 727 ± 292 | 5.4 |
| Apigenin (10 μM) | 4.0 ± 0.4* | 613 ± 66 | 6.7 |

[a]Protein from pooled human liver microsomes.

[b]Calculated using the Michaelis–Menten equation for noncompetitive inhibition ($V^{app}_{max} = V_{max}/(1+([I]/K_i))$, where $[I]$ is the flavonoid concentration (μM). An asterisk (*) indicates a significant inhibition compared with the incubation without inhibitor ($p < 0.001$).

*Note*. The control (no inhibitor) $V_{max}$ = 10 ± 1.4 nmol/min/mg microsomal protein, and $K_m$ = 688 ± 119 μM.

*Effect of Selected Flavonoid Mixtures on SULT-catalyzed Conversion of 7HC Into 7HCS and on Oxidation of 1′-Hydroxyestragole Into 1′-Oxoestragole*

Given that the diet may contain a variety of SULT inhibitors, experiments were performed to assess the effect of combined flavonoid exposure on SULT activity, as well as on oxidation of 1′-hydroxyestragole to 1′-oxoestragole. To this end, a test mixture was defined that mimics a realistic dietary flavonoid mixture and included four flavonoids which were found to be abundant in alkenylbenzene-containing herbs and spices and able to inhibit SULT activity, namely, quercetin, kaempferol, apigenin, and nevadensin, the latter being previously identified as a potent SULT inhibitor present in basil (Alhusainy *et al.*, 2010). The simultaneous addition of quercetin, kaempferol, apigenin, and nevadensin at concentrations equal to $0.2K_i$, $0.5K_i$, $1K_i$, $2K_i$, $5K_i$, or $10K_i$ of each flavonoid together with 25 μM of the substrate 7HC lowered the SULT activity in incubations with pooled male rat liver S9 (Figure 4). The reduction in the formation of 7HCS was similar to the reduction predicted by the Michaelis–Menten equation defined for a series of independent noncompetitive inhibitors (Figure 4), which verifies our assumption for a noncompetitive type of inhibition by each individual flavonoid on SULT enzyme activity, as well as an additive interaction between the flavonoids in the mixture (Figure 4).

With the exception of nevadensin that had no effect on 1′-hydroxyestragole oxidation in pooled human liver microsomal fractions (Alhusainy *et al.*, 2010), the other SULT inhibitors identified in alkenylbenzene-containing herbs and spices, namely, quercetin, kaempferol, myricetin, and apigenin, had an inhibiting effect on the oxidation of 1′-hydroxyestragole. Therefore, incubations for testing the inhibition of conversion of 1′-hydroxyestragole to 1′-oxoestragole by a mixture

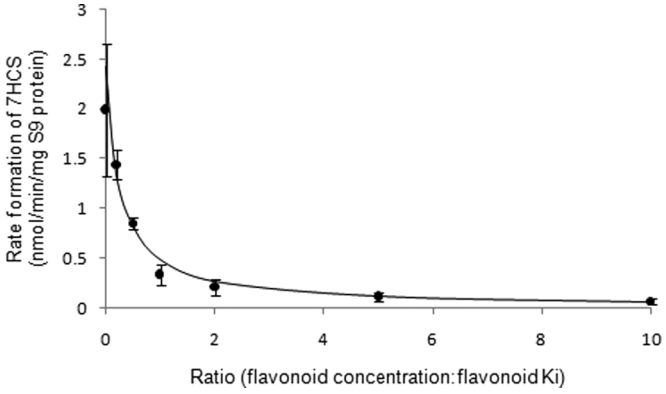


**FIG. 4.** SULT-dependent formation of 7HCS as measured in incubations with pooled male rat liver S9 in the presence of increasing concentrations of flavonoid mixtures containing quercetin, kaempferol, apigenin, and nevadensin at $0K_i$, $0.2K_i$, $0.5K_i$, $1K_i$, $2K_i$, $5K_i$, or $10K_i$ of each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

