REVIEW

In silico methods for physiologically based biokinetic models describing bioactivation and detoxification of coumarin and estragole: Implications for risk assessment

Ivonne M. C. M. Rietjens[1], Ans Punt[1], Benoît Schilter[2], Gabriele Scholz[2], Thierry Delatour[2] and Peter J. van Bladeren[1,2]*

[1] Division of Toxicology, Wageningen University, Wageningen, The Netherlands
[2] Nestlé Research Center, Lausanne, Switzerland

In chemical safety assessment, information on adverse effects after chronic exposure to low levels of hazardous compounds is essential for estimating human risks. Results from *in vitro* studies are often not directly applicable to the *in vivo* situation, and *in vivo* animal studies often have to be performed at unrealistic high levels of exposure. Physiologically based biokinetic (PBBK) modeling can be used as a platform for integrating *in vitro* metabolic data to predict dose- and species-dependent *in vivo* effects on biokinetics, and can provide a method to obtain a better mechanistic basis for extrapolations of data obtained in experimental animal studies to the human situation. Recently, we have developed PBBK models for the bioactivation of the alkenylbenzene estragole to its DNA binding ultimate carcinogenic metabolite 1′-sulfooxyestragole in both rat and human, as well as rat and human PBBK models for the bioactivation of coumarin to its hepatotoxic o-hydroxyphenylacetaldehyde metabolite. This article presents an overview of the results obtained so far with these *in silico* methods for PBBK modeling, focusing on the possible implications for risk assessment, and some additional considerations and future perspectives.

Received: May 8, 2009
Revised: June 11, 2009
Accepted: June 13, 2009

Keywords:
Coumarin / Estragole / Metabolism / Physiologically based biokinetic models / Risk assessment

1 Introduction

1.1 Estragole

Estragole is an alkenylbenzene that occurs in different herbs such as tarragon, basil, and fennel and is present in products derived from these herbs such as pesto and essential oils [1, 2]. Average daily intake of estragole was estimated to be 10–70 μg/kg bw.day [1, 3]. There is interest in the safety assessment of estragole as a food constituent, since estragole has been identified to be genotoxic *in vitro* and carcinogenic in rodent studies performed at high dose levels [4–6].

Based on disposition studies of ^{14}C-methoxy-labeled estragole in rats, mice, and humans and identification of the metabolites excreted, the principal metabolic pathways of estragole have been established [7, 8]. Figure 1 shows an overview of estragole metabolism including pathways for bioactivation to the proximate and ultimate carcinogenic metabolite and pathways for detoxification. The main phase I metabolic pathways include 1′-hydroxylation, O-demethylation, epoxidation and 3′-hydroxylation of estragole. The

Correspondence: Professor Ivonne M. C. M. Rietjens, Division of Toxicology, Wageningen University, Tuinlaan 5, 6703 HE Wageningen, The Netherlands
E-mail: ivonne.rietjens@wur.nl
Fax: +31-317-484931

Abbreviations: BMD, benchmark dose; **BMDL,** lower confidence bound of the benchmark dose; **EFSA,** European Food Safety Authority; **FEMA,** Flavor and Extract Manufacturers Association; **MOE,** margin of exposure; **oHPA,** o-hydroxyphenylacetaldehyde; **oHPAA,** o-hydroxyphenylacetic acid; **oHPE,** o-hydroxyphenylethanol; **PBBK,** physiologically based biokinetic; **SCF,** Scientific Committee on Food; **TDI,** tolerable daily intake; **VSD,** virtual safe dose

*Current address: KWR Watercycle Research Institute, P.O. Box 1072, 3430 BB Nieuwegein, The Netherlands.

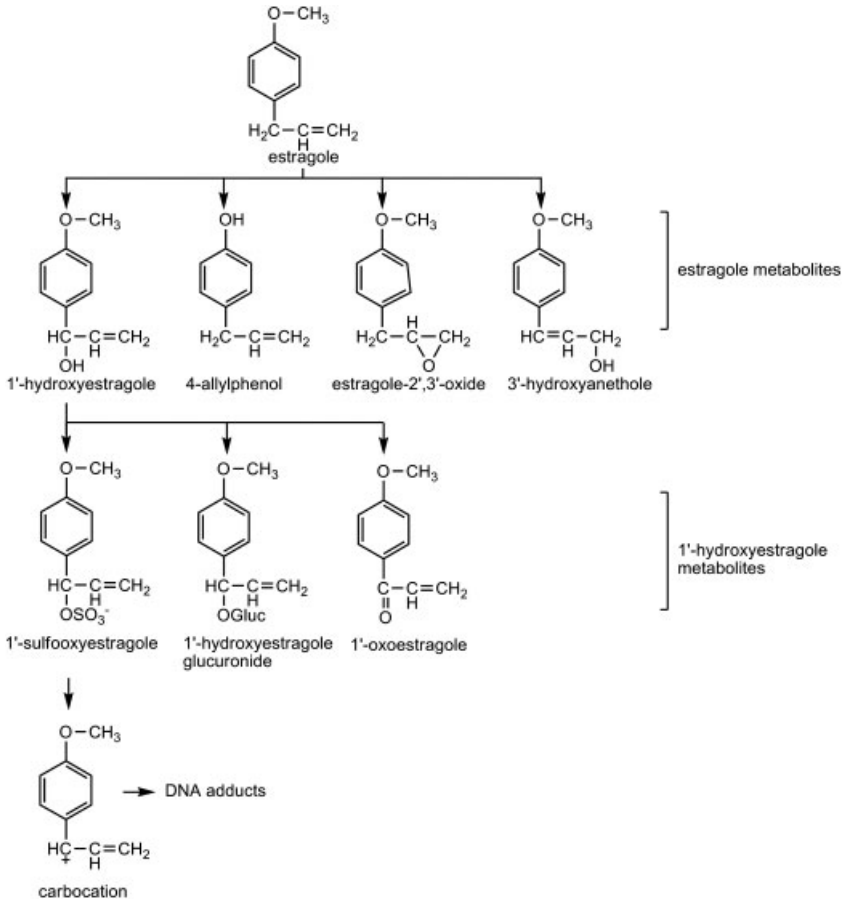

Figure 1. Metabolism of estragole with the bioactivation pathway proceeding by formation of the proximate carcinogen 1′-hydroxyestragole and the ultimate carcinogen 1′-sulfooxyestragole. Formation of the other metabolites eventually leads to detoxification and excretion.

main metabolic pathways of the proximate carcinogen 1′-hydroxyestragole are sulfonation to the ultimate carcinogen 1′-sulfooxyestragole, and detoxification through glucuronidation to 1′-hydroxyestragole glucuronide, or oxidation to 1′-oxoestragole.

Several evaluations have been performed to assess the safety of human exposure to estragole at low-dietary intake levels. In an evaluation performed by the Scientific Committee on Food (SCF) of the European Committee in 2001, it was concluded that estragole is genotoxic and carcinogenic and restrictions in use were indicated [3] (http://ec.europa.eu/food/fs/sc/scf/out104_en.pdf). The Expert Panel of the Flavor and Extract Manufacturers Association (FEMA) classified estragole in 1965 as Generally Recognized as Safe under the conditions of intended use as flavouring substance in food [9]. In 2002, the FEMA re-evaluated the data available for estragole and concluded again that exposure to estragole from food, mainly as spices or added as such, does not pose a significant cancer risk to humans [1]. In this conclusion, it was taken into account that there are experimental data suggesting a non-linear relationship between dose and profiles of metabolism and metabolic activation. In a more recent evaluation performed by the Joint FAO/WHO Expert Committee on Food Additives in 2008, it was indicated that although evidence of carcinogenicity to rodents given high doses of estragole exists, further research is needed to assess the potential risk to human health from low-level dietary exposure to estragole present in foods and essential oils and used as flavouring agents [10] (http://www.who.int/entity/ipcs/food/jecfa/summaries/summary69.pdf).

Overall, these different expert judgments reflect a general problem in cancer-risk assessment studies, which is a lack in scientific consensus on how to translate carcinogenicity data obtained in experiments with rodents at high levels of exposure to the situation for humans exposed to low levels. Determining the cancer risk in humans at low-dose-dietary intake levels requires extrapolation of the animal carcinogenicity data obtained with respect to species and dose. Uncertainties exist about the shape of the dose–response curve below the range of the animal experimental data, and about possible species differences in metabolism including metabolic activation and detoxification.

