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Study of the metabolism of estragole in humans consuming fennel tea

Annette Zeller [1], Kathie Horst [2], and Michael Rychlik[1, 2, *]

[1] Lehrstuhl für Lebensmittelchemie der Technischen Universität München, Lichtenbergstr. 4, D-85748 Garching, Germany

[2] BIOANALYTIK Weihenstephan, Z I E L Research Center for Nutrition and Food Sciences, Alte Akademie 10, D-85350 Freising, Germany

* Current Adress: Chair of Analytical Food Chemistry, Technical University of Munich, Alte Akademie 10, D-85354 Freising, Germany

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Phone +49-816171-3153 Fax +49-8161714216

E-mail michael.rychlik@wzw.tum.de

* to whom correspondence should be addressed

ABSTRACT

The metabolism of the potent carcinogen estragole was investigated in humans after consumption of fennel tea by analyses of its metabolites in blood plasma and urine. Stable isotope dilution assays based on LC-MS/MS detection revealed that 1'-hydroxylation of estragole happened very fast as the concentration of conjugated 1'-hydroxyestragole in urine peaked after 1.5 hours, whereas it was no longer detectable after 10 hours. Besides the formation of less than 0.41 % conjugated 1'-hydroxyestragole of the estragole dose administered, the further metabolite p-allylphenol was generated from estragole in a higher percentage (17 %). Both metabolites were also detected in blood plasma in less than 0.75 to 2.5 hours after consumption of fennel tea. In contrast to this, no estragole was present in these samples above its detection limit. From the results it can be concluded that an excess of the major fennel odorant *trans*-anethole principally does not interfere with estragole metabolism, whereas influences on the quantitative composition of metabolites cannot be excluded. The presence of a sulfuric acid conjugate of estragole could not be confirmed, possibly due to its high reactivity and lability.

INTRODUCTION

Estragole is a component of several herbs such as tarragon, basil, fennel, and anise. Of these, the fruits of fennel and anise serve as remedy against catarrh of the respiratory tract and gastro-intestinal disorders. Therefore, fennel extractions are the classical tea for nursing babies to prevent flatulence and spasms.

However, estragole as a ring-substituted allylbenzene along with the structurally similar safrole has been reported as a potent carcinogen in rodents (*1,2*). The reason for their hepatoxic properties is a specific metabolism of allylbenzenes leading to 1'-hydroxylation of the side chain. In consequence, conjugation of 1'-hydroxyestragole (OHE) with sulphuric acid is assumed to result in a higher carcinogenic activity as the sulfate might decompose readily to an electrophilic cation reacting easily with the DNA (3). In addition, the generated 1'-hydroxyestragole has been reported to have a higher carcinogenic potential than its precursor (*2*). However, due to its lability, the identity of the sulfate yet has not been unequivocally proved. Up to date, indications for its formation come from scavenging reactions indicating the formation of the sulfate (*4*) and from the decrease in incidence of hepatocellular carcinoma by application of the sulfotransferase inhibitor pentachlorphenol to rats (5). Further evidence for adverse effects comes from the detection of adducts of 1'-hydroxyestragole with the DNA by HPLC analysis (*6, 7*). Up to date, the metabolites p-allylphenol and 1'-hydroxyestragole (OHE) have only been detected as glucuronic acid conjugate and the intermediate sulphate ester of OHE has been postulated. In a recent study using biokinetic modelling and incubation of human liver microsomes with OHE, the oxidation product of OHE, namely 1'-oxoestragole, has been postulated as further important estragole metabolite (8). For estragole, 1'-hydroxylation competes with the demethylation of the methoxy group leading to p-allylphenol and is dose dependent (*9*). Recently, the dose dependent metabolism of

estragole also has been confirmed by biokinetic modelling using kinetic data from rats (10). As these studies have been performed only with estragole as pure compound, the question arises whether other compounds in natural products, such as the high abundant *trans*-anethole interfere with estragole in doses usually administered with tea.

In contrast to extensive rodent studies, the metabolism of estragole in humans has only been investigated by administration of ^{14}C-labelled estragole and detection of its metabolites in the urine by radioisotope dilution or elimination of $^{14}CO_2$ in the expired air (*11*). Because of the hazardous oral intake of radioactive substances, the goal of the present study was to develop stable isotope dilution assays for the detection of metabolites in body fluids. Still, a further aim of the present study was to investigate the metabolism of estragole in the human body after administration of plant products containing estragole along with other, higher abundant odorants. Among all dietary sources, common fennel has been estimated to contribute to almost 27 % of the overall intake of estragole from food (12), and, therefore, is an important and relevant food model for evaluating the metabolism of this odorant.

Therefore, the objective of the present investigation was to quantify the metabolites of estragole in humans after the consumption of fennel tea in order to obtain further data for a risk assessment of this odorant.

MATERIALS AND METHODS

Chemicals

Estragole and *trans*-anethole were purchased from Aldrich (Steinheim, Germany). [1'',1'',1''-$^{2}H_3$]-Estragole (*13*), p-allylphenol (*13*) and p-methoxycinnamyl alcohol (*14*) were synthesized as described in the literature cited. 1'-Hydroxyestragole and [1'',1'',1''-$^{2}H_3$]-1'-hydroxyestragole were synthesized by the following procedures.

Synthesis of 1'-hydroxyestragole (Figure 1; 3a): The Grignard reaction of anisaldehyde (2a) with vinyl magnesium chloride according to Laabs (*15*) gave 1'-hydroxyestragole (3a) in a satisfactory yield. In detail, vinyl magnesium chloride (4 ml, 1.7 mol/L in THF, 8.8 mmol, Fluka, Neu-Ulm, Germany) was added to a solution of anisaldehyde (500 mg, 3.2 mmol, Aldrich, Steinheim, Germany) in dry diethylether (50 ml, dried over NaH) under argon atmosphere at 0°C. Subsequently, the mixture was stirred at room temperature for one hour and finally refluxed at 35°C. The reaction was stopped after 1 hour by adding water (30 ml) after cooling to room temperature. Saturated ammonium chloride solution was added until all solids were dissolved. Subsequently the organic phase was washed with aqueous sodium hydrogen carbonate (1 mol/L) and water, then dried over anhydrous sodium sulfate and finally the solvent was completely evaporated. The product was then purified by silica column chromatography (silica 60, 230-400 mesh, Merck, Darmstadt, Germany) with a pentane/diethylether gradient ranging from 80/20 (v/v) to 50/50 (v/v) followed by completely removing the solvent giving a total yield of 220 mg 1'-hydroxyestragole (1.3 mmol) with ^{1}H-NMR purity of 98%. NMR data were identical to those reported by Iyer (*16*).

