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Quantitation of estragole by stable isotope dilution assays

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ABSTRACT

Various calibration strategies for the quantitation of the phenylpropane estragole by gas chromatography-mass spectrometry were developed and compared. For application in stable isotope dilution assays, two deuterium labelled estragole isotopologues were synthesized. Of these, [3',3'-$^{2}H_{2}$]estragole was prepared by Wittig reaction of 4-methoxy-phenylacetaldehyde with [$^{2}H_{3}$]methyltriphenylphosphonium bromide, whereas [1'',1'',1''-$^{2}H_{3}$]estragole was obtained by demethylation of estragole and deuteromethylation of the resulting 4-allylphenole.

Besides estragole isotopologues, 1,2,4-trimethoxybenzene and 4-propylanisole were also tested as internal standards (I.S.) for the determination of estragole in fennel tea.

[1'',1'',1''-$^{2}H_{3}$]estragole, 1,2,4-trimethoxybenzene, and 4-propylanisole revealed linear calibration functions and, therefore, were suitable for estragole quantitation. In contrast to this, [3',3'-$^{2}H_{2}$]estragole could only be applied as I.S. if it was added to the extracts in stoichiometric deficiency compared to unlabelled estragol. Moreover, due to its different chemical and physical properties, 1,2,4-trimethoxybenzene showed a recovery as low as 77%, whereas the other I.S. revealed recovery rates close to 100 %. Considering the "real" values of estragole in fennel tea, the choice of the I.S. obviously is less important than the way of preparing the tea. In contrast to the common method for tea preparation, squeezing of the tea bags increased the estragole content significantly by 50%.

Key words: estragole; fennel; fennel tea; stable isotope dilution assay

1

1. INTRODUCTION

The phenylpropane estragole is a component existing in many herbs such as tarragon, basil, fennel, and anise. Recently, estragole has become a cause for concern, as methyleugenol, which has a similar structure to estragole, has been found to be a potent carcinogen (National Toxicology Program, 2000). For this reason, a new legal limit for estragole of 10 mg/kg in non-alcoholic beverages is discussed in the European Union (EU) (Commission of the EC, 2006), which will have to be monitored by accurate analytical methods.

The most commonly used method to quantify estragole is gas chromatography coupled either to flame ionization detection or to mass spectrometry. As the estragole content mainly is determined as its relative amount in the essential oil, its concentration in food is calculated indirectly from the content of essential oil (Fehr, 1982).

However, this methodology is susceptible to incomplete recovery or discrimination and is restricted to samples containing estragole in the mg/kg range or when simple matrices such as teas are analyzed. These obstacles can be overcome, when suitable internal standards are added to the respective food prior to or during extraction in order to compensate for losses during clean-up and detection. However, if the internal standard has a different structure than the analyte, discriminations or losses are likely to occur. The superiority of stable isotopically labelled analogues over other internal standards has been proved for numerous GC-MS (e. g. Bancon-Montigny, Maxwell, Yang, Mester & Sturgeon, 2002) or LC-MS (e. g. Stokvis, Rosing & Beijnen, 2005) applications. In general, recovery, specifity and precision is improved, as only an isotopologic ratio has to be measured, which usually is inert against physical or chemical processes.

The benefits of stable isotope dilution methodology has also been reported for other odorants (Rychlik & Bosset, 2001), mycotoxins (Rychlik & Schieberle, 2001), or vitamins (Rychlik & Freisleben, 2002).

The aim of the present study was, therefore, (1) to synthesize stable isotopologues of estragole and (2) to compare these compounds with structurally different internal standards for quantitation of estragole.

2. MATERIALS AND METHODS

Chemicals

Estragole was purchased from Aldrich (Steinheim, Germany). The following compounds were obtained commercially from the sources given in parentheses: 4-propylanisol (Lancaster, Mühlheim am Main, Germany) and 1,2,4-trimethoxybenzene (Aldrich, Steinheim, Germany). [1'',1'',1''-$^2H_3$]Estragole and [3',3'-$^2H_2$]estragole were synthesized by the following procedures.

Synthesis of [1'',1'',1''-$^2H_3$]estragole (Figure 1,): Estragole was demethylized by using a boron-tribromide-methyl sulfide-complex according to Williard and Fryhle (1980) and subsequently methylized with [$^2H_3$]methyl iodide (Masanetz, 1998).