The aim of our physiologically based biokinetic (PBBK) studies for estragole was to obtain quantitative insight into dose- and species-dependent differences in the bioactivation and detoxification of estragole.

1.2 Coumarin

Coumarin is a naturally occurring compound that was first isolated from Tonka beans, and is found at high levels in some essential oils, particularly cassia leaf oil, cinnamon leaf oil, cinnamon bark oil and in lavender oil and peppermint oil. Coumarin is also found in fruits (bilberry), green tea and other foods, such as chicory, and in personal care products [11–13]. Chronic exposure to coumarin by the oral route has been reported to result in liver adenomas and carcinomas in rats and liver adenomas in mice [11–15]. Recently, the European Food Safety Authority (EFSA), based on the results of a study on DNA-adduct formation in kidney and liver of rats demonstrating that coumarin does not bind covalently to DNA, concluded that coumarin induces liver tumors by a non-genotoxic mode of action. A tolerable daily intake (TDI) of 0.1 mg coumarin/kg bw.day was established [13]. The theoretical maximum daily intake of coumarin was calculated to be about 4.1 mg/day or 0.07 mg/kg bw.day for a 60-kg person [11, 13].

Figure 2 shows an overview of coumarin metabolism. The major route of coumarin bioactivation is 3,4-epoxidation to coumarin epoxide, which is followed by subsequent rearrangement of the epoxide to o-hydroxyphenylacetaldehyde (oHPA) [16] which is considered to be the hepatotoxic intermediate [11, 17–19]. Coumarin epoxide may also be conjugated to glutathione both chemically and enzymatically, the latter route being especially efficient in rats and mice [18]. oHPA can be detoxified by reduction to o-hydroxyphenylethanol (oHPE), but especially by oxidation to o-hydroxyphenylacetic acid (oHPAA) [11, 18, 20–22].

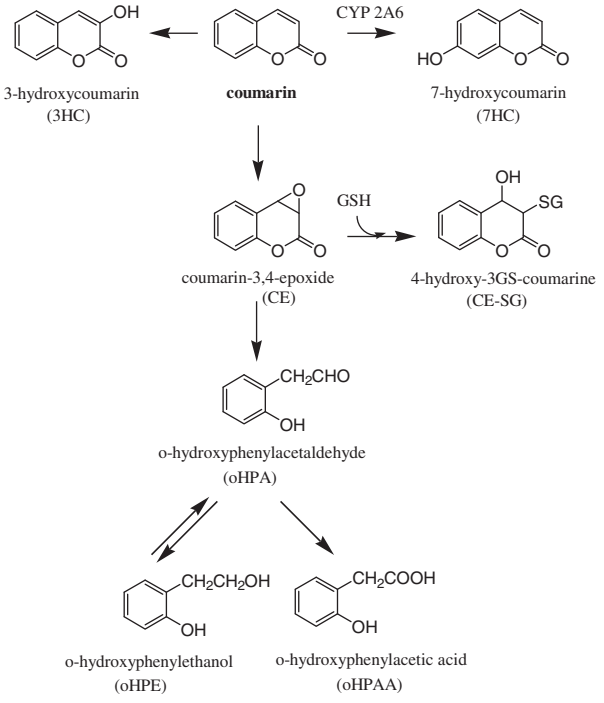

Figure 2. Metabolism of coumarin including the bioactivation pathway proceeding by formation of oHPA. Formation of the other metabolites eventually leads to detoxification and excretion.

Significant species differences between rat and man exist in coumarin bioactivation *via* the 3,4-epoxide pathway. In rats and mice, the 3,4-epoxidation pathway appears to be the major route of coumarin biotransformation, whereas in humans the detoxifying coumarin 7-hydroxylation predominates, a reaction catalyzed by CYP2A6 [11, 16, 19, 20, 23–27]. Furthermore, detoxification of oHPA to oHPAA was shown to be more efficient in humans than in rats [18].

Based on these species differences in biotransformation Felter *et al.* [19] argued that the uncertainty factor for interspecies variation used for definition of the TDI could be reduced from 10 to 2.5 leaving only the factor 2.5 for toxicodynamic differences but taking out the factor 4 for toxicokinetics. However, also of importance is that in man a genetic polymorphism has been identified for CYP2A6, the P450 enzyme catalyzing the detoxifying 7-hydroxylation of coumarin [28–30].

The aim of our PBBK studies for coumarin was to quantify the metabolic pathway(s) replacing the 7-hydroxycoumarin formation in homozygous CYP2A6-deficient subjects and to estimate computationally the expected consequences of the CYP2A6 deficiency for oHPA formation in the liver of human.

1.3 PBBK models

As outlined above, an overall problem in current risk-assessment strategies is the need to extrapolate experimental data obtained in animal experiments at high dose levels to a low-dose human situation. Uncertainties about the shape of the dose–response curve at dose levels relevant for dietary human intake, and about species differences in metabolic activation and detoxification, make it difficult to perform such extrapolations. PBBK modeling can provide a method to obtain a better mechanistic basis for extrapolations of data obtained in experimental animal studies to the human situation [31–33].

A PBBK model is a set of mathematical equations that together describe the absorption, distribution, metabolism and excretion characteristics of a compound within an organism on the basis of three types of parameters [34–37]. These parameters include physiological parameters (*e.g.* cardiac output, tissue volumes, and tissue blood flows), physico-chemical parameters (*e.g.* blood/tissue partition coefficients), and kinetic parameters (*e.g.* kinetic constants for metabolic reactions) [34–37]. Solution of the PBBK equations produces outcomes that are an indication of, for example, the tissue concentration of a compound or its metabolite in any tissue over time at any dose, allowing analysis of effects at both high but also more realistic low dose levels. Furthermore, such PBBK models can be developed for different species, which can facilitate interspecies

extrapolation. In addition, by incorporating equations and kinetic constants for metabolic conversions by individual human samples and/or specific isoenzymes, modeling of interindividual variations and genetic polymorphisms becomes feasible [38].

For the development of a PBBK model for a specific compound, model parameters need to be obtained. The physiological parameters of a species (*e.g.* blood flow rates and tissue volumes) can be obtained from the literature [39]. Tissue–blood partition coefficients might be obtained experimentally *in vitro* using vial equilibration techniques or equilibrium dialysis techniques [40, 41], but can also be obtained using *in silico* methods. Several *in silico* models have been published by which tissue–blood partition coefficients of a compound can be calculated based on their octanol–water partition coefficients [42]. Biochemical parameters for PBBK models, including metabolic parameters, are most often obtained by making preliminary assumptions about metabolic routes and optimizing the kinetic constants by fitting the model to available *in vivo* data [34, 35]. Alternatively, metabolic parameters might also be derived from *in vitro* experiments with tissue fractions, primary cell cultures, or tissue slices of organs involved in the metabolism of the compound [43]. Lipscomb and Poet [43] have pointed out some advantages of using *in vitro* metabolic parameters to define PBBK models, which include the ability to separately define and analyze individual metabolic processes, such as phase I metabolism and phase II metabolism, or bioactivation and detoxification, and to compare contributions from individual conversions with the overall metabolism across species and between individuals, when limited *in vivo* data are available as is often the case for humans [43].

2 Materials and methods

In the present studies, the metabolic parameters for the relevant biotransformation reactions for estragole and coumarin, shown in Figs. 1 and 2, were determined using *in vitro* experiments with tissue fractions [18, 20, 44–46]. PBBK models for estragole and coumarin in rat and human were developed based on these *in vitro* metabolic data. For estragole in rat and human, the models defined consisted of seven compartments including blood, liver, kidney, lung, fat, richly perfused tissue, and slowly perfused tissue [44, 47]. For coumarin in rat and human, the models defined consisted of five compartments including blood, liver, fat, richly perfused tissue, and slowly perfused tissue [45, 47]. A schematic diagram of both PBBK models is shown in Fig. 3. The values for the physiological parameters and partition coefficients that have been used in the models can be found in the literature [44–48]. The physiological parameters were obtained from the literature [39]. The partition coefficients were estimated from the log K_{ow} based on a method of DeJongh *et al.* [49]. Log K_{ow} values were estimated with the software package ClogP version 4.0 (Biobyte, Claremont, CA, USA). 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.