Mass spectrum (EI): m/z (relative intensity): 164 (100), 109 (94), 121 (75), 135 (70), 137 (67), 163 (67), 77 (50), 133 (40), 55 (33), 108 (30), 94 (27);

Mass spectrum (CI, methanol): m/z (relative intensity): 147 (100), 148 (11), 109 (11) 164 (2);

Mass spectrum (ESI+): m/z (relative intensity): 147 (100), 148 (10);

Mass spectrum (ESI+, MS/MS energy of collision 34 V): m/z (relative intensity): 103(100), 115 (98), 78 (95), 91 (75), 77 (71), 65 (44), 131 (39);

NMR spectrum: 1.9 (1, singulet, OH); 3.8 (3, singulet, H-1''); 5.2 (2, double-duplet, H-3'); 5.3 (1, duplet, H-1'); 6.0 (1, multiplet, H-2'); 6.8 –7.3 (4, multiplet, H-2, H-3, H-5, H-6)

Synthesis of [1'',1'',1''-$^2H_3$]-1'-hydroxyestragole (3b): p-Hydroxybenzaldehyde (1) was methylized using [$^2H_3$]-methyl iodide (*13*). The subsequent Grignard reaction with vinyl magnesium chloride as described before resulted in the formation of labelled 1'-hydroxyestragole. For methylation, p-hydroxybenzaldehyde (500 mg, 4 mmol, Acros Organics, Geel, Belgium) and potassium carbonate (1.5 g) were dissolved in acetone (20 ml) in a sealed flask and stirred at room temperature. After some minutes, [$^2H_3$]-methyl iodide (1.2g, 8.4 mmol, Acros Organics, Geel, Belgium) was added followed by closing the flask again and stirring the mixture for 20 hours. The reaction was stopped by adding water (20 ml), the labelled anisaldehyde (2b) was extracted with diethylether (50 ml) and the organic phase was dried over anhydrous sodium sulfate. Subsequently, the Grignard reaction with the crude aldehyde and vinyl magnesium chloride as well as purification by silica column chromatography was performed as described for the unlabelled 1'-hydroxyestragole. The total yield of (3b) was 346 mg (2 mmol) with a purity of 97% determinded by ^{1}H-NMR.

Mass spectrum (EI): m/z (relative intensity): 124 (100), 167 (90), 112 (75), 140 (60), 138 (50), 111 (48), 166 (45), 55 (43), 77 (38), 149 (30), 133 (30), 94 (30);

Mass spectrum (CI, methanol): m/z (relative intensity): 150 (100), 151 (12), 112 (11), 167 (3);

Mass spectrum (ESI+): m/z (relative intensity): 150 (100), 151 (10);

Mass spectrum (ESI+, MS/MS energy of collision 34 V): m/z (relative intensity): 103(100), 78 (97), 115 (62), 77 (48), 131 (44), 92 (40), 116 (39);

NMR spectrum: 1.9 (1, singulet, OH); 5.2 (2, double-duplet, H-3'); 5.3 (1, duplet, H-1'); 6.0 (1, multiplet, H-2'); 6.8 –7.3 (4, multiplet, H-2, H-3, H-5, H-6)

Quantification of Estragole in Fennel Tea made from Fennel Fruits

Quantification of estragole in fennel tea was performed as described recently (*8*). Fennel tea was prepared by extracting 2.5 g broken fennel fruits (*Foeniculum vulgare* Mill. ssp. vulgare var. vulgare, Martin Bauer, Vestenbergsgreuth, Germany) with 150 ml boiling water for 10 minutes and subsequently filtering the extract. The hot aqueous infusion was cooled to room temperature, then a solution of [$^2H_3$]-estragole in dichloromethane was added and stirred for one hour before extraction with dichloromethane (2 x 50 ml) in a separation funnel. The organic phase was dried over anhydrous sodium sulfate, concentrated to 2 ml and analyzed by HRGC-MS in CI-modus. Estragole was quantified by relative area counts of analyte and IS using the response factor detailed previously (*13*).

Design of the Human Studies

The protocol of the study was approved by the Ethics Committee of the Faculty of Medicine of the Technische Universität München. The test persons were 3 male and 4 female healthy non-smoking volunteers, aged 22 – 41 years, with body weights ranging from 49 to 70 kg. They abstained from estragole-containing products for 3 days prior to the study. On an empty stomach, all subjects drank 500 ml of a fennel

tea made from freshly broken fruits within 10 minutes. Then, after a fasting period of 2.5 hours, a meal without spices was supplied. Apart from the other test persons, one female volunteer drank in separate trials another 1000 ml and 250 ml of the fennel tea, respectively, with intervals of at least 2 months between the single testings.

Blank urine samples were taken in the morning prior to the administration of fennel tea. Then, urine samples were collected 0 - 1.5, 1.5 - 4, 4 - 8, 8 - 14 and 14 - 24 hours after dosing. Each sample was stored at –30 °C until analysis.

Only from one female test person blood samples 0.75, 1.5, 2 and 2.5 hours after dosing were drawn into 10 ml vacutainers (Sarstedt, Nümbrecht, Germany). Clotting was prevented by heparine. Whole blood was then separated into plasma and blood cells by centrifugation at 3000 g for 15 minutes at 5 °C and both fractions were stored separately at –30 °C.

Sample Preparation for Stable Isotope Dilution Assay (SIDA)

Analyses of blood plasma: 5 ml blood plasma were spiked with the internal standards [$^2H_3$]-estragole and [$^2H_3$]-1'-hydroxyestragole and stirred for one hour. Then, plasma was either analyzed by extracting the metabolites with 2 x 4 ml diethylether or, prior to the solvent extraction, incubated with β-glucuronidase and sulfatase. For enzymatic incubation, plasma was acidified with hydrochloric acid to pH 5 and 100 µl enzyme solution (from Helix pomatia, β-glucuronidase and sulfatase activity of 100000 units/ml and 7500 units/ml, respectively, Sigma-Aldrich Chemie, Taufkirchen, Germany) was added. Thereafter, the acidified plasma was incubated for 4 hours at 37 °C in a water bath and subsequently extracted with 2 x 4 ml diethylether.

All solvent extracts were washed with water and dried over anhydrous sodium sulfate. After evaporating the solvent, the residue was dissolved in methanol and

membrane filtered for LC-MS/MS or GC-GC-MS analyses. Estragole and its metabolites were quantified by relative area counts of analyte and IS using the response factors given below.

Analyses of urine: 200 ml urine were spiked with the internal standards [$^2H_3$]-estragole and [$^2H_3$]-1'-hydroxyestragole and stirred for one hour. An aliquot of 100 ml was acidified with hydrochloric acid to pH 5 and either 200 µl mixed enzyme solution (from Helix pomatia, β-glucuronidase and sulfatase activity of 100000 units/ml and 7500 units/ml, respectively, Sigma-Aldrich Chemie, Taufkirchen, Germany) or 200 µL pure glucuronidase solution (from E. coli, β-glucuronidase activity of 100000 units/ml, Sigma-Aldrich Chemie, Taufkirchen, Germany) was added. Incubation at 37 °C was stopped after 4 hours by addition of diethylether.

The metabolites were extracted with diethylether (2 x 50 ml) in both aliquots of urine (2 x 100 ml) and the organic phases were purified by the following procedures.

For analysis of 1'-hydroxyestragole the solvent extract was dried over anhydrous sodium sulfate, concentrated to 500 µl and loaded on a silica column (silica 60, 230-400 mesh, Merck, Darmstadt, Germany). The metabolite was eluted with 20 ml diethylether, the eluate evaporated to dryness and the residue dissolved in 300 µl methanol. After membrane filtration the extract was analyzed by LC-MS/MS. 1'-hydroxyestragole was quantified by relative area counts of analyte and IS using the response factor determined below.

For analyses of estragole, *trans*-anethole and p-allylphenol, volatiles were separated from nonvolatile compounds in the diethylether extract by solvent assisted flavor evaporation (SAFE) (*17*) at 40 °C. The distillates were dried over anhydrous sodium sulfate and the solvent was removed completely. The residue was dissolved in 300 µl

methanol and analyzed by GC-GC-MS. Quantification was performed by relative area counts of analyte and IS using the response factors detailed previously (*13*).