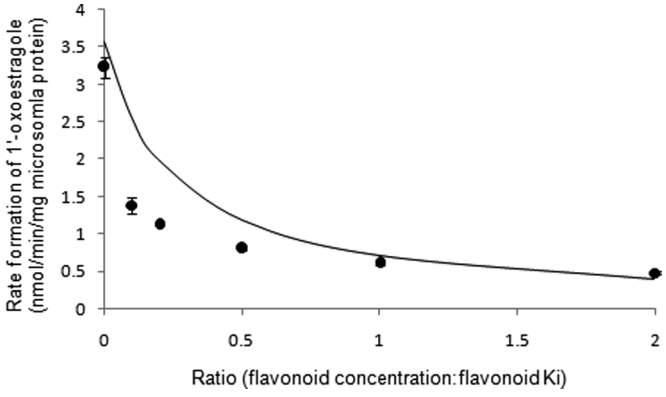


**FIG. 5.** Formation of 1′-oxoestragole as measured in incubations with pooled human liver microsomes in the presence of increasing concentrations of flavonoid mixtures containing quercetin, kaempferol, myricetin, and apigenin at $0K_i$, $0.1K_i$, $0.2K_i$, $0.5K_i$, $1K_i$, or $2K_i$ of each flavonoid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

of quercetin, kaempferol, myricetin, and apigenin were performed. Figure 5 shows that the simultaneous addition of quercetin, kaempferol, myricetin, and apigenin at concentrations equal to $0.1K_i$, $0.2K_i$, $0.5K_i$, $1K_i$, or $2K_i$, for each flavonoid together with 400 μM of the substrate 1′-hydroxyestragole reduced the formation of 1′-oxoestragole. The observed inhibition in the formation of 1′-oxoestragole in the presence of the flavonoid mixture at concentrations higher than their $K_i$ values matched the predicted values of the derived Michaelis–Menten formula defined for a series of independent noncompetitive inhibitors, whereas at concentrations lower than their $K_i$ values, the predicted values were somewhat higher than the values actually observed, which may be due to different types of enzymes playing a role in the oxidation of

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

*Effect of Flavonoid Mixtures on Conversion and DNA Binding of 1′-Hydroxyestragole in HepG2 Cells*

To investigate whether the inhibition of bioactivation and detoxification pathways of 1′-hydroxyestragole would also be observed in an intact cellular system, cells from the human HepG2 hepatoma cell line were exposed to 1′-hydroxyestragole in the absence or presence of different flavonoids and their combinations, followed by detection of the level of 1′-hydroxestragole oxidation, glucuronidation, and sulfonation, the latter reflected by the formation of the major estragole DNA adduct (E-3′-N2-dGuo). Table 4 summarizes the different flavonoid mixtures and/or pure flavonoids which were investigated in the HepG2 model system. The outcomes of these studies will reveal whether selected flavonoids and/or their mixtures are able to inhibit SULT-mediated bioactivation of 1′-hydroxyestragole in this cellular system, resulting in an ultimate shift of metabolism in favor of detoxification at the cost of bioactivation. The compounds were not cytotoxic to HepG2 cells under the conditions used in these experiments as observed by MTT activity measurements (data not shown). Fig. 6A reveals that a significant reduction in the formation of E-3′-N2-dGuo compared with control (no flavonoid) is observed in the human HepG2 cells following co-administration of 50 µM of the substrate 1′-hydroxyestragole and 13, 5.4, 2.3, 1.5, and 0.34 µM of a flavonoid mixture containing quercetin, kaempferol, myricetin, apigenin, and luteolin respectively (each at a concentration corresponding to its relative contribution in the diet, Table 4). E-3′-N2-dGuo formation in the HepG2 cells is also inhibited significantly by 100 µM of the same flavonoids in a mixture (each at 20 µM), by 50 µM kaempferol, or by 100 µM quercetin (Fig. 6A). The data indicate that the flavonoids are able to pass the cell membrane and exert an intracellular effect on SULT mediated bioactivation of 1′-hydroxyestragole.

HPLC-UV analysis of the media revealed a significant reduction in the formation of 1′-oxoestragole (measured as GS-1′-oxoestragole, Fig. 6B) and a significant increase in the formation of 1′-hydroxyestragole glucuronide (Fig. 6C). Altogether, the data indicate a shift metabolism from sulfonation and oxidation to glucuronidation, which is a detoxification pathway for 1′-hydroxyestragole.

