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

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A B S T R A C T

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

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1. Introduction

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

The genotoxic and carcinogenic activity of alkenylbenzenes has been ascribed to their bioactivation by cytochromes P450, leading to the formation of 1-hydroxyxymetabolites, and the subsequent
sulfonation of these 1'-hydroxymetabolites by sulfotransferases (SULTs), generating unstable DNA reactive 1'-sulfoxy metabolites (Fig. 1) (Anthony et al., 1987; Drinkwater et al., 1976; Miller et al., 1983; Sangster et al., 1987; Solheim and Scheline, 1973; Wiseman et al., 1987). Interestingly, a previous study (Jeurissen et al., 2008) showed that a methanolic basil extract caused a reduction in the level of DNA-adduct formation in incubations with DNA and rat or human liver S9 as well as in human hepatoma HepG2 cells exposed to 1'-hydroxyestragole, demonstrating the importance of the botanical matrix. The major compound in the methanolic basil extract responsible for this in vitro inhibition of the SULT-mediated bioactivation of estragole was found to be the flavonoid nevadensin (Alhusainy et al., 2010). Recently, it was demonstrated that nevadensin is also able to significantly inhibit the SULT-mediated bioactivation of estragole in vivo, resulting in reduced levels of estragole DNA-adducts in the livers of rats exposed simultaneously to estragole and nevadensin as compared to the estragole DNA-adduct levels in the livers of rats exposed to estragole alone (Alhusainy et al., 2013).

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

2. Materials and methods

2.1. PFS and chemicals

Five basil-containing PFS were selected for which a risk assessment was previously made based on the MOE approach, indicating that use of all these basil-containing PFS results in MOE values in the range of 1–1000 pointing at a high priority for risk management actions and a potential risk for human health (van den Berg et al., 2011b). Two of the selected PFS consisted of powdered basil material and three of the selected PFS were prepared from the essential oil of basil. Characteristics of the selected PFS are presented in Table 1. Basil-containing PFS were purchased via the internet.

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

2.2. Preparation of methanolic PFS extracts

The selected PFS consisting of powdered basil material were extracted as described previously (Alhusainy et al., 2010, 2012). In short, 5 g of powdered PFS material was extracted with a mixture of methanol, water and acetic acid (ratio 80:19:1) by stirring the powdered PFS material twice for 2 h at room temperature, each time using 100 ml of the extraction mixture. After filtration of the extract solution through a folded filter (Schleicher & Schuell), the filtrates of each PFS were pooled and evaporated to dryness using a rotary evaporator (Heidolph LABOROTA 4000 efficient, Metrohm, USA). The extraction yields were found to range between 21.6% and 23.8%. The dried extracts were subsequently dissolved in methanol to obtain concentrations of 2, 5, and 20 mg/ml. PFS containing basil essential oil were directly dissolved in methanol to obtain a concentration of 20 mg/ml followed by sonication (Bandelin Sonorex RK 100) for 10 min at room temperature. Thereafter, dilution series were prepared in methanol to make aliquots of 2 and 5 mg/ml. All stocks were filtered using a 0.2 μm cellulose acetate filter membrane (VWR international) and stored at −20°C until use in the SULT assay.