Determination of Detection and Quantification Limits

Human blood plasma and urine, which were both devoid of the analytes under study, were used for determination of the detection limit (DL) and quantification limit (QL). The following amounts of analytes were added to the respective matrices: 0.3, 0.9, 1.8 and 9 µg/kg plasma of 1'-hydroxyestragole for LC-MS/MS; 20, 40, 100 and 200 µg/kg plasma of estragole for GC-GC-MS; 0.4, 0.9, 1.8 and 4.4 µg/l urine of 1'-hydroxyestragole for LC-MS/MS and 9, 25, 50 and 100 µg/l urine of estragole for GC-GC-MS. Each sample was analyzed in triplicates by SIDA as detailed before. DL and QL were determined according to the method of Hädrich and Vogelgesang (*18*) as described for patulin by Rychlik and Schieberle (*19*). DL is the addition value referring to the 95 % confidence limit of the calibration line at the zero addition level. QL is the addition level which lowers the 95 % confidence limit to meet the upper 95 % confidence limit of the addition level at the DL.

Stability Tests of 1'-Hydroxyestragole

For analysis of the stability of 1'-hydroxyestragole, the latter was treated with various acids. On the one hand, 150 µg 1'-hydroxyestragole was incubated with 1 ml phosphorus buffer at pH 5 at room temperature, which was compared with a treatment at 37 °C in a water bath. On the other hand, 150 µg 1'-hydroxyestragole was treated with 1 ml trichloroacetic acid (20 % in water) to test the stability at pH 2. Formation of adducts with glutathione were tested by incubation of 50 µg 1'-hydroxyestragole in 1 ml isotonic phosphate buffer (1.42 g disodium hydrogen phosphate, 7.27 g sodium chloride and 0.19 g disodium-EDTA in 1l water and

acidified to pH 7.4 with hydrochloric acid) with glutathione solution (3 ml, 1 mmol/l). either at room temperature or at 37 °C. All mixtures were analyzed by HPLC-UV at 270 nm and area counts were used to determine degradation of the title compound.

High Resolution Gas Chromatography and Mass Spectrometry (HRGC-MS)

For quantification of estragole in fennel tea a gas chromatograph (CP 3800, Varian, Darmstadt, Germany) coupled with an ion trap detector (Saturn 2000, Varian, Darmstadt, Germany) running in the CI mode with methanol as reactant gas was used. The samples were injected on-column at 40 °C and the compounds were separated on a DB-FFAP capillary (30 m x 0.32 mm i.d., film thickness 0.25 µm, J & W Scientific, Fisons, Germany). The temperature was raised by 8 °C/min to 230 °C and helium with a flow rate of 2 ml/min was used as the carrier gas.

Mass traces for estragole and [$^2H_3$]-estragole were m/z 149 and m/z 152, respectively. The response factor of 1.05 was determined according to Rychlik and Schieberle (*19*).

Two Dimensional HRGC and Mass Spectrometry (GC-GC-MS)

Estragole, *trans*-anethole and p-allylphenol in blood plasma and urine extracts were analyzed by two dimensional HRGC/MS using [$^2H_3$]-estragole as internal standard. In the first dimension, compounds were separated by capillary DB-FFAP (30 m x 0.32 mm i.d., film thickness 0.25 µm, J & W Scientific, Fisons, Germany) after on-column injection at 40 °C in a gas chromatograph (Trace GC, 2000 series, Thermo Finnigan, Bremen, Germany). After 1 min the oven temperature was raised by 10 °C/min to the final temperature of 230 °C. Helium was used as the carrier gas and the flow rate was set to 2 ml/min. The effluent was quantitatively transferred to the second dimension by a moving column stream switching system (MCSS, Thermo Finnigan).

As the second dimension a DB-1701 column (30 m x 0.32 mm i.d., film thickness 0.25 µm, J & W Scientific, Fisons, Germany) was run in a gas chromatograph (CP 3800, Varian, Darmstadt, Germany) coupled with an ion trap detector (Saturn 2000, Varian, Darmstadt, Germany) running in the CI mode with methanol as reactant gas. In the second dimension the injection temperature of 40 °C was held for 1 min, then raised by 8 °C/min to the final temperature of 230 °C.

For quantification the respective mass traces and response factors r_f given in parentheses were used: estragole (m/z 149, r_f = 1.05), *trans*-anethole (m/z 149, r_f = 0.98), p-allylphenol (m/z 135, r_f = 0.70), [$^2H_3$]-estragole (m/z 152, internal standard).

High Pressure Liquid Chromatography and UV Spectrometry (HPLC-UV)

HPLC was performed by injecting 30 – 100 µl of the extract onto an Aqua RP 18 column (250 x 4.60 mm, i.d. 5 µm, Phenomenex, Aschaffenburg, Germany) in an HPLC type 522 (BIO-TEK Instruments, Bad Friedrichshall, Germany). Elution was performed at a flow rate of 0.8 ml/min with variable mixtures of methanol and 0.1 % aqueous formic acid (solvent B). A gradient starting from 70 % B was programmed within 30 min to 100 % methanol, held for a further 10 min. The effluent was monitored at 235 nm and 270 nm using an UV detector 535 (BIO-TEK Instruments, Bad Friedrich, Germany)

High Pressure Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS)

1'-Hydroxyestragole was quantified by LC-MS/MS, which was performed by means of an HPLC system (Surveyor HPLC System, Thermo Finnigan, Dreieich, Germany) coupled to a triple quadrupole MS system (TSQ Quantum Discovery, Thermo Finnigan, Dreieich, Germany) equipped with an Aqua RP 18 column (150 x 2.0 mm,

i.d. 5 µm, Phenomenex, Aschaffenburg, Germany). The column was flushed with aqueous formic acid (0,1 %, solvent A) for 10 min before injection. Then, 10 µl of the samples were injected and chromatographed at a flow of 0.2 ml/min with varying mixtures of A and methanolic formic acid (0.1 %, solvent B). The programmed gradient started from 100 % A and reached 80 % B within 30 min. Then, the concentration of B was raised immediately to 100 % within 2 min, maintained for further 10 min and then lowered to 0 % to equilibrate the column for the next injection.

For tandem mass spectrometry of 1'-hydroxyestragole the mass transitions (m/z precursor ion / m/z product ion) 147/103 and 147/115 for the unlabelled and 150/103 and 150/115 for the labelled compound, respectively, were chosen. The voltages applied to the precursor ion to obtain the product ions m/z 103 and m/z 115 were 34 V and 16 V, respectively. The mass spectrometer was operated in the positive electrospray mode with a spray needle voltage of 3.5 kV. The temperature of the capillary was 300 °C and the capillary voltage was 35 V. The sheath and auxillary gas nitrogen nebulized the effluent with flows of 25 and 15 arbitrary units, respectively. Each scan event was recorded within 200 ms.

Response factors were determined by mixing the labelled and unlabelled 1'-hydroxyestragole in concentrations ranging from 0.1 to 10. The mixtures were analyzed by LC-MS/MS and factors were calculated as reported recently (*19).*

^{1}H-NMR Spectroscopy

^{1}H-NMR spectra were recorded on a Bruker AMX 400 (Bruker, Karlsruhe, Germany) at 297K in $CDCl_3$ and TMS as internal standard (δ = 0 ppm)

RESULTS AND DISCUSSIONS

Method validation for stable isotope dilution assays (SIDA)

In physiological studies with human test persons only small amounts of potentially hazardous substances may be administered. Therefore, detection methods for analyses of metabolites in human fluids require high sensitivity and reproducibility. In former studies sensitive analysis of estragole was accomplished by applying radiolabelled analytes to humans and detecting radioactivity in the expired air or in body fluids (*11, 20*). A less risky and still sensitive alternative is the quantitation by stable isotope dilution assays (SIDA), which is based on the addition of stable isotopically labelled analogues of the analytes to the test material prior to extraction. Because of their structural similarity to the analytes, isotopologues show best accordance of chemical and physical properties. Therefore, losses during extraction, clean-up or detection are best compensated for.