*p-Allylphenole (**2**):* estragole (750 mg, 5 mmol) was added to a solution of boron-tribromide-methyl sulfide-complex (6 g, 19 mmol, Fluka, Neu Ulm, Germany) in 1,2-dichloroethane (100 ml, Merck, Darmstadt, Germany) under N_2-atmosphere. The yellow solution was refluxed at 89°C while the color changed from yellow to green and finally to black in the first 8 hours. The process of demethylation was monitored by thin layer chromatography (TLC; stationary phase: silica with fluorescence indicator, mobile phase: pentane/diethyl ether (80/20, v/v) , detection by fluorescence eradication) and the reaction was stopped after 30 hours refluxing by adding water (100 ml) after cooling to room temperature. Diethyl ether (100 ml freshly distilled, Merck, Darmstadt, Germany) was added and the mixture was stirred another 20 minutes followed by washing the organic phase successively with water and aqueous sodium hydrogencarbonate (1 mol/L). The synthesized allylphenol was isolated from the organic phase by extraction with aqueous sodium hydroxide (100 mL, 1 mol/L). After acidification of the alkaline phase to pH 2, (2) was extracted with diethyl ether. The product was then purified by column chromatography (silica 60, 230-400 mesh,

Merck, Darmstadt, Germany) using a pentane/diethyl ether gradient ranging from 95/5 (v/v) to 50/50 (v/v) followed by completely removing the solvent. The yield of **2** was 370 mg (2.8 mmol).

Mass spectrum (EI): m/z (relative intensity) 134 (100), 77 (80), 133 (78), 107 (60), 105 (50), 79 (30), 91 (28), 78 (25), 115 (20), 103 (17);

NMR spectrum: 3.3 (2, duplet, H-1'); 4.5 (1, singulet, OH); 5.0 (2, double-duplet, H-3'); 5.9 (1, multiplet, H-2'); 6.8 –7.1 (4, multiplet, H-2, H-3, H-5, H-6)

*[1'',1'',1''-$^{2}H_3$]estragole (**3**):* For methylation, p-allylphenole (320 mg, 2.4 mmol) and potassium carbonate (2.5 g, Aldrich, Steinheim, Germany) were dissolved in acetone (20 ml, LiChrosolv, Merck, Darmstadt, Germany) in a sealed flask and stirred at room temperature. After some minutes, [$^{2}H_3$]methyl iodide (1g, 6.9 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) and the title compound **3** was extracted with diethyl ether (50 ml) and purified by silica column chromatography (silica 60, 230-400 mesh, Merck) with a pentane/diethyl ether gradient ranging from 95/5 (v/v) to 70/30 (v/v) giving a total yield of 240 mg (1.6 mmol) with ^{1}H-NMR purity of 98%

Mass spectrum (EI): m/z (relative intensity) 151 (100), 150 (60), 124 (40), 117 (35), 77 (33), 78 (27), 105 (25), 79 (23);

Mass spectrum (CI, methanol): m/z (relative intensity) 152 (100), 151 (19), 153 (11), 150 (8), 124 (3)

NMR spectrum: 3.3 (2, duplet, H-1'); 5.0 (2, double-duplet, H-3'); 5.9 (1, multiplet, H-2'); 6.8 –7.1 (4, multiplet, H-2, H-3, H-5, H-6)

Synthesis of [3',3'-$^{2}H_2$]estragole: Oxidation of 2-(4-methoxyphenyl)-ethanol according to Dess and Martin (1983) yielded 4-methoxy-phenylacetaldeyde which

was converted into [3',3'-$^{2}H_2$]estragole in a Wittig reaction using [$^{2}H_3$]methyl-triphenyl-phosphonium bromide (Engel, 2002; figure 1).

*4-Methoxy-phenylacetaldeyde (***5***):* A suspension of 152 mg 2-(4-methoxyphenyl)-ethanol (1 mmol, Acros Organics) in dichloromethane (10 ml, freshly distilled, Merck, Darmstadt, Germany) was added dropwise to a solution of Dess-Martin-periodinane (424 mg ,1 mmol, 1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1H)-one, Lancaster) in dichloromethane (10 ml). The mixture was stirred for 2 hours at room temperature. Addition of sodium thiosulfate solution (20 ml, 1 mol/L in a saturated aqueous solution of sodium hydrogencarbonate) transferred the reduced Dess-Martin periodinane into a water soluble form. The organic phase was washed successively with saturated sodium hydrogencarbonate and water, then dried over anhydrous sodium sulfate and finally the solvent was completely removed. The obtained **5** was purified by silica column chromatography (silica 60, 230-400 mesh, Merck) with a pentane/diethyl ether gradient ranging from 95/5 (v/v) to 60/40 (v/v) to give a total yield of 60 mg (0.4 mmol).