2.3. SULT assay

The inhibition of SULT activity by the different methanolic basil-containing PFS extracts was measured as described by Alhusainy et al. (2010). Since the sulfonated 1′-hydroxymethabolite of estragole is unstable in an aqueous environment, hampering its direct detection by means of HPLC analysis, 7-hydroxy coumarin (7HC) was used as a substrate as previously described (Alhusainy et al., 2010, 2012). Incubations were performed in a total volume of 100 μl with 0.1 M Tris–HCl (pH 7.4) containing (final concentrations) 0.4 mg/ml commercially obtained pooled liver S9 from male Sprague–Dawley rats or mixed gender pooled human liver S9, 0.1 mM 3′-phosphoadenosine-5′-phosphosulfate (PAPS) and 10, 25 or 100 μg/ml of a methanolic PFS extract (added from a 200 times concentrated stock in methanol). Prior to the addition of 25 μM 7HC (added from a 200 times concentrated stock solution in DMSO), samples were pre-incubated for 1 min at 37°C. Reaction mixtures were incubated for 10 min at 37°C and terminated by adding 25 μl ice-cold acetonitrile. Incubations performed in the absence of PFS extract or in the presence of 25 μM of the known SULT inhibitor pentachlorophenol (PCP) (added from a 200 times concentrated stock solution in DMSO) were used as controls. In addition, blank incubations were performed in the absence of the co-factor PAPS or S9 liver homogenates. In all incubations, the total amount of methanol and/or DMSO was kept below 1% (v/v). All samples were centrifuged at 5°C for 5 min at 16,000 g and the supernatant was stored at −20°C until HPLC analysis. Experiments were performed in triplicate.

2.4. HPLC analysis and quantification of 7HCS

For quantification of 7HCS, 50 μl of each sample obtained in the SULT assay was subjected to HPLC analysis as described previously (Alhusainy et al., 2010, 2012). Chromatographic analysis was performed using a Waters 2695 separation module liquid chromatography system, connected to a 2998 photodiode array detector and an Altima C18 5 μm column, 150 mm × 4.6 mm (Grace Alltech, Breda, The Netherlands). The gradient was made with ultrapure water containing 0.1% (v/v) acetic acid and acetonitrile. The flow rate was 1 ml/min and a gradient was applied from 0% acetonitrile to 20% acetonitrile in 2 min, increased to 21% acetonitrile over 10 min and further increased to 100% acetonitrile in 2 min at which it was kept for 1 min. Starting conditions were reached in the next 2 min and retained for another 10 min. Under the conditions applied, 7HCS eluted at 6.2 min.

2.5. Fractionation of methanolic basil-containing PFS extracts

All methanolic basil-containing PFS extracts were subjected to fractionation based on the method described previously (Alhusainy et al., 2010). In short, 50 μl of a concentrated PFS extract (i.e. 150 mg/ml) dissolved in methanol were injected into a Waters 600 controller liquid chromatography system connected to a Waters 996 photodiode array detector. Chromatographic separation was performed using

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

ND: not detected, NC: not calculated.
an Alltima C18 5 µm column 150 mm × 4.6 mm (Grace Alltech, Breda, The Netherlands). The gradient was made with ultrapure water containing 0.1% (v/v) acetic acid and methanol. The flow rate was 1 ml/min. The mobile phase started with 0% methanol and was increased to 100% methanol within 60 min at which it was kept for 10 min. Starting conditions were reached in 10 min and retained in the next 10 min. In total, 60 fractions with a 1 min interval were collected into Eppendorf tubes. Subsequently, the methanolic solvent present in the collected fractions was evaporated under a stream of gaseous nitrogen and the residual water was removed by freeze drying using a Christ Alpha RVC freeze dryer at 0.8–1.2 mbar. For each basil-containing PFS, fractionation was repeated five times and the dried fractions obtained of each replicate were pooled by reconstituting them in 50 µl methanol. All fractions were tested in the SULT activity assay for their SULT-inhibitory potential.

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

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

For quantification of nevadensin, the major SULT-inhibiting constituent in the methanolic basil-containing PFS extracts prepared in this study (see results section), 3.5 µl of each methanolic extract was subjected to UPLC analysis (n = 3). UPLC analysis was performed on a Waters ACQUITY UPLC H-Class system connected to an ACQUITY UPLC photodiode array detector and a quaternary solvent manager. Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 1.7 µm column, 2.1 × 50 mm. The column was kept at 30 °C and the sample manager was set at 10 °C. The gradient was made with nanopure water containing 0.1% (v/v) TFA and acetonitrile. The flow rate was 0.6 ml/min and a gradient was applied from 0% acetonitrile to 20% acetonitrile in 0.2 min, increased to 75% acetonitrile over 1.8 min and further increased to 100% acetonitrile in 0.2 min at which it was kept for 0.6 min. Starting conditions were reached in the next 0.2 min and retained for another 1.2 min. The levels of nevadensin were quantified by comparing the peak areas to those in the calibration curve of nevadensin defined using a commercially available reference compound.