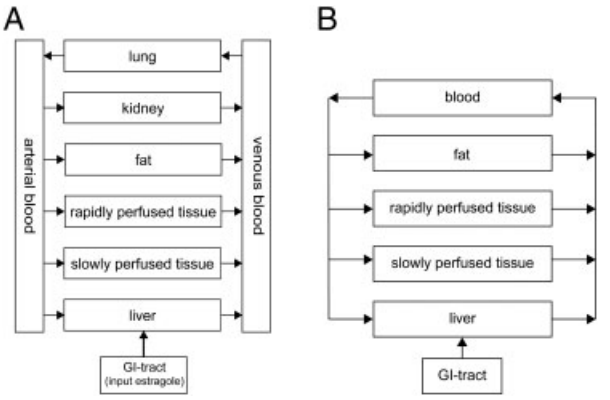

Figure 3. Schematic diagram of the PBBK model for (A) estragole and (B) coumarin [44, 45].

The V_{max} values for the different phase I metabolic pathways in the liver, expressed as nmol/min mg microsomal protein, were scaled to the liver using a microsomal protein yield of 32 mg/g liver [50]. The V_{max} values for the different phase I metabolic pathways in the lung and kidney were scaled accordingly using a microsomal protein yield of 20 mg/g lung, and 7 mg/g kidney [50–52]. The V_{max} values for sulfonation and glucuronidation of 1′-hydroxyestragole, expressed as nmol/min mg S9 protein, were scaled to the liver using a S9 protein yield of 143 mg/g liver [51]. The apparent *in vitro* K_m values were assumed to correspond to the apparent *in vivo* K_m values. The uptake of estragole and coumarin from the gastrointestinal tract was described by a first-order process, assuming direct entry from the intestine to the liver compartment. The absorption rate constant (K_a) was set to 1.0/h, resulting in a rapid absorption of estragole or coumarin from the gastrointestinal tract [7].

3 Results

3.1 Estragole

As an example Fig. 4 shows the estragole concentration-dependent rate of formation of the different estragole phase I metabolites by rat and human liver microsomes. From these curves, V_{max} and K_m values could be derived [44]. V_{max} and K_m values were also determined for glucuronidation and sulfation of 1′-hydroxyestragole [44, 45] using rat as well as human samples [44, 47].

Based on the *in vitro* kinetic data for the different bioactivation and detoxification reactions catalyzed by rat and human tissue fractions PBBK models for estragole metabolism in rat and human were developed [45, 47]. With

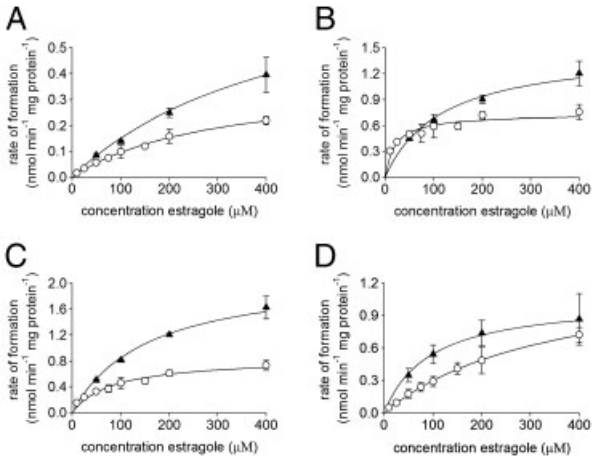

Figure 4. Estragole concentration-dependent rate of formation of 4-allylphenol (A), hydroxyestragole (B), estragole-2′,3′-oxide (C), and 3′-hydroxyestragole (D) by rat (▲) and human (○) liver microsomes. In the plots each point represents the mean (±SD) of three replicates.

these models predictions were made on formation of different metabolites in human liver in time and at different oral doses.

In male rat O-demethylation of estragole appeared to be a major metabolic route at low doses of estragole, occurring mainly in the lung and kidney. Due to saturation of the O-demethylation pathway in lung and kidney, formation of the proximate carcinogenic metabolite 1′-hydroxyestragole, which was shown to occur mainly in the liver of male rat, becomes relatively more important at higher doses of estragole. The PBBK model predicted that formation of this metabolite increased from 16% of the dose at a dose of 0.07 mg/kg bw to 29% of the dose at a dose of 300 mg/kg bw. This relative increase in formation of 1′-hydroxyestragole leads to a relative increase in formation of 1′-hydroxyestragole glucuronide, 1′-oxoestragole, and 1′-sulfooxyestragole, the latter being the ultimate carcinogenic metabolite of estragole. The formation of 1′-sulfooxyestragole predicted by the PBBK model increased from 0.08% of the dose at a dose of 0.07 mg/kg bw to 0.16% of the dose at a dose of 300 mg/kg bw. Overall, these results indicate that the relative importance of different metabolic pathways of estragole may vary in a dose-dependent way, leading to a relative increase in bioactivation of estragole at higher doses.

The findings of the PBBK model for male rat were in good agreement with observations in the literature, revealing dose-dependent effects on the biokinetics for estragole in female Wistar rats *in vivo*. In these *in vivo* studies, the proportion of O-demethylation was observed to decrease with increasing doses (as determined by the percentage of exhalation as $^{14}CO_2$), whereas the proportion of the dose excreted as 1′-hydroxyestragole glucuronide in the urine increased from 1.3 to 5.4% of the dose in the range of 0.05–50 mg/kg bw to 11.4–13.7% in the dose range of 500–1000 mg/kg bw [7]. The PBBK model provided insight in the mechanism underlying this dose-dependent effect observed *in vivo*, which was identified to be a result of saturation of the O-demethylation pathway in the lung and kidney.

Based on the PBBK model for estragole, dose-dependent effects on bioactivation and detoxification of estragole in humans could be studied as well. In humans no relative increase in formation of 1′-sulfooxyestragole was identified to occur with increasing dose levels. The PBBK model even predicted that the relative formation of this metabolite decreased from 0.19% of the dose at a dose of 0.07 mg/kg bw to 0.08% of the dose at a dose of 300 mg/kg bw, due to saturation of the 1′-hydroxylation pathway in the liver. Further analysis revealed that this difference between the rat and the human model, showing respectively an increase *versus* a decrease in the relative formation of 1′-sulfooxyestragole with increasing dose, was due to the fact that in the human model efficient O-demethylation in lung and kidney was absent, whereas in the rat these conversions reduced the relative formation of 1′-sulfooxyestragole at low dose levels. The human PBBK model also revealed that at a dose range within one order of magnitude of the estimated average dietary human intake of 0.07 mg/kg bw, these dose-dependent effects on the relative percentage of the dose converted to 1′-sulfooxyestragole were not significant.

The performance of the PBBK model defined for estragole in human could, to some extent, be evaluated against available *in vivo* data on the disposition of 0.001 mg/kg bw [methoxy-^{14}C]-labeled estragole in two human volunteers described by Sangster *et al.* [8]. The PBBK model predicted formation of 1′-hydroxyestragole glucuronide, corresponding to 2.0% of the dose after 24 h, is comparable to the reported *in vivo* level of this metabolite being ~0.5% of the dose [8]. The predicted formation of 4-allylphenol, corresponding to 2.4% of the dose after 8 h, is fourfold lower than the reported *in vivo* level of ~10% of the dose after 8 h [8]. These results indicate that the PBBK model predicts the formation of these metabolites within the same order of magnitude as the reported levels.

Figure 5 shows an overview of the PBBK-based predictions for the dose-dependent formation of 4-allylphenol, resulting from O-demethylation, 1′-hydroxyestragole, the proximate carcinogenic metabolite, 1′-sulfooxyestragole, the ultimate carcinogenic metabolite, 1′-hydroxyestragole glucuronide, and 1′-oxoestragole in the liver of rat and human at dose levels up to 300 mg/kg bw.