**TABLE 4**
**Flavonvoid Treatments Tested in Human HepG2 Cells Exposed to 50 µM of 1′-Hydroxyestragole for 22 h at 37°C**

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

*PBBK-Model-Based Predictions for Estragole Metabolism in the Presence of Selected SULT-inhibiting Flavonoids*

Figure 7A illustrates the PBBK-model-based predictions for the formation of 1′-sulfooxyestragole in the liver of rat and human after co-administration of 0.01 mg/kg bw/day of estragole, representing the estragole dose considered relevant for human dietary exposure from herbs and spices mainly (Smith *et al.*, 2002), and a mixture of the flavonoids quercetin, kaempferol, myricetin, and apigenin at a total EDI level of 20.0 ± 6.3 mg/day (Lugasi *et al.*, 2003; Mullie *et al.*, 2008), which corresponds to 0.33 mg flavonoids/kg bw/day for a person of 60 kg. The results presented in Fig. 6A reveal that the formation of 1′-sulfooxyestragole in the liver of rat after 24 h is predicted to decrease by 0.18%, 1.7%, 8%, and 14.4% compared with control when assuming, respectively, 1%, 10%, 50%, and 100% uptake of the flavonoid mixture, whereas in the liver of human, the formation of 1′-sulfooxyestragole was predicted to decrease by, respectively, 0.5%, 5%, 18%, and 27%. In addition, the possible consequences of consumption of estragole together with a flavonoid food supplement containing 500 mg of quercetin, representing food supplements actually available on the market, was also studied. The PBBK model predictions indicated that the intake of 500 mg quercetin, which corresponds to 8.3 mg querectin/kg bw/day for a person of 60 kg, together with 0.01 mg/kg bw of estragole may decrease

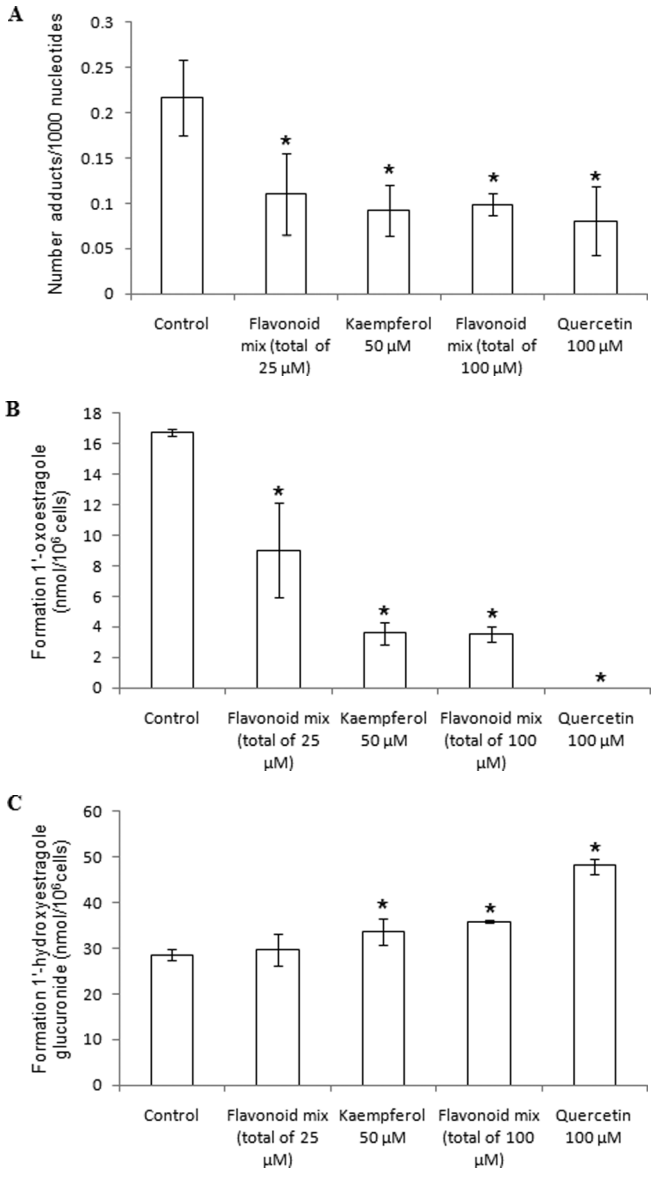


**FIG. 6.** Formation of (A) E-3′-N2-dGuo, (B) 1′-oxoestragole (measured as GSH adducts), and (C) 1′-hydroxyestragole glucuronide in HepG2 cells exposed for 22 h to 50 µM 1′-hydroxyestragole in the absence or presence of a mixture of the following flavonoids: quercetin, kaempferol, myricetin, apigenin, and luteolin (13, 5.4, 2.3, 1.5, and 0.34 ,µM respectively), 100 µM of the same flavonoids in a mixture (each at 20 µM), 50 µM kaempferol, or 100 µM quercetin.