2.7. PBK modeling, BMDL90 analysis and updated risk assessment using the MOE approach

An updated risk assessment of basil-containing PFS was made including the matrix-derived combination effect of nevadensin on the bioactivation of estragole. The reduction in the formation of estragole DNA-adducts (N²-(trans-isoestragol-3-yl)- 2’-deoxyguanosine (E-3’-N²-dGuo)) in the liver of rat following the combined exposure to estragole and nevadensin was predicted based on the PBK models previously developed for the bioactivation and detoxification of estragole (Punt et al., 2009) including a sub-model describing the absorption, distribution, metabolism and excretion of nevadensin (Alhusainy et al., 2013). The binary estragole–nevadensin PBK model used in this study was previously validated based on an in vivo study in which rats were exposed to estragole and nevadensin showing that the PBK model based predictions adequately match the measured DNA-adduct levels in the liver of rats (Alhusainy et al., 2013). In the present study, the estragole–nevadensin model (Alhusainy et al., 2013) was run for a period of 24 h using the estragole dose levels administered to mice (Miller et al., 1983) and a relative ratio of estragole and the SULT inhibitor nevadensin reflecting their occurrence in the specific PFS.

Based on the outcomes of the PBK model, the incidence of hepatomas as reported by Miller et al. (1983) was refined assuming a linear relationship between the reduced formation of E-3’-N²-dGuo DNA-adducts and the reduction in the hepatoma incidence. In a next step, refined BMDL90 (i.e. the lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence) values were calculated by performing a BMD (Benchmark Dose) analysis of the newly refined hepatoma incidences using EPA BMD software (version 2.1.2) as previously described (van den Berg et al., 2011b). MOE values were calculated by dividing the refined BMDL90 values by the estimated daily intake of estragole resulting from the use of each specific basil-containing PFS of interest (Table 1) (van den Berg et al., 2011b).

3. Results

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

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

3.2. Fractionation of methanolic extracts of basil-containing PFS

The methanolic extracts of basil-containing PFS were subjected to fractionation using HPLC. Fig. 3 displays the inhibition of the SULT-catalysed conversion of 7HC to 7HCS by all fractions obtained upon fractionation of methanolic extracts of the powdered basil-containing PFS (i.e. PFS1 and PFS2) (Fig. 3A and B) or from PFS containing basil-derived essential oil (i.e. PFS3, PFS4 and PFS5) (Fig. 3C–E). A potent SULT-inhibiting effect was shown for several fractions of the methanolic extracts prepared from PFS consisting of powdered basil material (Fig. 3A and B). Fraction 51 was found to exert the highest level of SULT-inhibiting activity for PFS1 and PFS2 (Fig. 3A and B). In fact, the formation of 7HCS was completely inhibited by the extracts prepared from PFS indicating that under these conditions 7HCS was not formed.

Fig. 2. Inhibition of the SULT-mediated conversion of 7HC to 7HCS by methanolic extracts of several basil-containing PFS. Incubations were performed using pooled rat (black bars) or human liver (grey bars) S9 homogenates, 7HC as a substrate and PAPS as a cofactor in the presence or absence of final concentrations of 10, 25 or 100 µg/ml of methanolic basil-containing PFS extracts or 25 µM PCP (positive control). Data are presented as a percentage of the amount of 7HCS formed in incubations without a SULT inhibitor (p < 0.05).
Fraction 50 of PFS1 also resulted in complete inhibition of SULT activity, whereas for PFS2 this fraction resulted in an almost complete SULT-inhibition of 92.9%. In addition, for both methanolic PFS extracts from powdered basil material, fraction 45 and 46 were found to contain potent SULT inhibitors resulting in a level of SULT-inhibition of 92.9–96.1%. Chromatographic analysis of fraction 50 and 51 demonstrated the presence of only one major compound in both fractions which was identified as nevadensin based on comparison of the retention time and UV spectra of these fractions to those of a
commercially available standard of nevadensin. Apigenin was identified in fraction 46 of both PFS consisting of powdered basil material (i.e. PFS1 and PFS2), although chromatographic analysis of this fraction also revealed the presence of other minor constituents.