The results obtained clearly reflect significant species-dependent differences in the relative importance of O-demethylation, being more important in male rat than in human (Fig. 5A), as well as in the major pathway for detoxification of 1′-hydroxyestragole, being glucuronidation to 1′-hydroxyestragole glucuronide in male rat (Fig. 5D) but oxidation to 1′-oxoestragole in human (Fig. 5E). These results also indicate that lower levels of urinary excretion of 1′-hydroxyestragole glucuronide in human than in male rat

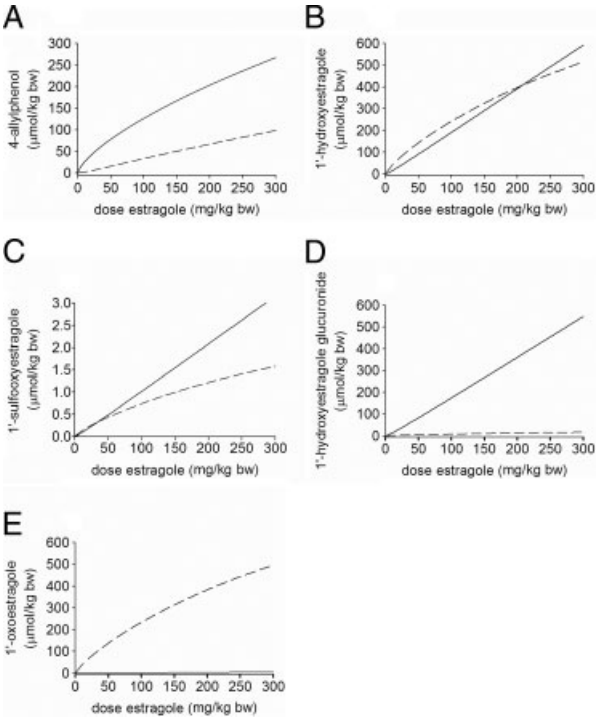

Figure 5. PBBK model-based predictions for the dose-dependent formation of (A) 4-allylphenol, (B) 1′-hydroxyestragole, (C) 1′-sulfooxyestragole, (D) 1′-hydroxyestragole glucuronide, and (E) 1′-oxoestragole in the liver of rat (solid line) and human (dotted line) at dose levels up to 300 mg/kg bw.

do not necessarily reflect lower levels of formation of the proximate and ultimate carcinogenic metabolites 1′-hydroxyestragole and 1′-sulfooxyestragole. The PBBK results obtained indicate that in spite of marked species differences in O-demethylation of estragole and in glucuronidation and oxidation of 1′-hydroxyestragole, the resulting species differences in formation of 1′-hydroxyestragole and 1′-sulfooxyestragole up to dose levels of 50 mg/kg bw are moderate and amount to less than a twofold species-dependent variation in bioactivation.

Formation of 1′-oxoestragole has not been considered to be an important metabolic route of 1′-hydroxyestragole before, mainly because in rat relatively small amounts of derivatives of this metabolite have been detected in the urine after exposure to estragole [53]. Based on the approach of identifying principal metabolic pathways of estragole in incubations with tissue fractions of relevant organs, it could be revealed that in human oxidation of 1′-hydroxyestragole is a major metabolic pathway, which was predicted by the PBBK model to account for 62.7% of the dose.

Altogether it is concluded that the species-dependent variation in bioactivation of estragole to 1′-sulfooxyestragole is smaller than the default factor of 4 generally assumed to reflect interspecies variation in kinetics (assuming that the default factor of 10 can be divided into a factor of 4 for kinetics and 2.5 for dynamics) [54] (http://www.inchem.org/documents/ehc/ehc/ehc210.htm).

3.2 Coumarin

Figure 6 shows the HPLC chromatograms of incubations of coumarin with microsomes from pooled human and rat liver reflecting the species-dependent differences in formation of 7-hydroxycoumarin, the major metabolite formed by human liver microsomes (Fig. 6A) and oHPA, the major metabolite formed by rat liver microsomes (Fig. 6B). In rat microsomal incubations, there was no formation of 7-hydroxycoumarin whereas in human microsomal incubations formation of oHPA was not observed to any significant extent.

The PBBK model defined for coumarin included the following: (i) uptake of coumarin from the intestine by passive diffusion; (ii) transport to the liver, fat, and all other organs lumped together as either rapidly perfused tissue or slowly perfused tissue; (iii) hepatic metabolism of coumarin to 7-hydroxycoumarin, oHPA, 3-hydroxycoumarin and 4-hydroxy-3-glutathionyl-coumarin; and (iv) conversion of oHPA to oHPAA and oHPE.

The PBBK model thus defined provided relative estimates of liver levels of oHPA, not only in man and rat, but also in humans deficient in coumarin 7-hydroxylation, at increasing levels of coumarin exposure (Fig. 7).

For rat liver a dose-dependent increase in the C_{max} for oHPA formation is observed (Fig. 7A). For human liver of wild-type CYP2A6 subjects (Fig. 7B dotted line) a dose-dependent increase in oHPA formation is only observed at dose levels above 15 mg/kg bw when 7-hydroxylation of coumarin becomes saturated and additional amounts of coumarin start to be metabolized through alternative biochemical pathways. For homozygous CYP2A6-deficient subjects, with V_{max} for coumarin 7-hydroxylation set to zero, there is a dose-dependent increase in the C_{max} for oHPA in the liver without an apparent threshold (Fig. 7B solid line). Nevertheless, comparison of Fig. 7B to Fig. 7A reveals that along the whole dose range modeled the predicted oHPA levels in liver of CYP2A6-deficient subjects remain at least tenfold lower than the C_{max} values predicted for oHPA in rat liver at similar dose levels.

4 Discussion

The results presented show that integrating *in vitro* metabolic parameters, using a PBBK model as a framework, provides a good method to evaluate the occurrence of dose-dependent effects and species differences in bioactivation and detoxification of estragole and coumarin. Using this approach, mechanisms underlying dose-dependent effects in bioactivation were revealed. Furthermore, insight was

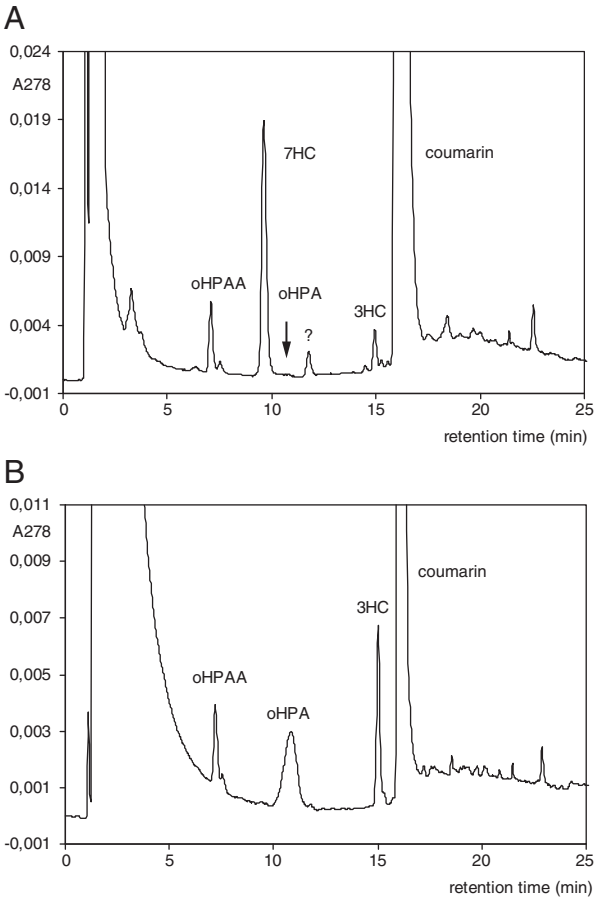

Figure 6. HPLC chromatograms of 30 min incubations of 1000 μM coumarin with (A) pooled human liver microsomes and (B) pooled rat liver microsomes. An unidentified metabolite peak is marked with a question mark [45].

obtained in the occurrence of species differences in metabolism and metabolic activation.