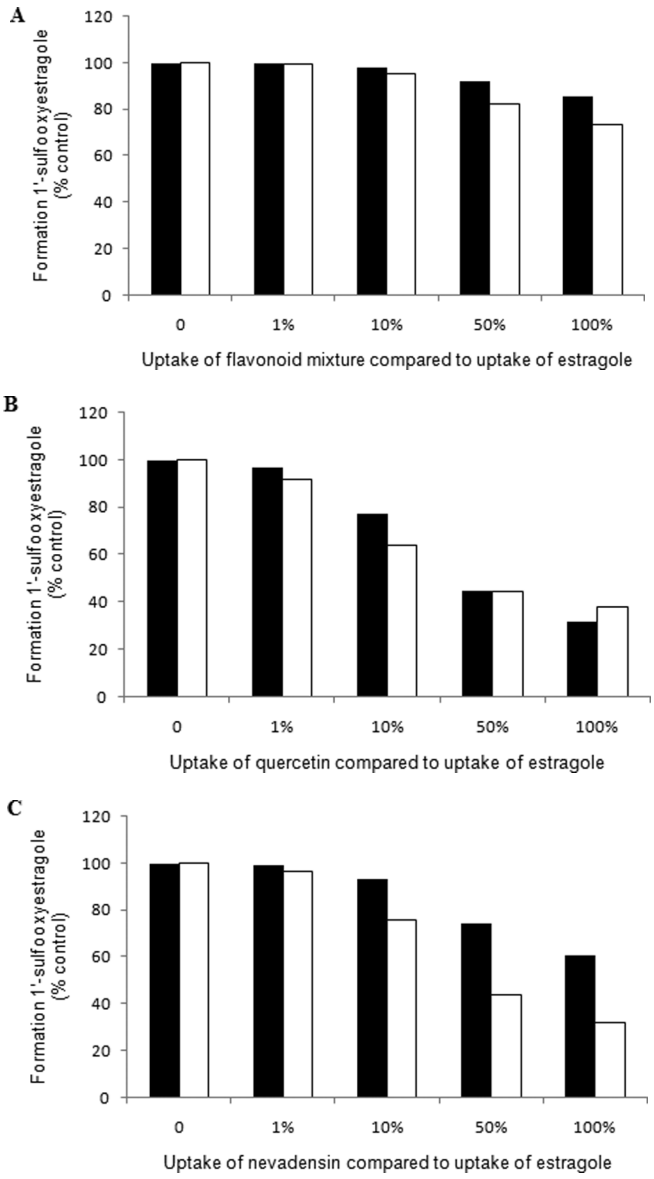


**FIG. 7.** PBBK-model-based predictions for the formation of 1′-sulfooxyestragole in the liver of rat (black bars) and human (white bars) after co-administration of 0.01 mg/kg bw/day of estragole and (A) a mixture of flavonoids at their EDI levels including 12 mg/day of quercetin, 4.6 mg/day of kaempferol,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

the formation of 1′-sulfooxyestragole in the liver of rat by 3%, 23%, 55%, and 69% when assuming 1%, 10%, 50%, and 100% uptake of quercetin, and in the liver of human, formation of 1′-sulfooxyestragole was predicted to decrease by, respectively, 8.6%, 36%, 56%, and 63% at these levels of quercetin uptake (Fig. 7B).

In a third PBBK modeling scenario, the protective effect of nevadensin against SULT-mediated bioactivation of estragole in consumers of basil was evaluated. Assuming that basil contains 0.5% of essential oil (Smith *et al.*, 2002) and that the essential oil of basil contains 22.7% of estragole (Sanda *et al.*, 1998), an EDI of 0.01 mg/kg bw/day of estragole would result from an intake of 529 mg basil/day. Based on our previous work (Alhusainy *et al.*, 2010), the amount of nevadensin in 529 mg of basil is expected to be 0.34 mg, and this would result in an EDI

for nevadensin of 0.0057 mg/kg bw/day for a person of 60 kg. At an estragole dose of 0.01 mg/kg bw/day and a nevadensin intake of 0.0057 mg/kg bw/day, the formation of 1′-sulfooxyestragole in the liver of rat is predicted to decrease by 0.77%, 7%, 26%, and 40% compared with control, whereas in the liver of human, the formation of 1′-sulfooxyestragole was predicted to decrease by 3.5%, 25%, 56%, and 68% when assuming, respectively, 1%, 10%, 50%, and 100% uptake of nevadensin (Fig. 7C).