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

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

PBK modeling was performed to predict the in vivo effect of the combined exposure to estragole and nevadensin on E-3′-N2-dGuo formation in rat liver. The binary estragole–nevadensin PBK model used for these analyses was previously validated based on an in vivo study in which DNA-adducts were quantified in the liver of rats simultaneously exposed to estragole and nevadensin (Alhusainy et al., 2013).

DNA-adduct formation was modeled using the binary estragole–nevadensin PBK model (Alhusainy et al., 2013) at estragole dose levels used in the estragole rodent bioassay (Miller et al., 1983) on which our previous risk assessment of the basil-containing PFS was based (van den Berg et al., 2011b). Table 2 shows that the level of estragole DNA-adduct formation in rat liver at dose levels of 54 and 107 mg/kg bw/day estragole are predicted by the PBK model to equal 1983 adducts in 10⁸ nt and 4114 adducts in 10⁸ nt in the absence of nevadensin. In the presence of nevadensin, at a ratio between estragole and nevadensin reflecting their levels in PFS1, a reduction in the formation of estragole DNA-adducts by 65% (i.e. 688 adducts in 10⁸ nt) and 66% (i.e. 1385 adducts in 10⁸ nt) was predicted for estragole dose levels of 54 and 107 mg/kg bw/day respectively. Table 2 also shows the predicted PBK model outcomes for the formation of estragole DNA-adducts following an exposure to 54 and 107 mg/kg bw/day estragole in the presence of nevadensin at levels reflecting the ratio between estragole and nevadensin in PFS2. Results reveal that, at 54 and 107 mg/kg bw/day estragole, the reduction of E-3′-N2-dGuo DNA-adduct formation is predicted by the PBK model to equal 89% and 88% following the co-administration of estragole and nevadensin in a ratio relevant for their occurrence in PFS2. Extracts of PFS consisting of basil essential oils were able to inhibit SULT activity only to a moderate extent and the effect of this SULT-inhibition was not further investigated. It should be noted that the rodent bioassay by Miller et al. (1983) was performed using dose levels of estragole that considerably exceed the levels of exposure to estragole resulting from the use of basil-containing PFS. The levels of DNA-adduct formation at levels of 4.6 and 13.9 μg/kg bw/day estragole, the estimated daily intake of estragole resulting from the use of PFS1 at the recommended daily intake of 1 or 3 capsules respectively, were predicted by the PBK model to equal 0.1 adducts per 10⁸ nt or 0.3 adducts per 10⁸ nt. Co-administration of estragole and nevadensin, at a dose reflecting their actual levels in PFS1, was predicted to result in a decrease of estragole DNA-adduct formation by 0.02% and 0.07% following exposure to 4.6 or 13.9 μg/kg bw/day estragole respectively. For PFS2, DNA-adduct formation was predicted by the PBK model to amount to 0.1 adducts in 10⁸ nt or 0.2 adducts in 10⁸ nt following exposure to 5.4 or 8.1 μg/kg bw/day estragole respectively, the estimated daily intake of estragole resulting from the use of PFS2 at the recommended daily intake of 4 and 6 capsules respectively. The formation of estragole DNA-adducts was predicted to decrease by 0.22% and 0.33% at estragole intake levels of 5.4 and 8.1 μg/kg bw/day in the presence of nevadensin at a dose reflecting their actual levels in PFS2 amounting to 6.4 or 9.6 μg/kg bw/day nevadensin respectively. These results demonstrate that the consequences of the in vivo matrix-derived combination effect are especially observed in rodent bioassays at estragole dose levels that cause significant cancer incidences but are predicted to be limited at exposure levels of estragole and nevadensin relevant for the daily use of PFS consisting of powdered basil material. For the extracts consisting of basil essential oil only the PBK predicted levels of DNA-adducts formed upon intake of estragole at dose levels resulting from intake of PSF3, PSF4 and PSF5 at the recommended daily dose levels were calculated and were found to amount to 128, 3–13 and 53–87 in 10⁸ nt respectively.