For analyses of estragole and its metabolites we developed SIDAs in human blood plasma and urine. In a prior study, the synthesis and successful usage of [$^2H_3$]-estragole as internal standard (IS) for quantitation of estragole in fennel tea has been reported (*13*). In addition, a SIDA was developed for 1'-hydroxyestragole (OHE) since it is the proximate carcinogen responsible for the mutagenic and hepatocarcinogenic effect of estragole (*2, 9*). The synthesis of [$^2H_3$]-1'-hydroxyestragole was accomplished by methylizing p-hydroxybenzaldehyde with [$^2H_3$]-methyl iodide followed by a Grignard reaction of the labelled anisaldehyde with vinyl magnesium chloride. ^{1}H-NMR and HRGC/MS analyses revealed a yield of 50 % and proved an isotopologic purity of [$^2H_3$]-OHE of about 100 %. For SIDA's method validation, response factors and calibration functions were determined by mass spectrometry. Analyses of 1'-hydroxyestragole by LC-MS/MS proved highest

sensibility as the protonated molecule easily eliminates water and forms a stabilized cation m/z 147 (**Figure 2**) as the precursor ion in the ion source.

During LC- tandem mass spectrometry the same product ion at m/z 115 for the labelled and unlabelled compound was generated from the respective precursor ion as the labelled methoxy group is lost during MS-MS. However, the linearity of the calibration curve proved the suitability of [$^2H_3$]-1'-hydroxyestragole for SIDA in mass ratios ranging from 0.1 to 10. The comparison of detection limits (DL) of the diethylether solution for injection using LC-MS/MS (DL 0.45 µg/l) and GC-GC-MS (DL 860 µg/l) indicates that sensitivity was much lower using GC-GC-MS.

By contrast, estragole could only be detected by GC-GC-MS due to its poor ionization during positive electrospray ionization.

Besides 1'-hydroxyestragole the furthermore proposed metabolite of estragole (*14, 21*) p-methoxycinnamyl alcohol would not be distinguishable by LC-MS/MS analyses because it yields the same cation at m/z 147 formed by water elimination in the ion source. Therefore, a chromatographic separation of both substances by HPLC was successfully developed.

Stability tests of 1'-hydroxyestragole

In prior studies, Zangouras et al. *(9*) and Drinkwater et al. (*2*) detected 1'-hydroxyestragole in urine of mice or rats only as conjugate with glucuronic acid after enzymatic incubation with β-glucuronidase. Therefore, in the present study an incubation of urine with the latter enzyme was inevitable to measure the complete excretion of OHE. Since the pH optimum of β-glucuronidase is slightly acidic (pH 5) and 1'-hydroxyestragole has been shown to isomerise to p-methoxycinnamyl alcohol (*6*) with increasing acidity (*22*), stability of OHE at pH 5 and pH 2 was tested. **Figure 3** shows a rapid degradation of 1'-hydroxyestragole at pH 5 and at the enzyme's

temperature optimum at 37 °C, while its stability at room temperature was still satisfactory. Strong acid conditions at pH 2 led to a nearly complete decomposition of 1'-hydroxyestragole within 4 hours. However, by addition of the stable isotopologue, the decomposition of the latter in course of a SIDA would compensate for the losses of OHE. Still, the decrease of both analyte and isotopologic standard would impair the sensitivity of the method. We concluded that enzymatic incubation of body fluids at pH 5 to detect conjugated 1'-hydroxyestragole should be performed during less than 4 hours in order to prevent high degradation of the analyte.

In human blood high amounts of glutathione (*22*) occur, which can easily form adducts with electrophiles as shown for patulin (*23*) and estragole-2',3'-epoxide (*24*). However, the reaction with glutathione at pH 7.4 and 37 °C showed no decrease of the UV peak areas of 1'-hydroxyestragole. Therefore, interference with analysis of OHE by formation of glutathione adducts during sampling and cleanup can be excluded.

Sample preparation of urine and blood plasma for quantitation of estragole and 1'-hydroxyestragole

For quantification of estragole and 1'-hydroxyestragole in urine and blood plasma the sample preparations was optimized in order to achieve best DL and QL. In blood plasma both substances could be analyzed easily by solvent extraction without further clean-up. For urine, the solvent extracts had to be purified more effectively for protection of the analytical columns and to increase sensitivity. During analysis of 1'-hydroxyestragole interfering compounds were removed by silica column chromatography. Unfortunately, during the latter cleanup estragole was lost as it was not eluted from the column. Therefore, solvent assisted flavor evaporation (SAFE) has to be applied as clean-up procedure for the quantification of estragole. The

analysis of estragole was performed by GC-GC-MS and 1'-hydroxyestragole by LC-MS/MS, respectively, which resulted in best sensitivity. **Figure 4** presents an example of the urinary LC-MS/MS chromatogram showing 1'-hydroxyestragole and its isotopically labelled isotopologue.

To determine the DL and QL, we applied the calibration procedure proposed by Hädrich and Vogelgesang (*18*). The respective matrix devoid of the analyte was spiked with increasing amounts of analyte and internal standard prior to analyses. DL for 1'-hydroxyestragole was 0.3 µg/kg plasma and QL 0.9 µg/kg, respectively. In urine, DL (0.06 µg/kg) and QL (0.18 µg/kg) were much lower due to a higher volume of sample material available for the analyses. For estragole the DL (5.5 µg/kg plasma, 17 µg/kg urine) and QL (16 µg/kg plasma, 50 µg/kg urine) were much higher because of the less sensitive detection by GC-GC-MS. Still, two dimensional gas chromatography resulted in better detection limits than single HRGC-MS.

As previously described, Zangouras et al. (*9*) and Drinkwater et al. (*2*) only detected 1'-hydroxyestragole when applying enzymatic incubation with β-glucuronidase to the body fluids. In addition, Miller and Miller (*7*) postulated conjugates with sulfuric acid. Therefore, in a first series of experiments, a mixture of β-glucuronidase and sulfatase was used. The amount of enzymatic solution was first chosen as recommended by Drinkwater et al. (*2*). Still, the use of the double amount was investigated but did not influence the concentration of liberated 1'-hydroxyestragole from the conjugate. As described before, it proved to be more important that the time period of the incubation should not exceed 4 hours in order to minimize degradation of 1'-hydroxyestragole. Incubation periods of longer than 4 h resulted in decreasing signal abundance of 1'-hydroxyestragole isotopologues, whereas their ratio remained stable. Therefore, we concluded that 4 h were sufficient to liberate all bound 1'-hydroxyestragole.