Mass spectrum (EI): m/z (relative intensity) 121 (100), 150 (15), 77 (14), 91 (10), 122 (9), 78 (9)

NMR spectrum: 3.6 (2, duplet, H-1'); 3.8 (3, singulet, H-1'') 6.8 –7.1 (4, multiplet, H-2, H-3, H-5, H-6), 9.7 (1, singulet, CHO)

*[3',3'-$^{2}H_2$]estragole (***6***):* A suspension of [$^{2}H_3$]methyl-triphenyl-phosphonium bromide (330 mg, 0.92 mmol, Aldrich) in dry diethyl ether (30 ml, dried over NaH) was stirred under a nitrogen atmosphere. Then, a solution of butyllithium in hexane (350 µl, 0.88 mmol, 2.5 mol/l, Acros Organics) was added dropwise at room temperature and stirring continously for 10 minutes until the mixture turned deeply orange. Finally, 4-methoxy-phenylacetaldehyde (60 mg, 0.4 mmol) in dry diethyl ether (30 mL, freshly

distilled, Merck, Darmstadt, Germany) was added and the reaction mixture was stirred for 2 hours. A washing step with a solution of sodium dihydrogenphosphate (414 mg, 3 mmol, Merck, Darmstadt, Germany) in water (50 mL) stopped the reaction. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness at a temperature of 40°C. The crude product was purified by column chromatography on a RP 18 phase (LiChroprep RP 18, 25-40 µm, Merck) with a methanol/water gradient ranging from 30/70 (v/v) to 95/5 (v/v) to give a total yield of 308 µg (2 µmol) showing a purity of 95% determined by ^{1}H-NMR.

Mass spectrum (EI): m/z (relative intensity) 150 (100), 149 (94), 121 (50), 119 (37), 148 (32), 78 (31), 135 (25), 107 (25), 118 (23), 117 (18), 79 (17)

Mass spectrum (CI, isobutane): m/z (relative intensity) 151 (100), 150 (45), 149 (11), 152 (7), 121 (7)

NMR spectrum: 3.3 (2, duplet, H-1'); 3.9 (3, singulet, H-1''); 5.9 (1, triplet, H-2'); 6.8 – 7.1 (4, multiplet, H-2, H-3, H-5, H-6)

Quantification of estragole in fennel tea made from fennel fruits

Either 4-propylanisol, 1,2,4-trimethoxybenzene, [3',3'-$^{2}H_2$]estragole, or [1'',1'',1''-$^{2}H_3$]estragole were used as internal standards (I.S.) for quantification of estragole in fennel tea made from fennel fruits. Before analysis, the samples were analyzed for the I.S. to prove their absence in fennel. Fennel fruits (*Foeniculum vulgare* Mill. ssp. vulgare var. vulgare, Martin Bauer, Vestenbergsgreuth, Germany) were broken by a squeezer (Korn Quetsche, Eschenfelder, Hauenstein, Germany). Fennel tea was prepared by extracting 2.5 g broken fennel fruits with 150 ml boiling water for 10 minutes and subsequently filtrating the extract. The hot aqueous infusion was cooled to room temperature, solutions of the I.S. in dichloromethane were 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 and concentrated at 47 °C to 2 ml using a vigreux column (50 x 1 cm) and analyzed by HRGC/MS. Estragole was quantified by relative area counts of analyte and I.S. using the response factors presented in Table 1, which were determined by analyzing definite mixtures of analyte and I.S..