3.4. BMDL10 analysis and updated risk assessment using the MOE approach

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

Based on the refined hepatoma incidences obtained, a BMD analysis was performed resulting in BMDL10 values ranging between 21–25 mg/kg bw/day and 63–87 mg/kg bw/day when using the estragole to nevadensin ratios of PFS1 and PFS2 respectively (Table 4) (for details on BMD analysis see Table S1 in the Supplementary Materials). Subsequently, MOE values were calculated by comparing the newly defined BMDL10 values with the estimated

Table 2

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

<table>
<thead>
<tr>
<th>Dose estragole (mg/kg bw/day)</th>
<th>Dose nevadensin (mg/kg bw/day)</th>
<th>Adducts in 10⁸ nt in the absence of nevadensin as predicted by PBK modeling</th>
<th>Adducts in 10⁸ nt in the presence of nevadensin as predicted by PBK modeling</th>
<th>% reduction DNA-adduct formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basil-containing PFS1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>54</td>
<td>8</td>
<td>1983</td>
<td>688</td>
<td>65</td>
</tr>
<tr>
<td>107</td>
<td>15</td>
<td>4114</td>
<td>1385</td>
<td>66</td>
</tr>
<tr>
<td>Basil-containing PFS2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>54</td>
<td>64</td>
<td>1983</td>
<td>214</td>
<td>89</td>
</tr>
<tr>
<td>107</td>
<td>127</td>
<td>4114</td>
<td>496</td>
<td>88</td>
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</table>
daily exposure levels to estragole resulting from the use of the specific basil-containing PFS (i.e. 0.005–0.014 mg estragole/kg bw/day and 0.005–0.008 mg estragole/kg bw/day for PFS1 and PFS2 respectively). Table 4 shows the refined MOE values that were obtained in this way. For PFS1 the refined MOE values vary between 2000 and 5000 and for PFS2 MOE values were found to amount to 8000–20,000 (Table 4). These refined MOE values are considerably higher than the MOE values of 200–1000 and 400–1000 that were previously derived using the non-refined BMDL10 values from tumour data obtained in a rodent study using purified estragole without the natural (botanical) matrix being present (Miller et al., 1983; van den Berg et al., 2011b).

4. Discussion

In the presented paper, the consequences of combined exposure to estragole and other compounds present in basil-containing PFS on the SULT-mediated bioactivation of estragole were studied. The results demonstrated that methanolic extracts of PFS consisting of powdered basil material contain potent SULT inhibitors. In line with these findings, a significant inhibition of SULT activity was previously also shown for a methanolic extract prepared from powdered basil material containing potent SULT inhibitors. Based on these results, the effect of apigenin on the SULT-mediated bioactivation of estragole was likely to be lower when estragole is consumed in a matrix of basil-containing PFS compared to studies administering.

### Table 3

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

<table>
<thead>
<tr>
<th>Dose estragole (mg/kg bw/ day)</th>
<th>Dose nevadensin (mg/kg bw/ day)</th>
<th>Incidence hepatomas in the presence of estragole only (Miller et al., 1983)</th>
<th>% reduction DNA-adduct formation</th>
<th>Calculated incidence hepatomas after co-administration of estragole and nevadensin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basil-containing PFS1</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0/50</td>
<td>0</td>
<td>0/50</td>
</tr>
<tr>
<td>0.05</td>
<td>8</td>
<td>27/48</td>
<td>65</td>
<td>9/48</td>
</tr>
<tr>
<td>0.147</td>
<td>4.78</td>
<td>35/49</td>
<td>66</td>
<td>12/49</td>
</tr>
<tr>
<td>Basil-containing PFS2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0/50</td>
<td>0</td>
<td>0/50</td>
</tr>
<tr>
<td>0.05</td>
<td>64</td>
<td>27/48</td>
<td>89</td>
<td>3/48</td>
</tr>
<tr>
<td>0.147</td>
<td>127</td>
<td>35/49</td>
<td>88</td>
<td>4/49</td>
</tr>
</tbody>
</table>