In order to elucidate the possible formation of the sulfate ester of 1'-hydroxyestragole, the urine additionally was treated with recombinant glucuronidase which was free from sulfatase activity. The results of the latter tests gave identical contents of liberated 1'-hydroxyestragole compared to the incubation with the mixed glucuronidase and sulfatase ($p<0.01$) enzyme. Therefore, the presence of the sulfate ester in percentages exceeding 5 % (equivalent to 0.02 % of the estragole dose) of the total conjugate amount could be excluded in urine. This result can either be explained by a small rate of sulfate formation and/or by its fast decomposition and reaction with cellular nucleophiles. A possible minor rate of formation cannot be ruled out, although Punt et al. (10) calculated a sulfate formation of 0.16 % from a 100 mg/kg bw estragole dose in rats. However, the latter authors applied a dose over three orders of magnitude higher than that we used and 1'-hydroxylation and successive sulfate formation are known to decrease with lower doses. Moreover, the same authors reported earlier that in humans sulfatation of OHE is approximately 10 times slower and less effective than in rats (*3*). For these reasons, it is plausible that we could not detect the sulfate in our studies.

Quantification of estragole and trans-anethole in blood plasma and urine following consumption of fennel tea

Test persons consumed estragole by drinking fennel tea made from freshly broken fennel fruits on an empty stomach. Doses were adjusted by gavage of different amounts of fennel tea. Concentrations of estragole in fennel tea were determined by SIDA. All volunteers collected their urine in defined time periods and from one female subject blood samples were drawn after 0.75, 1.5, 2 and 2.5 hours and separated in plasma and erythrocytes by centrifugation. After consumption of 1l fennel tea, which contained 3.5 mg estragole, the plasma of this volunteer revealed no detectable

amount of estragole in any of the samples. Considering the mean blood content of 4.36 kg in females of the respective body weight (*25*) and a theoretical absorption rate of 100 %, the estragole concentration in plasma should amount to 807 µg/kg. Because less than 0.7 % of this amount (DL 5.5 µg/kg) were detected 0.75 hours after dosing, it can be concluded that distribution in body fluids and metabolism happened very fast.

Because of its structural similarity and its occurrence in higher concentrations in fennel tea (30 mg/l), *trans*-anethole was also analyzed in blood plasma. In all samples the *trans*-anethole content was above its DL and decreased slightly after 2.5 hours from 44 to 35 µg/kg (**Table 1**). The total amount of 0.27 - 0.35 % of the dose, calculated on the basis of a mean plasma content of 2.4 kg in females (*24*), proved the fast distribution and metabolism. As estragole exhibits similar solubility as *trans*-anethole (*26*), it could be assumed that the former was absorbed to a similar rate and similar percentage as the latter.

In accordance with the findings of Sangster et al. (*11*), no estragole or *trans*-anethole was detectable in human urine as metabolism is necessary for excreting both phenylpropanes due to their low hydrophilicity.

Quantification of 1'-hydroxyestragole in blood plasma and urine following consumption of fennel tea

After consumption of 3.5 mg estragole (68 µg/kg bw), free and conjugated 1'-hydroxyestragole could be detected in the blood plasma of the female volunteer under study (**Table 2**). However, the amounts of free 1'-hydroxyestragole were lower than its QL, which renders the obtained concentration data equivocal. In contrast, the glucuronic acid ester of 1'-hydroxyestragole was quantifyable. The calculated molar rates of formation in percent of the consumed estragole were very low. After 0.75

hours less than 0.1 % of the estragole dose could be detected as conjugated 1'-hydroxyestragole in plasma.

In contrast to blood, higher amounts of OHE were quantified as urinary metabolite. Although hardly any free 1'-hydroxyestragole was detected (only 2 % of the conjugated metabolite), its conjugate with glucuronic acid was excreted by up to 0.27 % of the estragole dose. Excretion happened very fast and after 10 hours no metabolite was detectable in the urine. Summing up the urinary excretion over 10 h, 0.39 % of 3.5 mg estragole was metabolized to its 1'-hydroxy derivate by the female volunteer. Additional amounts of the estragol dose bound to DNA or proteins after decomposition of the labile sulfate ester of 1'-hydroxyestragole might be postulated, but appear negligible as no traces of the sulfate ester have been detected. Analyses of DNA adducts after adaptation of a recently published method to human DNA samples (*3*) could clarify this question.

Changes in the estragole dosing showed variation in the rates of formation (**Figure 5**) as lower doses resulted in decreased yields of 1'-hydroxylation. This finding was in good accordance with the reports on rat studies performed by Zangouras et al. (*9*) and Anthony et al. (*14*), who found that doses of 1000 mg/kg bw resulted in a 1'-hydroxylation rate of 8 - 9.5 %, whereas lower doses of 0.05 mg/kg bw yielded only 0.3 - 1.3 % of OHE.

A physiological study with 3 male and 4 female volunteers, given a single estragole dose of 1.3 mg (19 – 27 µg/kg bw depending on body weights from 49 – 70 kg) showed similar formation rates of free and conjugated 1'-hydroxyestragole (**Table 3**). In the urine of three volunteers no free metabolite was detectable. The rates of formation of conjugated metabolite were 0.17 – 0.41 % of the estragole intake. This confirmed the general metabolism of estragole to OHE in humans although the amount of 1'-hydroxylation differed between the single individuals. In **Figure 6** the

chronological and cumulative formation rates for each volunteer are presented. In most cases excretion was completed 6 – 8 hours after dosing. The rapid metabolism of ^{14}C-estragole in humans has already been indicated by Sangster et al. (11), who reported an excretion of 50 % of the dose administered within the first 20 h. The remaining 50 % of the dose, however, could not be detected.

Likewise, the formation rates in our study were in good accordance with the findings of the latter authors, who detected 0.2 – 0.4 % of 1'-hydoxyestragole after dosing of 100 µg ^{14}C-estragole.

In a recent biokinetic modeling using kinetic data from microsomal preparations of livers at a dose of 0.07 mg/kg bw, which is equal to the dose in our study, Punt et al. (8) predicted a formation of 1.9 % OHE glucuronide. This predicted rate is slightly higher than our data and point to the need for a light adjustment of the model.

Quantification of p-allylphenol and p-methoxycinnamyl alcohol in blood plasma and urine

Since 1'-hydroxyestragole is a minor metabolite when lower doses of estragole are administered (*9*), the presence of the major metabolite (*21*), p-allylphenol, was investigated. Quantification was performed by GC-GC-MS because of the weak sensitivity in positive electrospray ionization during LC-MS/MS. The plasma samples without enzymatic incubation revealed no detectable amounts of free p-allylphenol, so after 0.75 hours less than 0.4 % of the estragole intake was detected as the free metabolite. In contrast to this, conjugated p-allylphenol was detected with a formation rate of 4 % of the estragole dose in human blood plasma drawn after 0.75 h.

In urinary samples the detected amounts of conjugated p-allylphenol were much higher after only 1.5 hours (17 % of the estragole intake) which again proved the fast metabolism of estragole in the human body. 8 hours after dosing this metabolite was

completely excreted to an extent of 20 % related to the estragole dose. However, no free p-allylphenol (less than 0.4 % of estragole intake) was detectable in the urine material. In comparison, Solheim and Scheline (*21*) detected about 40 % of the estragole dosing as conjugated p-allylphenol and likewise could not prove the presence of the free metabolite in the urine of rats.

Regarding the further proposed metabolite of estragole, namely p-methoxycinnamyl alcohol, in neither plasma nor urine samples the latter compound was detected.