4.1 Implications for risk assessment

4.1.1 Coumarin

For coumarin significant species differences exist in metabolism. In man, the 3,4-epoxidation of coumarin leading to the hepatotoxic oHPA is a minor route, whereas in rats the detoxifying 7-hydroxylation appears to be a minor route. Whether these species differences in toxicokinetics may provide an argument for the reduction of the interspecies safety factor when extrapolating from the animal studies to the human situation, as previously suggested by Felter *et al.* [19], is dependent on how these differences in kinetics together influence the levels of oHPA in the liver of rat and human. To provide some insight into this question, a PBBK model for coumarin for both rat and human was developed, taking into account coumarin 7-hydroxylation, coumarin

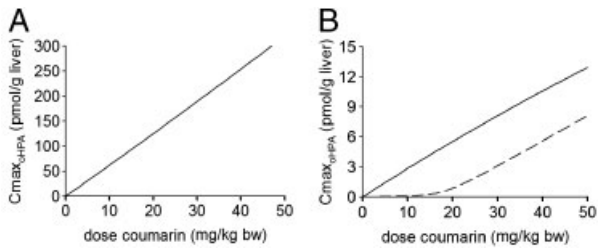

Figure 7. PBBK model predicted dose-dependent concentration of oHPA in the liver of (A) rat and (B) a human wild-type CYP2A6 subject (dotted line) and a human homozygous CYP2A6-deficient subject (V_{max} for coumarin 7-hydroxylation set to zero) (solid line).

3-hydroxylation, formation of the glutathione conjugate of coumarin 3,4-epoxide, formation of oHPA, detoxification of oHPA to oHPAA, and conversion of oHPA to oHPE. The PBBK models presented may not give insight in the absolute formation of oHPA in the liver of rat and human *in vivo* since the models were not validated against *in vivo* data. Nevertheless, the model simulations give insight in the relative differences in oHPA formation in the liver of rat and human, in order to assist extrapolation of rat data to the human situation.

The predicted C_{max} for oHPA in the liver of the average CYP2A6 wild-type human subject was predicted to be about three orders of magnitude lower than the C_{max} predicted for the liver of rats, representing a species sensitive to coumarin liver toxicity [19]. The PBBK models developed also allowed modeling of the kinetics in CYP2A6-deficient human subjects that are homozygous for the CYP2A6*2 allele (V_{max} for coumarin 7-hydroxylation set to zero). The PBBK model thus defined revealed that for these CYP2A6-deficient human subjects the C_{max} and AUC_{0-24h} the area under the concentration time curve for oHPA formation could amount to values that were, respectively, 70- and 500-fold higher than those predicted for the average CYP2A6 wild-type human subject. The increased AUC_{0-24h} is in line with observations reported before for an individual who was homozygous for the CYP2A6*2 allele in which approximately 50% of a 2 mg dose of coumarin was excreted as oHPAA [29], reflecting a significant increase in the percentage of coumarin excreted over time *via* the coumarin 3,4-epoxide pathway upon homozygous CYP2A6 deficiency. Assuming that all oHPAA formed will be excreted in the urine, the PBBK model predicts that upon dosing 2 mg (0.03 mg/kg bw for a 60 kg person), corresponding to the dose applied in the Hadidi *et al.* study [29] and within the range of estimated normal dietary exposure [15], the excretion of oHPAA will be 0.05% of the dose for a wild-type human subject and increases to 28% of the dose for a homozygous CYP2A6-deficient human subject. This increase in the predicted value of the dose excreted as oHPAA to 28% approximately explains the 50% reported by Hadidi *et al.* [29] and reveals that the model for the CYP2A6-deficient human subjects is in good agreement with the *in vivo* data obtained by Hadidi *et al.* [29].

C_{max} values predicted for oHPA formation in the liver of CYP2A6 wild-type human and of homozygous CYP2A6-deficient subjects were, respectively, about 1000- and 10-fold lower than C_{max} values predicted for rat liver. For wild-type human subjects and the subjects with completely deficient coumarin 7-hydroxylation the $AUC_{0-24\,h}$ values for oHPA in the liver were also, respectively, about 1000- and 10-fold lower than that for rat liver. This points at reduced chances on oHPA liver toxicity in humans as compared with rat even for homozygous CYP2A6-deficient subjects. The results obtained also demonstrated that this holds over a dose range from 0.1 mg/kg bw (the TDI) to 50 mg/kg bw (Fig. 7).

It is concluded that even in human subjects with complete deficiency in detoxifying 7-hydroxylation the chances on formation of the hepatotoxic coumarin metabolite oHPA will be lower than those expected in the liver of rats when exposed to a similar dose on a body-weight basis.

Clinical data and case reports have been interpreted to indicate that a subgroup within the human population might be especially sensitive to the hepatotoxic effects of coumarin, occurring a few weeks after treatment [55–57]. Our modeling results corroborate that the CYP2A6 polymorphism is unlikely to be the factor underlying the higher sensitivity of these individuals. This outcome agrees with the lack of a correlation between the hepatotoxic responses and the CYP2A6 genotype status of the patients [58], because the frequency of homozygous individuals with two defective alleles in the general population is estimated to be much lower than the frequency of study patients with elevated liver enzymes. The higher sensitivity in these individuals may be due to other as yet unsolved factors which may include the fact that these studies were generally not performed in healthy subjects, such as patients with chronic lymphedema [55], chronic venous insufficiency [56, 57], individuals with a history of hepatitis [56, 57], and/or upon concomitant exposure to troxerutin [56, 57]. Increased toxicity could also be due to bolus dosing rather than dietary administration. Therefore, it can be concluded that the human studies include several confounding factors and that the reason for the increased susceptibility to liver damage of some individuals within the groups of patients treated with coumarin remains to be established.

This could be a reason for taking not only these patient studies, but also animal studies into account in the safety assessment on coumarin. The PBBK results reveal that in human subjects, even when 7-hydroxylation is deficient, the chances on formation of the toxic oHPA metabolite will be significantly lower than those expected in the liver of rats when exposed to a similar dose on a body-weight base. This conclusion should be taken into account when extrapolating data from experimental studies in sensitive animals, *i.e.* rats, to the general human population, and could be a reason to reduce the uncertainty factor for interspecies variation used for the definition of the TDI from 10 to 2.5 leaving only the factor 2.5 for toxicodynamic differences but taking out the factor 4 for toxicokinetics, as suggested before [59] (http://www.advisorybodies.doh.gov.uk/coc/guideline04.pdf).

4.1.2 Estragole

Worldwide different approaches exist to assess the risk of compounds that are both genotoxic and carcinogenic. Numerical estimates of the risk associated with human exposure might be derived by extrapolation of carcinogenicity data obtained in animals at high dose levels to low dose levels relevant for the human situation. Many mathematical models have been proposed by which such an extrapolation below the available experimental data can be performed, of which linear extrapolation is the simplest form [60]. Extrapolating from animal tumor data at high doses using mathematical modeling in order to obtain estimates of the risk to humans at low-dose exposure levels has been much debated, since it is not known whether or not the model chosen actually reflects the underlying biological processes. In addition, it is argued that species differences are not taken into account and that obtaining numerical estimates may be misused or misinterpreted in further risk management and risk communication, where the uncertainties and inaccuracy connected to the model may not be communicated [59].

Considering these disadvantages the Scientific Committee of the EFSA recommends using a different approach, known as the margin of exposure (MOE) approach [59]. The MOE approach uses a reference point, usually taken from data from an animal experiment that represents a dose causing a low but measurable cancer response. It can be, for example the $BMDL_{10}$, the lower confidence bound of the benchmark dose that gives 10% (extra) cancer incidence (benchmark dose, BMD_{10}). The MOE is defined as the ratio between this reference point, the $BMDL_{10}$, and the estimated dietary intake in humans. When this MOE is higher than 10 000, the compound is considered to be of low priority for risk management actions [59–62]. This safety margin of 10 000 is applied to adequately allow for various uncertainties in the MOE approach, including:

(i) a factor of 100 for species differences and human variability in biokinetics and biodynamics;
(ii) a factor of 10 for interindividual human variability in cell cycle control and DNA repair; and
(iii) a factor of 10 for uncertainties in the shape of the dose–response curve outside the observed dose range.

To date, carcinogenicity data for estragole from which a $BMDL_{10}$, and thus a MOE, can be derived result from a long-term carcinogenicity study conducted in mice [5]. Table 1 summarizes the carcinogenicity data obtained for estragole in female mice in this study. A BMD analysis of these data using BMDS version 1.4.1c software was performed of which the results are summarized in Table 2. Based on the results summarized in Table 2, it is concluded

that the $BMDL_{10}$ value varies between 9 and 33 mg/kg bw.day.