Quantification of estragole in fennel tea made from teabags

1,2,4-trimethoxybenzene and [3',3'-$^{2}H_{2}$]estragole in dichloromethane were used as internal standards for quantification of estragole in fennel tea made from teabags. For [3',3'-$^{2}H_{2}$]estragole, a mass ratio of estragole to the standard ranging from 1:1 to 4:1 had to be adjusted. Extraction was performed as described by the official German method (BVL, 1999; Zeller & Rychlik, 2006). Five fennel teabags (PhytoLab, Vestenbergsgreuth, Germany) were extracted with 1 liter boiling water in a sealed flask. After 5 minutes of extraction time, the infusion was stirred once. When the extraction time of 10 minutes was completed, the teabags were removed. In one batch the teabags were squeezed for removing any residual water, in another batch only the dripping residual water from the teabags was combined with the infusion. After cooling, the loss of water was compensated by adding water to the infusion. A 200 ml tea aliquot of the 1 liter infusion was spiked with the internal standards and filled up to 250 ml in a volumetric flask. 2 g sodium chloride was dissolved in a 10 ml aliquot of the spiked infusion and volatile compounds were extracted with 2 ml *tert*-butyl methylether. The organic phase was dried over anhydrous sodium sulfate and analyzed by HRGC/MS.

Determination of response factors

Solutions of estragole and the respective I.S. were mixed in five mass ratios ranging from 0.2 to 4 and the mixtures were subjected to HRGC/MS as outlined below. Response factors R_f were calculated according to following equation:

$$R_f = \frac{A_{I.S.}\ m_{estragole}}{A_{estragole}\ m_{I.S.}}$$

where $A_{estragole}$ is the area of unlabelled estragole in mass trace *m/z* = 149; $A_{I.S.}$ is the area of the I.S. in the mass trace given in table 1; $m_{I.S.}$ is the amount of added internal standard; $m_{estragole}$ is the amount of added unlabelled estragole.

Calculation for estragole quantification

Estragole contents C in fennel tea were calculated using the following equation:

$$C = \frac{A_{estragole}\ m_{I.S.}\ R_f}{A_{I.S.}}$$

where $A_{estragole}$ is the area of unlabelled estragole in mass trace *m/z* = 149; $A_{I.S.}$ is the area of the I.S. in the mass trace given in table 1; $m_{I.S.}$ is the amount of added internal standard; R_f is the response factor given in table 1.

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

Two GC/MS equipments were used for analysis of estragole.

The first one consisted of a gas chromatograph 5890 series II (Hewlett-Packard, Waldbronn, Germany) connected to a sector field mass spectrometer type MAT 95 S

(Finnigan, Bremen, Germany) using DB-FFAP capillary (30 m x 0.32 mm i.d., film thickness 0.25 µm, Fisons, Mainz, Germany).The samples were injected on-column at 40 °C. After 1 min the oven temperature was raised by 8°C/min to 230 °C as the final temperature. Helium was used as the carrier gas and the flow rate was set to 2 ml/min. Electron ionization (EI) was performed at 70 eV and chemical ionization (CI) at 115 eV using isobutane as reactant gas. This system was used for the analysis of the isotopologic distribution of labelled estragole.

The second instrument was used for quantification of estragole in fennel tea and consisted of a gas chromatograph (CP 3800, Varian, Darmstadt, Germany) coupled with an ion trap detector (Saturn 2000, Varian) running in the CI mode with methanol as reactant gas. 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, Fisons). 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.

^{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 mg/kg)

3. RESULTS AND DISCUSSIONS

Odorants such as estragole in fennel are analyzed most sensitively and simply by gas chromatography coupled to flame ionization detection or to mass spectrometry. As injection volumes in GC are hardly reproducible, methods for quantitation of absolute contents require the use of internal standards (I.S.), which have to be added to the material under study prior or simultaneously to extraction. As the I.S. should be structurally as similar as possible to the analyte, stable isotopologues of the analytes show best accordance of chemical and physical properties. Therefore, losses during extraction, clean-up and detection are best compensated for.

In a previous report on the flavour of fennel tea (Zeller & Rychlik, 2006), we quantified estragole by using 4-propylanisol as I.S. due to its structural similarity to estragole. Besides, the structurally related 1,2,4-trimethoxybenzene is also used as I.S. in an official method for the analysis of estragole in fennel tea, laid down in the collection of methods according to section 64 of the German food and feed law (BVL, 1999). For a comparison of these internal standards with SIDA we synthesized [3',3'-$^{2}H_{2}$]estragole and [1'',1'',1''-$^{2}H_{3}$]estragole. The synthesis of the former was accomplished by oxidation of 2-(4-methoxyphenyl)-ethanol and followed by Wittig reaction with [$^{2}H_{3}$]methyl-triphenyl-phosphonium bromide (Figure 1). The yield of [$^{2}H_{2}$]estragole was as low as 0.5 % and, in addition, analysis of the isotopologic distribution by ^{1}H-NMR revealed 15% unlabelled or singly labelled estragole besides labelled [$^{2}H_{2}$]estragole. Therefore, we developed an alternative method for the synthesis of labelled estragole, which consisted of demethylizing unlabelled estragole followed by methylizing the resulting 4-allylphenole with [$^{2}H_{3}$]methyliodide. The improved yield was 38% and ^{1}H-NMR and HRGC/MS analysis proved that labelling of [$^{2}H_{3}$]estragole was almost complete showing about 100% of the [$^{2}H_{3}$]isotopologue.