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

### Table 4

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

<table>
<thead>
<tr>
<th>PFS</th>
<th>Daily Intake Estragole (mg/kg bw/day)</th>
<th>MOE using data from Miller et al. (1983) (van den Berg et al., 2011b)</th>
<th>Refined BMDL10 taking into account the matrix-derived combination effect (mg/kg bw/day)</th>
<th>Refined MOE taking into account the matrix-derived combination effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFS1</td>
<td>0.005–0.014</td>
<td>200–1000</td>
<td>21–25</td>
<td>2000–5000</td>
</tr>
<tr>
<td>PFS2</td>
<td>0.005–0.008</td>
<td>400–1000</td>
<td>63–87</td>
<td>8000–20,000</td>
</tr>
<tr>
<td></td>
<td>4.78</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.147–0.589</td>
<td>6–40</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2.275–3.414</td>
<td>1–3</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Details on BMD analysis can be found in Table S1 in the Supplementary Materials.

* Using the dose of PFS intake as recommended by the manufacturer of the basil-containing PFS and the chemically determined levels of estragole in the PFS, daily intake estimates of estragole were obtained assuming a body weight of 60 kg.

* Refined MOE = refined BMDL10 (mg/kg bw/day)/daily intake estragole (mg/kg bw/day).

* ND: Not Determined.

Miller et al. (1994) reported that basil essential oil, containing 88% estragole, was found to induce unscheduled DNA Synthesis (UDS) in the same concentration range as pure estragole showing that there was no protective effect of the remaining compounds present in the essential oil. The differences between the observed protective effects of PFS consisting of powdered basil material and PFS consisting of basil essential oils can be explained by the fact that PFS consisting of powdered basil material contain other compounds in addition to estragole that exert a substantial SULT-inhibiting activity whereas the presence of such modulating compounds in PFS consisting of basil essential oil is apparently limited. Thus, assessing the risk to human health resulting from the use of basil-containing PFS, one should take into account the specific processing and manufacturing conditions used since differences in these production processes can induce marked changes in composition, especially when extracting essential oils containing concentrated levels of individual (toxic) ingredients such as estragole.

In the present study, the flavonoids nevadensin and apigenin were identified in the methanolic extracts of PFS consisting of powdered basil material as the compounds responsible for inhibiting SULT activity. In line with these findings, both flavonoids were previously identified as the major SULT-inhibiting constituents in methanolic extracts of intact basil sold in the supermarket for use as a herb (Alhusainy et al., 2010, 2012). Other flavonoids that are reported to be present in some basil varieties have also been demonstrated to exert SULT-inhibiting activities (Alhusainy et al., 2012). These flavonoids include luteolin and catechin. However, it is important to realise that according to literature, nevadensin is one of the major flavonoids of basil and exists in almost all varieties of the genus Ocimum basilicum L. whereas apigenin, luteolin and catechin are present in relatively low quantities or are only present in some basil varieties (Grayer et al., 1996, 2004; Shan et al., 2005). For example, the level of apigenin in different varieties of the genus O. basilicum L. was estimated to be up to 71-fold lower in ‘lo and bud samples (Grayer et al., 2004). In addition, the K value for SULT-inhibition by apigenin was shown to be 175-fold higher than the K value for SULT-inhibition by nevadensin (i.e. 4 nm) (Alhusainy et al., 2010, 2012) corroborating nevadensin to be a more important SULT inhibitor. Based on these results, the effect of apigenin on SULT-mediated bioactivation of estragole can be expected to be significantly lower than that of nevadensin. Furthermore, nevadensin was not only demonstrated to be a potent inhibitor of SULT activity in vitro, but it was recently also shown to significantly inhibit the SULT-mediated bioactivation and DNA-adduct formation of estragole in vivo (Alhusainy et al., 2013).