The average *per* capita daily intake of estragole was estimated by the SCF to amount to about 4.3 mg *per* day (corresponding to 0.07 mg/kg bw.day for a 60-kg person) [3]. This estimation is based on a relative conservative method using theoretical maximum use levels of estragole in 28 food categories and consumption data for these food categories based on 7 days dietary records of adult individuals [3]. Using a different method, a lower average *per* capita daily intake of estragole was estimated by the Expert Panel of the FEMA [1]. This estimation was performed using production volume data of herbs, essential oils, and flavour substances containing estragole in the United States [1]. The FEMA estimated the daily *per* capita intake to be less than 10 μg/kg bw day [1].

Using the exposure assessment provided by the SCF [3] of 0.07 mg/kg bw.day and the $BMDL_{10}$ of 9–33 mg/kg bw.day, the MOE value would amount to 129 to 471, which is lower than 10 000, indicating that the consumption of estragole at these intake levels might be a high priority for risk management. Using the exposure assessment provided by Smith *et al.* [1] of 0.01 mg/kg bw.day and the $BMDL_{10}$ of 9–33 mg/kg bw.day, the MOE value would amount to 900–3300. Comparison of this MOE value to the value of 10 000 indicates that at these intake levels the use of estragole containing spices and their essential oils might also be considered a priority for risk management.

In the opinion of the EFSA it has been stated that the default MOE of 10 000 can be reduced or increased when appropriate chemical specific data are available [59]. The results of our PBBK modeling can provide insight in especially the applicability of the default safety factor for species differences in biokinetics used to define the value of 10 000. The outcomes of the PBBK models of this study reveal that species differences in bioactivation of estragole were observed to be about twofold and thus smaller than the default factor of 4 generally assumed to reflect interspecies variation in kinetics. However, a twofold reduction of the default value of 10 000 would not lead to a different conclusion on the priority for risk management.

A similar conclusion emerges from the approach in which linear extrapolation from a defined point of departure is used to derive a so-called virtual safe dose (VSD) at which the additional cancer risk upon life time exposure would be one in a million and considered negligible [63] (http://cfpub.epa.gov/ncea/raf/recordisplay.cfm?deid = 116283). Using the data and BMD analysis of the study of Miller *et al.* [5] (Tables 1 and 2) it can be concluded that in mice, a benchmark response of 10% extra tumor risk is observed at a BMD_{10} value of 13–41 mg/kg bw.day. By linear extrapolation from this point of departure, the VSD that results in an additional cancer risk of one in a million is calculated to amount to 0.13–0.41 μg/kg bw.day. Comparison of this estimated VSD to the estimated dietary human intake of 10–70 μg/kg bw.day [1, 3] indicates that dietary intake levels are about two orders of magnitude above the VSD, indicating a priority for risk management.

The results of the PBBK models developed for estragole for male rat and human indicate that kinetic data do not provide a reason to argue against such a linear extrapolation from the rat tumor data to the human situation. This is shown in Fig. 8, in which the PBBK model predicted dose-dependent formation of 1′-sulfooxyestragole in the liver of rat and human is displayed. Both curves appear to be quite linear from doses as high as the BMD_{10} at which actual increased tumor incidences are observed in rodent

Table 1. Overview of the data from Miller *et al.* [5] on the incidence of hematomas in female mice exposed for 12 months *via* the diet to estragole

Dose	Estimated dose (mg/kg bw. day)	No. of animals	No. of mice with hematomas	Incidence
0	0	43	0	0
0.23% in diet	150–300	48	27	56
0.46% in diet	300–600	49	35	71

Table 2. Results of a BMD analysis of the data from Miller *et al.* [5] on the incidence of hepatomas in female mice exposed for 12 months *via* the diet to estragole (Table 1), using BMDS version 1.4.1c and the default settings of extra risk, a benchmark response of 10% and a 95% confidence limit

Mice gender	Model	No. of parameters	Log likelihood	Accepted	BMD_{10} (mg/kg bw. day)	$BMDL_{10}$ (mg/kg bw. day)
Females	Null	1	−96.1243			
Females	Full	3	−62.2103			
Females	Two-stage	1	−62.7403	Yes	22.4	18.1
Females	γ	1	−62.7403	Yes	22.4	18.1
Females	Log logistic	1	−62.2124	Yes	13.1	9.2
Females	Log probit	1	−62.7928	Yes	40.7	32.7
Females	Weibull	1	−62.7403	Yes	22.4	18.1

To make a worst-case estimate the lowest dose levels of the range were used (*i.e.* 150 and 300 mg/kg bw day, respectively).

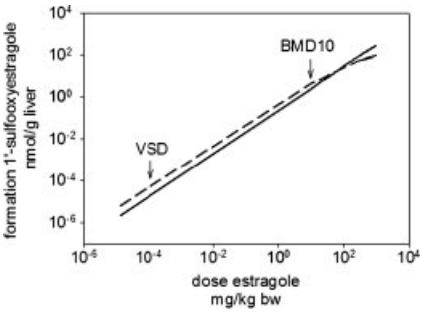

Figure 8. PBBK model predicted dose-dependent formation of 1′-sulfooxyestragole in the liver of rat (solid line) and human (dotted line).

bioassays, down to as low as the VSD, when plotted on a log–log scale as shown in Fig. 8 as well as on a linear scale (figure not shown). Since the BMD_{10} appears to be within the linear part of the curve and since the rat and human curves do not differ substantially, the PBBK results of this study support that possible non-linear kinetics and species differences in kinetics should not be used as arguments against using this linear low-dose extrapolation from high-dose animal data to the low-dose human situation.

All together, the results presented demonstrate that PBBK models provide a useful tool in risk assessment of foodborne chemicals when evaluating human risks.

4.1.3 Additional considerations

Although the risk assessments outlined above for estragole and coumarin take into account the predicted data on dose-dependent effects, species differences and interindividual differences in bioactivation, it should be noted that other factors might affect the risk assessment as well. The carcinogenic effects of estragole and coumarin will for instance also depend on toxicodynamic processes (*i.e.* processes of importance for the ultimate formation and development of tumors). This could be investigated in further detail by extending the PBBK models to so-called physiologically based biodynamic models in which dose levels and 1′-sulfooxyestragole or oHPA formation should be coupled to DNA adduct formation, considered a biomarker of exposure, or to toxicity and, ultimately, cancer incidence.

In addition it should be noted that whereas animal carcinogenicity experiments are conducted with a pure compound, human dietary exposure to estragole or coumarin occurs in a complex food matrix containing other (herbal) ingredients. In a complex food matrix, interactions can occur that can affect the bioavailability of food components [46, 64]. For example, a slow or incomplete release of estragole or coumarin from the matrix could result in a reduced bioavailability as compared with the bioavailability when dosed as a pure compound by oral gavage. In addition to the effect of the food matrix on the bioavailability, interactions with other herbal ingredients might occur at the level of metabolic activation and/or detoxification [46, 64]. It was, for instance, recently observed by Jeurissen *et al.* [65] that a methanolic basil extract is able to efficiently inhibit the sulfotransferase-mediated DNA adduct formation in HepG2 human hepatoma cells exposed to 1′-hydroxyestragole. These results suggest that the bioactivation of estragole and subsequent adverse effects of estragole are probably lower when estragole is consumed in a matrix of other basil ingredients than would be expected on the basis of experiments using estragole as a single compound. Whether this inhibition of DNA adduct formation by matrix ingredients could also occur *in vivo* was, however, not yet established and should be further explored.

In conclusion, the data presented show that PBBK modeling provides a good method to evaluate the occurrence of dose-dependent effects, species differences, and human variability in bioactivation and detoxification. The model predictions obtained can be used to provide a more mechanistic basis for the assessment of the effects in humans at low-dose dietary intake levels based on data obtained in experiments with rodents at high dose levels. However, for a complete assessment of the cancer risk at low-dose human intake scenarios additional information is still needed. For example, more insight will be needed in toxicodynamic processes that can affect the risk assessment, and more insight is needed in the modulating effects of herbal ingredients on the carcinogenic process resulting in a so-called matrix effect.