For calibration of the methods based on the different I.S., response curves were recorded by analyzing different mixtures of estragole with the respective I.S. by GC-MS. All curves apart from [$^2H_2$]estragole showed good linearity at least within mass ratios of estragole and its standard ranging from 0.2 to 5 and resulted in the response factors given in table 1.

Influence of different internal standards on the quantitation of estragole

After the absence of the I.S. in fennel tee made from broken fennel fruits has been proved, quantification of estragole revealed different recoveries using the four internal standards 4-propylanisol, 1,2,4-trimethoxybenzene, [$^2H_2$]estragole and [$^2H_3$]estragole (Table 1). The best recovery rate was achieved with [$^2H_3$]estragole (99 %) as I.S., whereas the use of 1,2,4-trimethoxybenzene resulted only in 77 % recovery of the estragole content in fennel tea. The latter value could be explained by the minor structural similarity of 1,2,4-trimethoxybenzene to estragole, which obviously causes a discrimination of estragole during extraction with dichloromethane. In Table 1 the response factors are given for all I.S.. Most I.S. were detected using response factors near the value 1.0, still [$^2H_2$]estragole showed a response factor of only 0.72 because of the incomplete isotopologic labeling. Moreover, we observed an additional spectrometric overlap as estragole's spectrum in chemical ionization revealed a 20 % abundance of the $[M]^+$ signal at *m/z* 148 relative to that of the protonated molecule $[M+1]^+$ at *m/z* 149 (figure 2). Therefore, the signal $[M]^+$ at *m/z* 149 of singly labelled estragole included in the [$^2H_2$]estragole material showed a total 10 % intensity and interfered with the signal $[M+1]^+$ of unlabelled estragole. This effect has a crucial impact when the standard is in stoichiometric excess, which forced us to use this standard only in stoichiometric deficiency compared to analyte amounts in the extracts. This problem could have

been solved by using extensive calculations, but we did not go to this trouble as we had access to the more suitably labelled [$^2H_3$]estragole (figure 2).

In fennel tea prepared from broken fruits the estragole content was determined in a range from 3.18 mg/l (4-propylanisol as I.S.) to 3.61 mg/l ([$^2H_2$]estragole as I.S.) including all recoveries (figure 3). Without considering the recovery rates, estragole contents were determined to range from 2.55 mg/l (1,2,4-trimethoxybenzene as I.S.) to 3.36 mg/l ([$^2H_2$]estragole as I.S.). Regarding precision, relative standard deviation ranged between 7 and 12 %. When recovery and precision data are considered, apparently, only for the analysis using 1,2,4-trimethoxybenzene as I.S., the recoveries have to be included in order to obtain a correct value for the estragole content.

In addition, the data were compared by the t-test, which assesses whether the means of two data sets are statistically different from each other (Gottschalk & Kaiser, 1982). The sets were obtained from quantifications using the different I.S. and included the respective recoveries (Table 2). A significant difference only can be seen between the use of [$^2H_2$]estragole and 4-propylanisol as I.S., whereas the other means do not differ significantly. The risk level of wrong testimony in these cases was slightly above or under 5%. Therefore, the use of different internal standards would not alter the correct quantitation of estragole in fennel tea.