Conclusions

In the present study the formation of 1'-hydroxyestragole in the human body after dosing of estragole could be shown by SIDA. The metabolite could be detected in blood plasma and urine after consumption of fennel tea. In comparison, the major metabolite p-allylphenol was analyzed and could also be detected in body fluids. In total, about 20 % of the estragole dose was detectable as the two urinary metabolites. The remaining 80 % of the dose may be assigned to the oxidation product of OHE, 1'-oxoestragole, and its glutathione adduct, which recently has been modeled as major metabolites (8). These and further reported metabolites (*21, 27*) such as p-methoxyhippuric acid, p-methoxybenzoic acid, p-methoxyphenyl lactic acid and 2',3'-epoxy estragole were not analyzed in the present study and remain to be investigated. Interestingly, the specific metabolites of estragole were still detectable when consuming a mixture of different odorants present in fennel tea. Therefore, we concluded that an excess of *trans*-anethole principally does not interfere with estragole metabolism, whereas influences on the quantitative composition of metabolites cannot be excluded. Regarding conclusions on the toxic risk due to fennel consumption it has to be emphasized that estragole is rapidly metabolized and 1'-hydroxyestragole is quickly excreted as its glucuronic acid conjugate. The

presence of a sulfuric acid conjugate could not be confirmed, possibly due to its high reactivity and lability. Further analyses of DNA adducts could clarify the question of its formation *in-vivo*. Finally it can be pointed out that studies about estragole metabolism in rodents can be transferred to man.

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Tables

Table 1: *Trans*-anethole concentrations in human blood plasma at different times after consumption of fennel tea containing 30.5 mg *trans*-anethole (587 µg/kg bw), quantified in SIDA using GC-GC-MS

time [h]	*trans*-anethole concn [a)] [µg/kg plasma]	% of dose [b)] [mol%]
0.75	44	0.35
1.25	42	0.32
2.5	35	0.27

[a] concentrations were calculated by two measurements of two replicate analyses using [$^2H_3$]-estragole as internal standard. Relative standard deviation did not exceed 15%. Detection limit was 5.5 µg/kg and quantification limit was 16 µg/kg estragole in blood plasma

[b] in 2.4 kg blood plasma corresponding to a single *trans*-anethole intake of 30.5 mg

Table 2: 1'-Hydroxyestragole concentrations in human blood plasma and urine at different times after consumption of fennel tea containing 3.5 mg estragole (68 µg/kg bw), quantified in SIDA using LC-MS/MS

		free 1'-hydroxyestragole		**conjugated 1'-hydroxyestragole**	
	time [h]	concn [a)] [ng/kg]	% of dose [b)] [mol%]	concn [a)] [ng/kg]	% of dose [b)] [mol%]
plasma	0.75	710	0.04	1040	0.07
	1.25	380	0.02	700	0.04
	2.0	n.d [c)]	-	570	0.03
	2.5	n.d [c)]	-	520	0.03
urine	0 - 1.5	620	0.006	27600	0.27
	1.5 - 2.25	81	0.002	3500	0.07
	2.25 - 3.25	n.d [c)]	-	2260	0.04
	3.25 - 7.5	n.d [c)]	-	940	0.01
	7.5 - 10.5	n.d [c)]	-	< 60	-
	10.5 - 20.5	n.d [c)]	-	< 60	-

[a] concentrations were calculated by 2-4 measurements of two replicate analyses using [$^2H_3$]-1'-hydroxyestragole as internal standard. Relative standard deviation did not exceed 15%. Detection limit was 300 ng/kg in plasma and 60 ng/kg in urine and quantification limit was 900 ng/kg in plasma and 180 ng/kg in urine [b] in 2.4 kg blood plasma or 400 – 800 g urine corresponding to a single estragole intake of 3.5 mg [c] not detectable

Table 3: Total 1'-hydroxyestragole concentrations in human urine after consumption of fennel tea containing 1.3 mg estragole, quantified in SIDA using LC-MS/MS

				free 1'-hydroxyestragole		conjugated 1'-hydroxyestragole	
		urine	dose	total concn [a]	total % of dose [b]	total concn [a]	total % of dose [b]
		[ml]	[µg/kg bw]	[ng/kg]	[mol%]	[ng/kg]	[mol%]
test person	1	1576	19	320 [c]	0.01	9200	0.27
	2	1316	27	106 [c]	0.003	9800	0.23
	3	1428	24	93 [c]	0.002	15700	0.37
	4	1577	25	n.d. [d]	-	11200	0.17
	5	1536	25	130 [c]	0.004	11600	0.41
	6	2385	19	n.d. [d]	-	11000	0.34
	7	1962	19	n.d. [d]	-	8000	0.20

[a] total concentrations were calculated as the sum of urine samples from 0 – 10 h. Each sample was measured 2-4 times of two replicate analyses using [$^2H_3$]-1'-hydroxyestragole as internal standard. Relative standard deviation did not exceed 15%. [b] in urine corresponding to a single estragole intake of 1.3 mg [c] only concentrations up to 1.5 h were detected [d] not detectable

Table 4: p-Allylphenol concentrations in human blood plasma and urine at different times after consumption of fennel tea containing 3.5 mg or 1.7 mg estragole, quantified in SIDA using GC-GC-MS

		free p-allylphenol		**conjugated p-allylphenol**	
	time [h]	concn [a)] [µg/kg]	% of dose [b)] [mol%]	concn [a)] [µg/kg]	% of dose [b)] [mol%]
plasma 3.5 mg estragole intake	0.75	n.d. [c)]	-	55	4.1
	1.25	n.d. [c)]	-	52	3.8
	2.5	n.d. [c)]	-	52	3.8
urine 1.7 mg estragole intake	0 - 1.5	n.d. [c)]	-	600	16.6
	1.5 - 4	n.d. [c)]	-	104	3.3
	4 - 8	n.d. [c)]	-	n.d. [c)]	-
	8 - 14	n.d. [c)]	-	n.d. [c)]	-
	14 - 22	n.d. [c)]	-	n.d. [c)]	-

[a] concentrations were calculated by 2-4 measurements of two replicate analyses using [$^2H_3$]-estragole as internal standard. Relative standard deviation did not exceed 15%. Detection limit was 5.5 µg/kg estragole in plasma and 17 µg/kg in urine and quantification limit was 16 µg/kg estragole in plasma and 50 µg/kg in urine [b] in 2.4 kg blood plasma or 400 – 500 g urine corresponding to a single estragole intake of 3.5 mg or 1.7 mg [c] not detectable

LEGEND TO THE FIGURES

Figure 1: Synthetic pathways leading to 1'-Hydroxyestragole (R = OCH_3) and [1'',1'',1''-$^2H_3$]-1'-hydroxyestragole (R = OCD_3).

Figure 2: Formation of a stabilized cation of 1'-hydroxyestragole in the positive electrospray ion source leading to the precursor ion at *m/z* 147 for collision-induced dissociation in LC-MS/MS.

Figure 3: Degradation of 1'-hydroxyestragole at pH 5 37°C, pH 5 room temperature (RT) and pH 2 RT (with detection limit = DL) showing its lability at low pH and elevated temperature.

Figure 4: LC-MS/MS chromatogram of an urine sample containing [$^2H_3$]-1'-hydroxyestragole as internal standard. Measurements were performed in Single Ion Monitoring (SIM) and Selected Reaction Monitoring (SRM). Analyte and standard are unequivocally detected in their respective SIM and SRM traces at identical retention time.

Figure 5: Chronological cumulative rate of formation of conjugated 1'-hydroxyestragole in human urine samples of one volunteer corresponding to different estragole doses. With higher doses the rate of formation increases, which is in accordance with the literature (*6, 10*).

Figure 6: Chronological cumulative rate of formation of conjugated 1'-hydroxyestragole in human urine samples of 7 different test persons.