Part of the work on coumarin was supported by the Dutch Ministry of Economic Affairs (Innovation Vouchers G071064 and G062238), J. S. Polak Koninklijke Specerijenmaalderij b.v., and the Vereniging voor de Bakkerij- en Zoetwarenindustrie (VBZ). Part of the work on estragole was supported by the Nestlé Research Center Lausanne, Switzerland.

The authors have declared no conflicts of interest.

5 References

[1] Smith, R. L., Adams, T. B., Doull, J., Feron, V. J. *et al.*, Safety assessment of allylalkoxybenzene derivatives used as flavouring substances – methyl eugenol and estragole. *Food Chem. Toxicol.* 2002, *40*, 851–870.

[2] Siano, F., Ghizzoni, C., Gionfriddo, F., Colombo, E. *et al.*, Determination of estragole, safrole and eugenol methyl ether in food products. *Food Chem.* 2003, *81*, 469–475.

[3] SCF (Scientific Committee on Food), Opinion of the scientific committee on food on estragole (1-allyl-4-methoxybenzene), 2001.

[4] Drinkwater, N. R., Miller, E. C., Miller, J. A., Pitot, H. C., Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1′-hydroxyestragole in the mouse and muta-

genicity of 1′-acetoxyestragole in bacteria. *J. Natl. Cancer Inst.* 1976, *57*, 1323–1331.

[5] Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L. *et al.*, Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res.* 1983, *43*, 1124–1134.

[6] Wiseman, R. W., Miller, E. C., Miller, J. A., Liem, A., Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F_1mice. *Cancer Res.* 1987, *47*, 2275–2283.

[7] Anthony, A., Caldwell, J., Hutt, A. J., Smith, R. L., Metabolism of estragole in rat and mouse and influence of dose size on excretion of the proximate carcinogen 1′-hydroxyestragole. *Food Chem. Toxicol.* 1987, *25*, 799–806.

[8] Sangster, S. A., Caldwell, J., Hutt, A. J., Anthony, A., Smith, R. L., The metabolic disposition of [methoxy-14C]-labelled trans-anethole, estragole and p-propylanisole in human volunteers. *Xenobiotica* 1987, *17*, 1223–1232.

[9] Hall, R. L., Oser, B. L., Recent progress in the consideration of flavoring ingredients under the food additives amendment III. GRAS substances. *Food Technol.* 1965, *253*, 151–197.

[10] JECFA, *Sixty-ninth Meeting*, Rome, Italy, 17–26 June 2008.

[11] Lake, B. G., Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. *Food Chem. Toxicol.* 1999, *37*, 423–453.

[12] SCF (Scientific Committee on Food), Opinion on coumarin (a constituent of natural flavouring source materials limited by annex II of flavourings directive 88/388/EC) expressed on 16 December 1994. Reports of the Scientific Committee on Food. Thirty-sixth series, 1997, pp. 13–20.

[13] EFSA, Opinion of the Scientific Panel on Food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the commission related to coumarin. Question number EFSA-Q-2003-118. *EFSA J.* 2004, *104*, 1–36.

[14] NTP (National Toxicology Program), Toxicology and carcinogenesis studies of coumarin (CAS No. 91-64-5) in F344/N rats and $B6C3F_1$ mice (gavage studies). Technical Report No. NTP TR 422. NIH Publication No 93-3153, US Department of Health and Human Services, NIH, Research Triangle Park, NC 1993.

[15] Carlton, B. D., Aubrun, J.-C., Simon, G. S., Effects of coumarin following perinatal and chronic exposure in Sprague–Dawley rats and CD-1 mice. *Fundam. Appl. Toxicol.* 1996, *30*, 145–151.

[16] Born, S. L., Rodriguez, P. A., Eddy, C. L., Lehman-McKeeman, L. D., Synthesis and reactivity of coumarin 3,4-epoxide. *Drug Metab. Dispos.* 1997, *25*, 1318–1324.

[17] Lake, B. G., Gray, T. J. B., Evans, J. G., Lewis, D. F. V. *et al.*, Studies on the mechanism of coumarin-induced toxicity in rat hepatocytes: comparison with dihydrocoumarin and other coumarin metabolites. *Toxicol. Appl. Pharmacol.* 1989, *97*, 311–323.

[18] Vassallo, J. D., Hicks, S. M., Daston, G. P., Lehman-McKeeman, L. D., Metabolic detoxification determines species differences in coumarin-induced hepatotoxicity. *Toxicol. Sci.* 2004, *80*, 249–257.

[19] Felter, S. P., Vassallo, J. D., Carlton, B. D., Daston, G. P., A safety assessment of coumarin taking into account species-specificity of toxicokinetics. *Food Chem. Toxicol.* 2006, *44*, 462–475.

[20] Born, S. L., Caudill, D., Smith, B. J., Lehman-McKeeman, L. D., *In vitro* kinetics of coumarin 3,4-epoxidation: application to species differences in toxicity and carcinogenicity. *Toxicol. Sci.* 2000, *58*, 23–31.

[21] Fentem, J. H., Fry, J. R., Whiting, D. A., o-Hydroxyphenylacetaldehyde: a major novel metabolite of coumarin formed by rat, gerbil and human liver microsomes. *Biochem. Bioophys. Res. Commun.* 1991, *179*, 197–203.

[22] Lake, B. G., Osborne, D. J., Walters, D. G., Price, R. J., Identification of o-hydroxyphenylacetaldehyde as a major metabolite of coumarin in rat hepatic microsomes. *Food Chem. Toxicol.* 1992, *30*, 99–104.

[23] Born, S. L., Api, A. M., Ford, R. A., Lefever, F. R., Hawkins, D. R., Comparative metabolism and kinetics of coumarin in mice and rats. *Food Chem. Toxicol.* 2003, *41*, 247–258.

[24] Van Iersel, M., Walters, D. G., Price, R. J., Lovell, D. P., Lake, B. G., Sex and strain differences in mouse hepatic coumarin 7-hydroxylase activity. *Food Chem. Toxicol.* 1994, *32*, 387–390.

[25] Van Iersel, M. L., Henderson, C. J., Walters, D. G., Price, R. J. *et al.*, Metabolism of [3-14C]coumarin by human liver microsomes. *Xenobiotica* 1994, *24*, 795–803.

[26] Draper, A. J., Madan, A., Parkinson, A., Inhibition of coumarin 7-hydroxylase activity in human liver microsomes. *Arch. Biochem. Biophys.* 1997, *341*, 47–61.

[27] Bogan, D. P., Deasy, B., O'Kennedy, R., Smyth, M. R., Interspecies differences in coumarin metabolism in liver microsomes examined by capillary electrophoresis. *Xenobiotica* 1996, *26*, 437–445.

[28] Fernandez-Salguero, P., Hoffman, S. M. G., Cholerton, S., Mohrenweiser, H. *et al.*, A genetic polymorphism in coumarin 7-hydroxylation: sequence of the human CYP2A genes and identification of variant CYP2A6 alleles. *Am. J. Human Genet.* 1995, *57*, 651–660.

[29] Hadidi, H., Zahlsen, K., Idel, J. R., Cholerton, S., A single amino acid substitution (Leu160His) in cytochrome P450 CYP2A6 causes switching from 7-hydroxylation to 3-hydroxylation of coumarin. *Food Chem. Toxicol.* 1997, *35*, 903–907.

[30] Hadidi, H., Irshaid, Y., Vågbø, C. B., Brunsvik, A. *et al.*, Variability of coumarin 7- and 3-hydroxylation in a Jordanian population is suggestive of a functional polymorphism in cytochrome P450 of CYP2A6. *Eur. J. Clin. Pharmacol.* 1998, *54*, 437–441.

[31] Andersen, M. E., Krishnan, K., Physiologically based pharmacokinetics cancer risk assessment. *Environ. Health. Persp.* 1994, *102*, 103–108.

[32] Clewell, H. J., Gentry, P. R., Gearhart, J. M., Allen, B. C., Andersen, M. E., Comparison of cancer risk estimates for vinyl chloride using animal and human data with a PBPK model. *Sci. Total Environ.* 2001, *274*, 37–66.