Influence of different extraction methods on the quantitation of estragole

After the tests of different I.S., the influence of different extraction methods was analyzed by quantifying estragole in a fennel tea made from teabags. The resulting tea was not comparable with that made from broken fruits as the origin and dosage of the used fennel was different. The fennel tea from tea bags was prepared by extracting the bags with boiling water for 10 minutes, then the teabags were removed

either (1) by including the residual water from the dripping teabags or (2) by collecting the residual water after squeezing the teabags. The results of the quantitations are presented in Figure 4. In fennel tea, made from teabags by squeezing the bags before removing, the estragole content was found to be 0.83 mg/l (1,2,4-trimethoxybenzene as I.S.) and 0.79 mg/l ([$^2H_2$]estragole as I.S.), respectively. Furthermore, the content of estragole in fennel tea made from teabags that were not squeezed ranged from 0.56 mg/l (1,2,4-trimethoxybenzene as I.S.) to 0.55 mg/l ([$^2H_2$]estragole as I.S.), respectively. (In all quantifications the recoveries were included.) The relative standard deviation was only 4 % using 1,2,4-trimethoxybenzene as I.S., but 13 % and 11 % using [$^2H_2$]estragole as I.S.. The data show an additional extraction of 45 % of estragole when the teabags were squeezed before they were removed. Considering this fact, the influence of the internal standard seems to be less important, which was further evidenced by t-tests. The different extraction methods, but also the use of different I.S., was examined and the results are given in Table 3. Whether the teabags were squeezed or not squeezed could be distinguished significantly by a risk level lower than 1%. The use of different internal standards had no influence on the quantification of estragole (risk level higher than 5%). Although the latter results apparently are in contrast to the earlier measurements, showing a difference when using 1,2,4-trimethoxybenzene or [$^2H_2$]estragole as I.S. with a risk level lower than 5 %, the findings can be explained by the number of replicate analysis.

Conclusion

Our results indicate that for simple matrices such as fennel tea the choice of the internal standard is less important for an accurate quantitation. By contrast, for obtaining correct data for a tea prepared from herbs, the method of preparation and

extraction has a much bigger impact. Furthermore, for quantitation in more complex matrices such as bread or pesto, the labelled standards can be supposed to be a significant improvement in estragole analysis.

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Table 1: Fragment ions, response factors and recovery rate used for quantification of estragole (analyte) in fennel tea

Internal standard	m/z used for quantification in CI mode		response factor	recovery rate
	analyte	internal standard		
1,2,4-Trimethoxybenzene	149	169	0.88	77 %
4-Propylanisole	149	151	0.98	107 %
[3',3'-$^2H_2$]Estragole	149	151	0.72	93 %
[1'',1'',1''-$^2H_3$]Estragole	149	152	1.01	99 %

Table 2: Statistic evaluation by t-test of differences between the use of different internal standards

Comparison of two different internal standards (I.S.)		p [a)]	n [b)]	difference [c)]
1st I.S.	2nd I.S.			
1,2,4-Trimethoxybenzene	4-propylanisole	> 0.05	14	not detectable
1,2,4-Trimethoxybenzene	[$^2H_2$]estragole	< 0.05	14	apparent
1,2,4-Trimethoxybenzene	[$^2H_3$]estragole	> 0.05	14	not detectable
[$^2H_3$]Estragole	4-propylanisole	< 0.05	14	apparent
[$^2H_2$]Estragole	4-propylanisole	< 0.01	14	significant
[$^2H_2$]Estragol	[$^2H_3$]estragole	> 0.05	14	not detectable

[a] risk level of wrong testimony (Gottschalk & Kaiser, 1982) [b] number of replicate analyses [c] significance level (Gottschalk & Kaiser, 1982)

Table 3: Statistic evaluation by t-test of differences between the use of two different extraction methods and two different internal standards (I.S.)

Comparison of two different extraction methods and I.S.		p [a)]	n [b)]	difference [c)]
1st Method (I.S.)	2nd method (I.S.)			
Teabags squeezed (1,2,4-Trimethoxybenzene)	teabags not squeezed (1,2,4-trimethoxybenzene)	< 0.001	5	highly significant
Teabags squeezed ([$^2H_2$]Estragole)	teabags not squeezed ([$^2H_2$]estragole)	< 0.01	5	significant
Teabags squeezed (1,2,4-Trimethoxybenzene)	teabags squeezed ([$^2H_2$]estragole)	> 0.05	5	not detectable
Teabags not squeezed (1,2,4-Trimethoxybenzene)	teabags not squeezed ([$^2H_2$]estragole)	> 0.05	5	not detectable

[a] risk level of wrong testimony (Gottschalk & Kaiser, 1982) [b] number of replicate analyses [c] significance level (Gottschalk & Kaiser, 1982)

LEGEND TO THE FIGURES

Figure 1: Synthetic pathways leading to [1'',1'',1''-$^{2}H_3$]estragole (**3**) and [3',3'-$^{2}H_2$]estragole (**6**)

Figure 2: Mass Spectra of estragole (left), [3',3'-$^{2}H_2$]estragole (middle), and [1'',1'',1''-$^{2}H_3$]estragole (right) in chemical ionization (CI).