Since nevadensin can markedly reduce estragole DNA-adduct formation in vivo (Alhusainy et al., 2013), the adverse effects of estragole are likely to be lower when estragole is consumed in a matrix of basil-containing PFS compared to studies administering.
pure estragole. Thus, performing a risk assessment for basil-containing PFS as described previously (van den Berg et al., 2011b), by using the BMDL10 data obtained from a rodent study with high dose levels of the pure estragole (Miller et al., 1983), may result in an overestimation of the priority for risk management and the potential risk for human health. Therefore, an updated risk assessment of basil-containing PFS using the MOE approach was made in the present study. To this end, studies in rodents should ideally be performed with the botanical or the botanical preparation of interest, allowing the calculation of specific BMDL10 values. However, obviously, performing rodent bioassays for all basil-containing PFS is practically unfeasible as the generation of such data is expensive, labour-intensive and above all requires extensive animal testing. Therefore, the present study focused on the chemical identification of the most potent SULT inhibitor in basil-containing PFS and the use of the previously developed and validated estragole–nevadensin PBMOL model (Alhusainy et al., 2013) to predict the possible in vivo reduction in estragole DNA-adduct formation, taking for each PFS the relative ratio between its levels of estragole and nevadensin into account. To obtain this refined estimate, the possible reduction in the values for the incidence of hepatocellular carcinomas reported by Miller et al. (1983) was predicted using the PBMOL model, subsequently allowing the calculation of refined BMDL10 data. Based on this approach, MOE values could be calculated for the individual PFS. These MOE values were found to be considerably higher than the MOE values that were previously calculated not taking the matrix-derived combination effect into account by using the non-refined BMDL10 values (van den Berg et al., 2011b). The results now presented indicate a lower priority for risk management actions for the powdered basil-containing PFS when considering the consequences of the botanical matrix on the bioactivation of estragole. These results indicate that for these PFS the use of BMDL10 data derived from long-term carcinogenicity studies using pure estragole results in an overestimation of the potential risk for human health. In contrast, it was concluded that the matrix-derived combination effect of PFS consisting of basil essential oil is only of minor importance suggesting that the use of BMDL10 data from studies using pure estragole accurately reflects the potential risk for human health resulting from the use of these PFS.

It should be noted that the estragole doses tested by Miller et al. (1983) are not representative for the human situation, since these are dose levels high enough to cause detectable tumor incidences in groups of 50 experimental animals. Risk assessment by the MOE approach is done using rodent tumor data and is thus based on dose levels actually inducing a significant tumor incidence and not necessarily on realistic human intake levels. At these high dose levels used in the Miller et al. study the concomitant dose of nevadensin is also high and apparently high enough to reach nevadensin levels in the liver that are in the range of the Kt value of 4 nM for SULT-inhibition (Alhusainy et al., 2010). The presented study however also gives an estimate of the effects at estragole and nevadensin dose levels representing a realistic human intake. The matrix-derived combination effect of nevadensin on the bioactivation of estragole was predicted to be limited at dose levels of estragole and nevadensin relevant for the use of PFS consisting of powdered basil-material. At these dose levels the corresponding dose of nevadensin is not high enough to reach the Kt of nevadensin in vivo. This illustrates that testing compounds in the presence of their natural matrix in rodent bioassays may not necessarily provide better BMDL10 values for risk assessment, and that the importance of a matrix-derived combination effect for risk assessment of individual compounds should be done on a case-by-case basis also taking into account analysis of dose-dependent effects on the interactions detected. On the basis of the results now available and our previous results showing MOE values ranging between 200–1000 and 1–40 for PFS consisting of powdered basil material or its essential oil, respectively (van den Berg et al., 2011b), it is still concluded that the use of some basil-containing PFS is of priority for risk management actions and a potential risk for human health.

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

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.08.019.

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naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res. 43, 1124–1134.