[33] Clewell, H. J., III, Andersen, M. E., Barton, H. A., A consistent approach for the application of pharmacokinetic modeling in cancer and non-cancer risk assessment. *Environ. Health Perspect.* 2002, *110*, 85–93.

[34] Krewski, D., Withey, J. R., Ku, L. F., Andersen, M. E., Applications of physiologic pharmacokinetic modeling in carcinogenic risk assessment. *Environ. Health. Perspect.* 1994, *102*, 37–50.

[35] Krishnan, K., Andersen, M. E., Physiologically based pharmacokinetic modeling and toxicology. In: Hayes, A. W. (Ed.), *Principles and Methods of Toxicology*, Raven Press, New York 2001 pp. 193–241.

[36] Chiu, W. A., Barton, H. A., DeWoskin, R. S., Schlosser, P. *et al.*, Evaluation of physiologically based pharmacokinetic models for use in risk assessment. *J. Appl. Toxicol.* 2007, *27*, 218–237.

[37] Clewell, R. A., Clewell, H. J., III, Development and specification of physiologically based pharmacokinetic models for use in risk assessment. *Regul. Toxicol. Pharmacol.* 2008, *50*, 129–143.

[38] Bogaards, J. J. P., Hissink, E. M., Briggs, M., Weaver, R. *et al.*, Prediction of interindividual variation in drug plasma levels *in vivo* from individual enzyme kinetic data and physiologically based pharmacokinetic modeling. *Eur. J. Pharm. Sci.* 2000, *12*, 117–124.

[39] Brown, R. P., Delp, M. D., Lindstedt, S. L., Rhomberg, L. R., Beliles, R. P., Physiological parameter values for physiologically based pharmacokinetic models. *Toxicol. Indust. Health* 1997, *13*, 407–484.

[40] Gargas, M. L., Burgess, R. J., Voisard, D. E., Cason, G. H., Andersen, M. E., Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharmacol.* 1989, *98*, 87–99.

[41] Jepson, G. W., Hoover, D. K., Black, R. K., McCafferty, J. D. *et al.*, Partition coefficient determination for non-volatile and intermediate volatility chemicals in biological tissues. *Toxicologist* 1992, *12*, 262.

[42] Payne, M. P., Kenny, L. C., Comparison of models for the estimation of biological partition coefficients. *J. Toxicol. Environ. Health A* 2002, *65*, 897–931.

[43] Lipscomb, J. C., Poet, T. S., *In vitro* measurements of metabolism for application in pharmacokinetic modeling. *Pharmacol. Ther.* 2008, *118*, 82–103.

[44] Punt, A., Delatour, T., Scholz, G., Schilter, B. *et al.*, Tandem mass spectrometry analysis of N2-(trans-isoestragole-3′-yl)-2′ as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1′-hydroxyestragole. *Chem. Res. Toxicol.* 2007, *20*, 991–998.

[45] Punt, A., Freidig, A., Delatour, T., Scholz, G. *et al.*, A physiologically based biokinetic (PBBK) model for estragole bioactivation and detoxification in rat. *Toxicol. Appl. Pharmacol.* 2008, *231*, 248–259.

[46] Rietjens, I. M. C. M., Zaleska, M., Boersma, M. G., Punt, A., Differences in simulated liver concentrations of toxic coumarin metabolites in rats and different human populations evaluated through physiologically based biokinetic (PBBK) modeling. *Toxicol. In Vitro* 2008, *22*, 1890–1901.

[47] Punt, A., Paini, A., Boersma, M. G., Freidig, A. *et al.*, Use of physiologically based biokinetic (PBBK) modeling to study estragole bioactivation and detoxification in human as compared to male rats. *Toxicol Sci.* 2009, *110*, 255–269.

[48] Ramsey, J. C., Andersen, M. E., A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. *Toxicol. Appl. Pharmacol.* 1984, *73*, 159–175.

[49] DeJongh, J., Verhaar, H. J., Hermens, J. L., A quantitative property-property relationship (QPPR) approach to estimate *in vitro* tissue-blood partition coefficients of organic chemicals in rats and humans. *Arch. Toxicol.* 1997, *72*, 17–25.

[50] Barter, Z. E., Bayliss, M. K., Beaune, P. H., Boobis, A. R. *et al.*, Scaling factors for the extrapolation of *in vivo* metabolic drug clearance from *in vitro* data: reaching a consensus on values of human microsomal protein and hepatocellularity per gram of liver. *Curr. Drug Metab.* 2007, *8*, 33–45.

[51] Medinsky, M. A., Leavens, T. L., Csanády, G. A., Gargas, M. L., Bond, J. A., *In vivo* metabolism of butadiene by mice and rats: a comparison of physiological model predictions and experimental data. *Carcinogenesis* 1994, *7*, 1329–1340.

[52] Beierschmitt, W. P., Weiner, M., Age-related changes in renal metabolism of acetaminophen in male Fischer 344 rats. *Age* 1986, *9*, 7–13.

[53] Solheim, E., Scheline, R. R., Metabolism of alkenebenzene derivatives in the rat. I. p-Methoxyallylbenzene (Estragole) and p-methoxypropenylbenzene (Anethole). *Xenobiotica* 1973, *3*, 493–510.

[54] WHO, International Programme on Chemical Safety (IPCS): Assessing human health risks of chemicals: principles for the assessment of risk to human health from exposure to chemicals, Environmental Health Criteria 210, World Health Organisation, Geneva 1999.

[55] Loprinzi, C. L., Kugler, J. W., Sloan, J. A., Rooke, T. W. *et al.*, Lack of effect of coumarin in women with lymphedema after treatment for breast cancer. *New Engl. J. Med.* 1999, *340*, 346–350.

[56] Schmeck-Lindenau, H. J., Naser-Hijazi, B., Becker, E. W., Henneicke-von Zepelin, H. H., Schnitker, J., Safety aspects of a coumarin–troxerutin combination regarding liver function in a double-blind placebo-controlled study. *Int. J. Clin. Pharmacol. Therapeut.* 2003, *41*, 193–199.

[57] Vanscheidt, W., Rabe, E., Naser-Hijazi, B., Ramelet, A. A. *et al.*, The efficacy and safety of a coumarin-/troxerutin-combination (SB-LOT) in patients with chronic venous insufficiency: a double blind placebo-controlled randomised study. *Vasa* 2002, *31*, 185–190.

[58] Burian, M., Freudenstein, J., Tegtmeier, M, Naser-Hijazi, B. *et al.*, Single copy of variant CYP2A6 alleles does not confer susceptibility to liver dysfunction in patients treated with coumarin. *Int. J. Clin. Pharmacol. Therapeut.* 2003, *41*, 141–147.

[59] COC (Committee on Carcinogenicity of Chemicals in Food, consumer products and the environment), Guidance on a strategy for the risk assessment of chemical carcinogens, 2004.

[60] EFSA, Opinion of the scientific committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. *EFSA J.* 2005, *282*, 1–31.

[61] Barlow, S., Renwick, A. G., Kleiner, J., Bridges, J. W. *et al.*, Risk assessment of substances that are both genotoxic and carcinogenic report of an International Conference organized by EFSA and WHO with support of ILSI Europe. *Food Chem. Toxicol.* 2006, *44*, 1636–1650.

[62] Dybing, E., O'Brien, J., Renwick, A. G., Sanner, T., Risk assessment of dietary exposures to compounds that are genotoxic and carcinogenic, an overview. *Toxicol. Lett.* 2008, *180*, 110–117.

[63] EPA, Guidelines for carcinogen risk assessment and supplemental guidance for assessing susceptibility from early-life exposure to carcinogens, Environmental Protection Agency, Washington, DC, USA 2005.

[64] Schilter, B., Andersson, C., Anton, R., Constable, A. *et al.*, Guidance for the safety assessment of botanicals and botanical preparation for use in food and food supplements. *Food Chem. Toxicol.* 2003, *41*, 1625–1649.

[65] Jeurissen, S. M. F., Punt, A., Delatour, Th., Rietjens, I. M. C. M., Basil extract inhibits the sulfotransferase mediated formation of DNA aducts of the procarcinogen 1′-hydroxyestragole by rat and human liver S9 homogenates and in HepG2 human hepatoma cells. *Food Chem. Toxicol.* 2008, *46*, 2296–2302.