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

**TABLE 5**
**Input Data for PBBK Models Representing Three Possible Intake Scenarios of Flavonoids and Estragole at 0.01 mg/kg bw/day**

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

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

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

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

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

## DISCUSSION

The alkenylbenzene estragole is an important natural constituent of herbs and spices. However, several studies have shown both genotoxic and carcinogenic properties for this compound in rodents (Drinkwater *et al.*, 1976; Miller, 1983). These experiments were performed using the pure compound dosed by gavage in high doses, whereas in herbs and spices, estragole is present in relatively lower amounts together with other constituents in a food matrix, which can influence its biochemical fate. The present study reports that methanolic extracts from different alkenylbenzene-containing herbs and spices were able to inhibit SULT activity involved in the formation of the proximate hepatocarcinogen 1′-sulfooxyestragole. Flavonoids including nevadensin, quercetin, kaempferol, myricetin, and apigenin were the major constituents responsible for this inhibition of SULT activity with $K_i$ values in the nano- to micromolar range. Apart from SULT inhibitors present in herbs and spices and identified in the present study, the regular human diet is rich in other SULT inhibitors ranging from a number of natural dietary chemicals, such as polyphenols (Eaton *et al.*, 1996), to a number of food additives such as (±)-catechin, (+)-catechin, 4-chlorobenzoic acid, aspartame, benzoic acid, erythrosine, gallic acid, octyl gallate, *p*-hydroxybenzoic acid, propyl gallate, protocatechuic acid, saccharin, tannic acid, tartrazine, and vanillin, which have all been shown to be capable to inhibit the sulfonation of a number of xenobiotics and endobiotics in human liver cytosol to varying extents (Bamforth *et al.*, 1993). A great volume of literature data supports the plausibility of noncompetitive inhibition of sulfonation of various substrates by different flavonoids such as quercetin, genistein, (+)-catechin, kaempferol, apigenin, and diadzein (Ghazali and Waring, 1999; Mesía-Vela and Kauffman, 2003; Walle *et al.*, 1995). The various flavonoids tested in this study were also shown to be able to significantly inhibit estragole DNA-adduct formation in human HepG2 cells exposed to the proximate carcinogen 1′-hydroxyestragole (50% reduction compared with control) even at concentrations as low as 25 μM of the flavonoid mixture. The results of the present work also show that at levels of flavonoids sufficient to inhibit SULT activity, they can also inhibit the oxidation of 1′-hydroxyestragole, which is the major phase-II pathway of 1′-hydroxyestragole in the liver of human. Additional results of the present study revealed that in the human HepG2 cells exposed to 1′-hydroxyestragole in the presence of flavonoids, reduction in the activity of sulfonation and oxidation results in a metabolic shift toward glucuronidation, which serves as a detoxification pathway for 1′-hydroxyestragole.

In a next step, the kinetics for SULT inhibition were incorporated in the PBBK models for estragole in rat and human to predict the effect of co-exposure to estragole and (mixtures of) the different flavonoids on the bioactivation *in vivo*. The PBBK model-based predictions indicate that the reduction of estragole bioactivation in rat and human by co-administration of the flavonoids is dependent on whether the intracellular liver concentrations of the flavonoids can reach their $K_i$ values determined in the present study. For example, high intake levels of SULT inhibitors (*e.g.* quercetin) at levels present in food

supplements currently available on the market might result in significant inhibition of the formation of 1′-sulfooxyestragole even at 10% uptake of the flavonoid. Interestingly, for human liver, a dose of nevadensin of only 0.34 mg/day was predicted to result in comparable inhibition of 1′-sulfooxyestragole formation as a dose of quercetin of 500 mg/day (Figs. 6B and C). This clearly illustrates that although nevadensin is not a major flavonoid constituent in the diet, its contribution to the SULT mediated modulation of estragole is already achieved at relatively lower dose levels due to its $K_i$ value of 4 nM (Alhusainy *et al.*, 2010), which is 2–3 orders of magnitude lower than that for other flavonoids. Furthermore, the inhibitory effect of nevadensin *in vivo* may also be more easily achieved than that of the other flavonoids because nevadensin is a methylated flavonoid, and methylated flavones such as nevadensin have been demonstrated to be more metabolically stable and have a higher intestinal absorption than their unmethylated analogues increasing the oral bioavailability of methylated flavones compared with their unmethylated analogues (Wen and Walle, 2006).