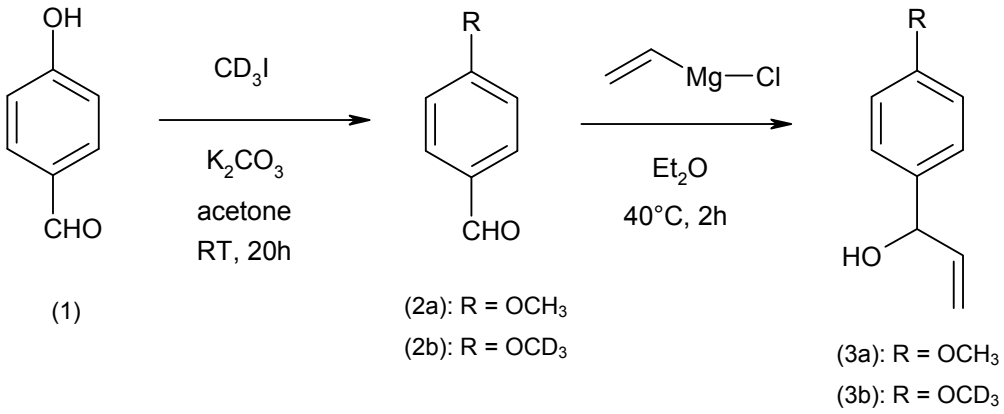

Figure 1

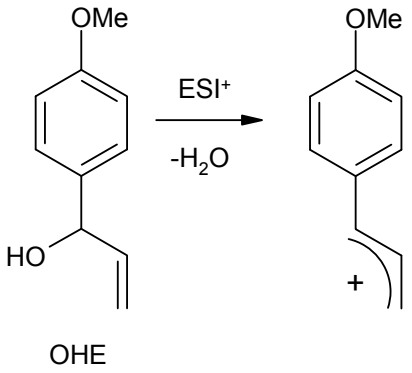

Figure 2

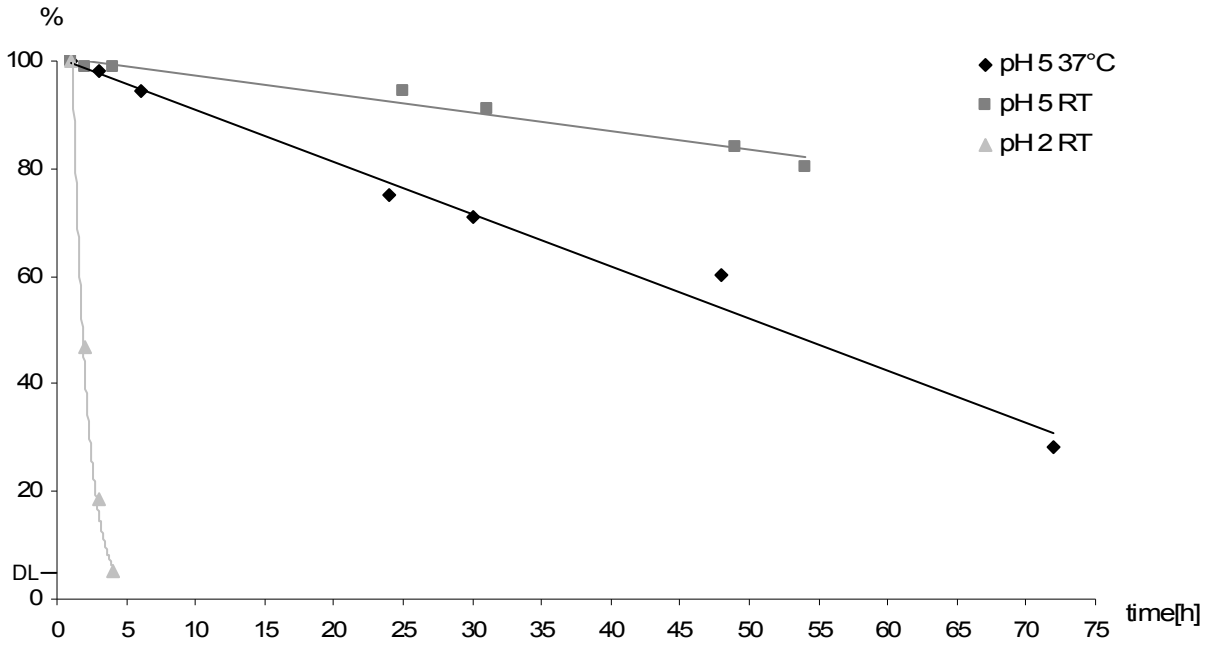
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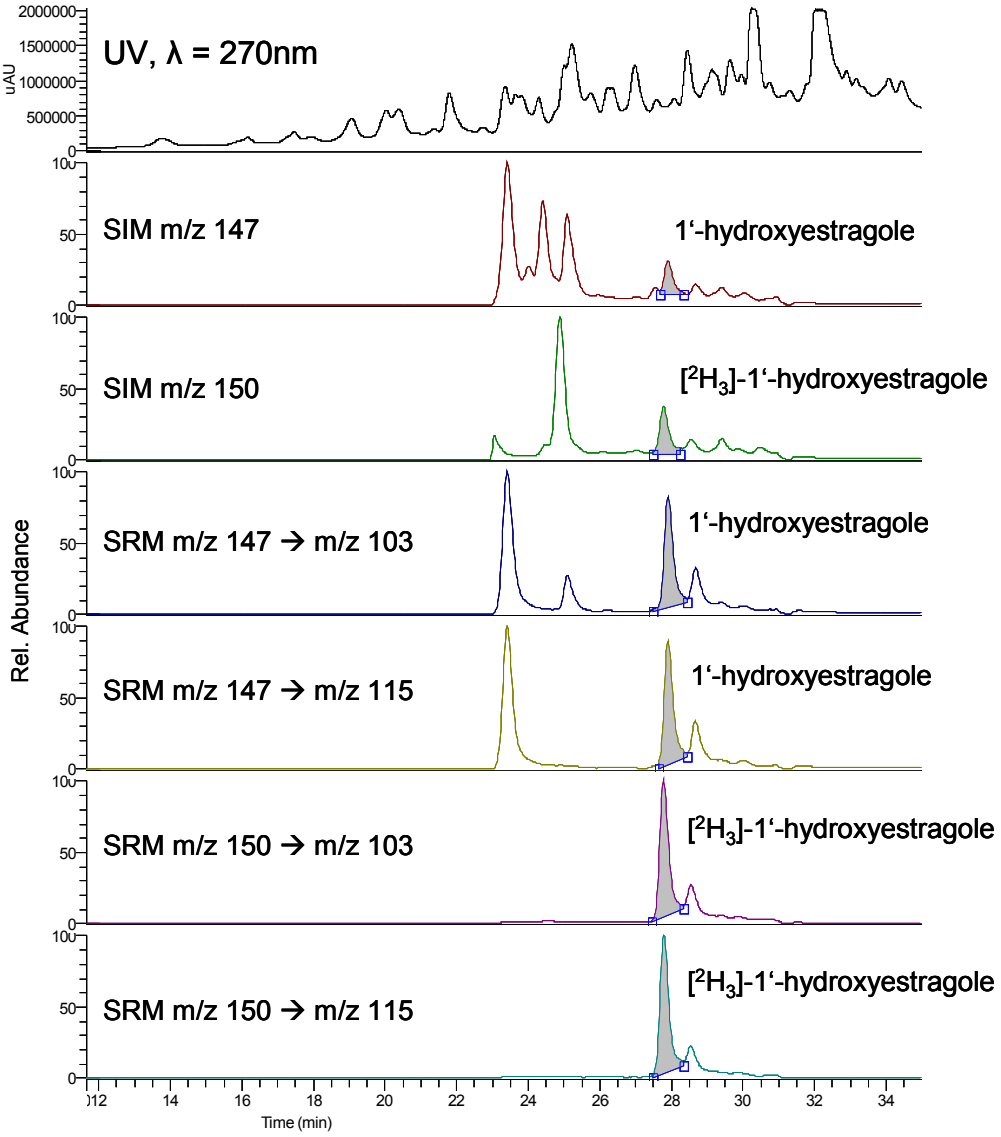
Figure 3


UV, λ = 270nm
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SIM m/z 147
1‘-hydroxyestragole
SIM m/z 150
[2H3]-1‘-hydroxyestragole
SRM m/z 147 → m/z 103
1‘-hydroxyestragole
SRM m/z 147 → m/z 115
1‘-hydroxyestragole
SRM m/z 150 → m/z 103
[2H3]-1‘-hydroxyestragole
SRM m/z 150 → m/z 115
[2H3]-1‘-hydroxyestragole
Rel. Abundance
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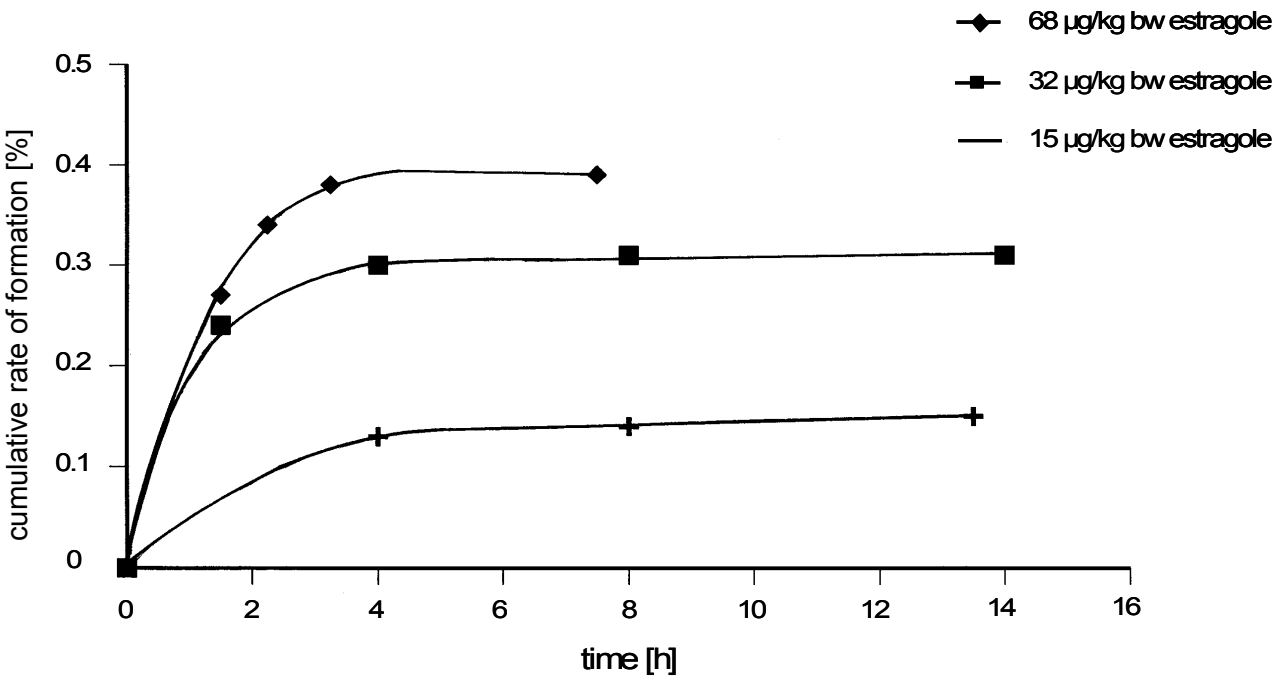
34
Time (min)

Figure 4


68 µg/kg bw estragole
32 µg/kg bw estragole
15 µg/kg bw estragole
cumulative rate of formation [%]
0.5
0.4
0.3
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0.1
0
0
2
4
6
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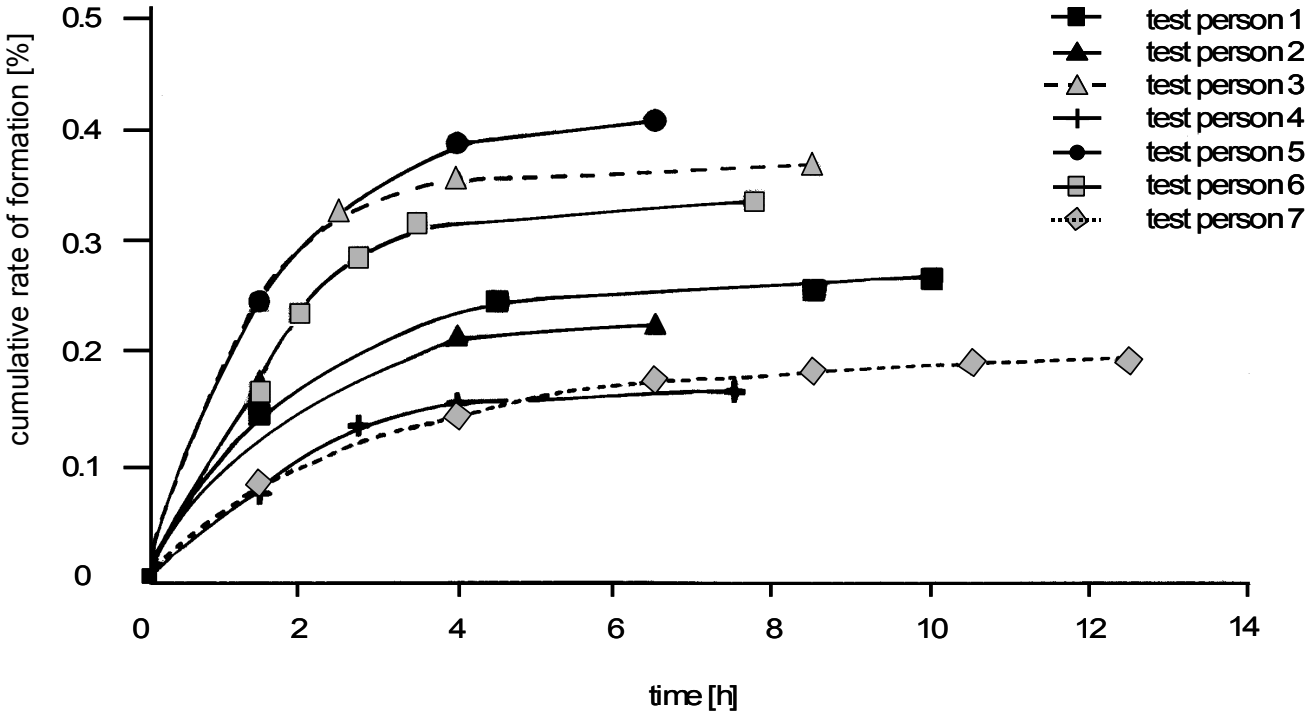
12
14
16
time [h]

Figure 5


cumulative rate of formation [%]
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time [h]
test person 1
test person 2
test person 3
test person 4
test person 5
test person 6
test person 7

Figure 6