In this context, it is also of interest to take into account the reported intracellular and circulating concentrations of the different flavonoid-type SULT inhibitors. Mean plasma levels of quercetin, for example, reached 7.6 µM in human volunteers after consuming a supplement of 160 g stewed and homogenized onions, which provided 331 µmol of quercetin glucosides (Graefe *et al.*, 2001), a plasma level which is sufficient to inhibit SULT activity giving a $K_i$ for quercetin-mediated SULT inhibition of 1.5 µM as determined in the present study. Moreover, after a single oral dose of 200 mg/kg bw of *Chrysanthemum morifolium* extract to rats apigenin reached maximum plasma levels of 16 µM (Chen *et al.*, 2007). Clearly, plasma levels of quercetin and apigenin can reach levels higher than their $K_i$. Data on human plasma levels of other flavonoids tested in the present study are lacking. However, plasma levels for other flavonoids, namely, the isoflavones genistein and daidzein, may exceed 0.5 µM in women who eat a traditional Japanese diet (Uehara *et al.*, 2000) and may even reach 1 µM in individuals consuming some dietary supplements (Gooderham *et al.*, 1996), both levels are sufficient to inhibit SULT1A1 and SULT1EI significantly given the $IC_{50}$ for inhibition of SULT1A1 and SULT1EI by genistein of 0.5 and 1 µM, respectively (Harris *et al.*, 2004). Moreover, plasma levels of other flavonoids, such as hesperetin, may reach 1–3 µM after ingestion of large amounts of fruit and vegetables (Erlund *et al.*, 2002), which are concentrations sufficient to inhibit both SULT1A1 and SULT1E1 (Harris *et al.*, 2004). It is important to note, however, that *in vivo* flavonoids undergo extensive conjugation to their corresponding glucuronic acid and sulfate conjugates, and that in most *in vivo* studies, flavonoids are measured after deconjugation. Therefore, the reported plasma concentrations may reflect total flavonoid concentration in the conjugated and unconjugated forms instead of the concentrations of the aglycon. This raises the question if in these conjugated forms the flavonoids are still able to interact with SULTs. In this regard, it is worth to note that conjugated metabolites of flavonoids can retain the biological activity of the parent compound (Harris *et al.*, 2004). This is highly dependent on the molecular site of conjugation. Moreover, studies in which cells *in vitro* were exposed to glucuronidated and sulfonated conjugates of quercetin demonstrated that within the cells, the flavonoid was no longer conjugated due to efficient extra- and/or intracellular deconjugation (Lee-Hilz *et al.*, 2008). Another issue of interest is if these flavonoids reported in literature can inhibit the same SULT isoforms which are involved in the sulfonation of the substrate 1′-hydroxyestragole, which have so far not been identified.

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

Finally, it is important to note that the PBBK model analysis of the effect of the inhibitors on estragole bioactivation *in vivo* as presented is a fist approximation. More refined models could take into account (1) submodels for the ADME characteristics of the individual flavonoids and/or (2) nonadditive modes of interactions for the flavonoids in mixture on the conversion of 1′-hydroxyestragole to 1′-oxoestragole which better fit the experimental *in vitro* data for this inhibition. However, development of such refined models was beyond the scope of the present study.

In conclusion, dietary flavonoids may modulate the bioactivation and thus the ultimate cancer risk posed by estragole provided that the physiological liver concentrations of the inhibitors reach their $K_i$ values. Whether *in vivo* levels of flavonoids can reach their $K_i$ values and whether conjugated forms of the flavonoids can still inhibit SULT activity are important issues that need further investigation. Overall, an *in vivo* modulation for estragole bioactivation is highly plausible given the facts that (1) SULT inhibitors are abundant in the diet, (2) their inhibition constants ($K_i$) for SULT activity are in the nano- to micromolar range, and (3) their combined effect on SULT enzyme activity was shown to be additive. If the PBBK-based predicted data can be validated *in vivo*, which is an important issue for future research, this would imply that the likelihood of bioactivation and subsequent adverse effects might be lower when alkenylbenzenes are consumed in a matrix of food items containing SULT inhibitors than what would be expected on the basis of experiments using alkenylbenzenes as pure compounds. The safety of alkenylbenzene-containing botanicals could consequently be judged and regulated on the basis of the EDIs of the different alkenylbenzenes compared with the EDI of the different respective SULT inhibitors which are present in the diet. Further research investigations are directed at investigating efficiency of SULT inhibitors in reducing-DNA adduct formation and the subsequent carcinogenic risk *in vivo*.

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## SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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