Figure 3: Estragole content in fennel tea from broken fruits depending on different internal standards (I.S.)

Figure 4: Estragole content in fennel tea from teabags depending on different extraction methods and internal standards (I.S.)

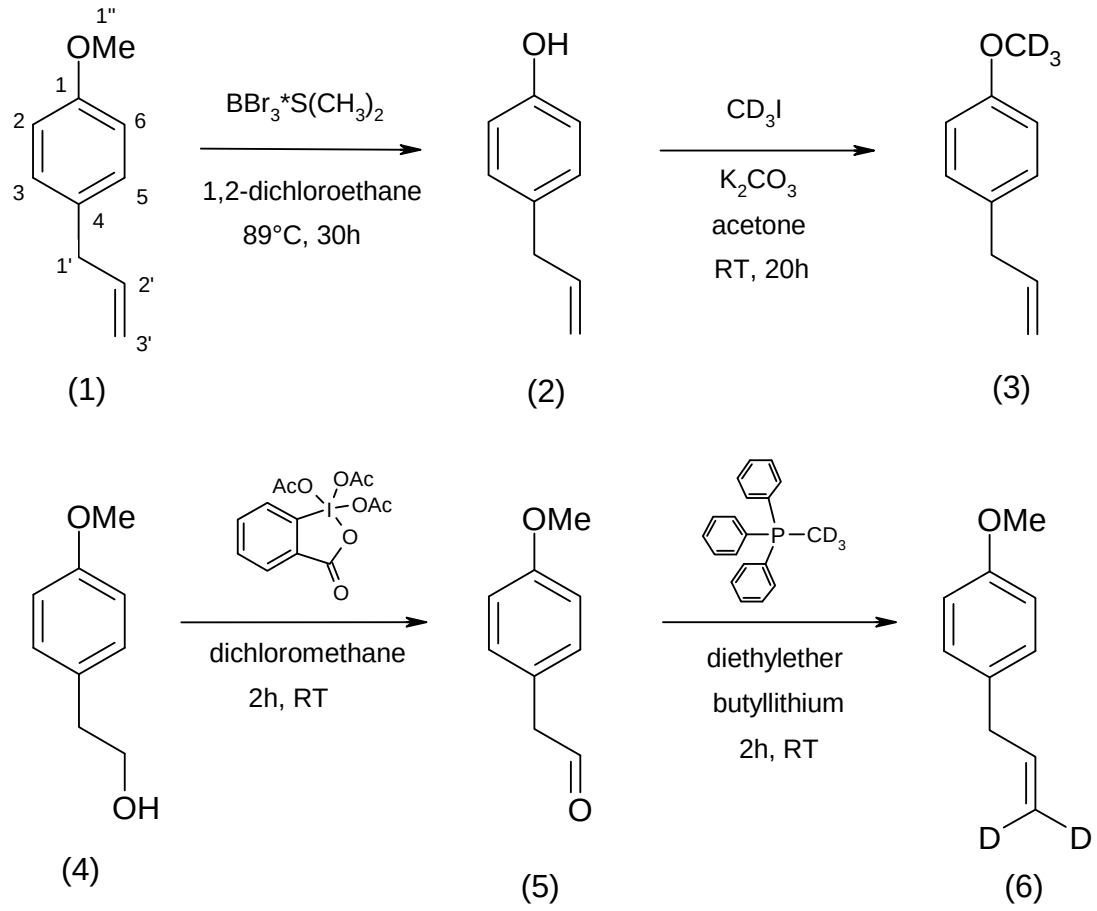
1"
OMe
1
2
6
3
5
4
1'
2'
3'
BBr3*S(CH3)2
1,2-dichloroethane
89°C, 30h
OH
CD3I
K2CO3
acetone
RT, 20h
OCD3
(1)
(2)
(3)
OMe
AcO
OAc
OAc
I
O
O
dichloromethane
2h, RT
OMe
P
CD3
diethylether
butyllithium
2h, RT
OMe
OH
O
D
D
(4)
(5)
(6)

Figure 1

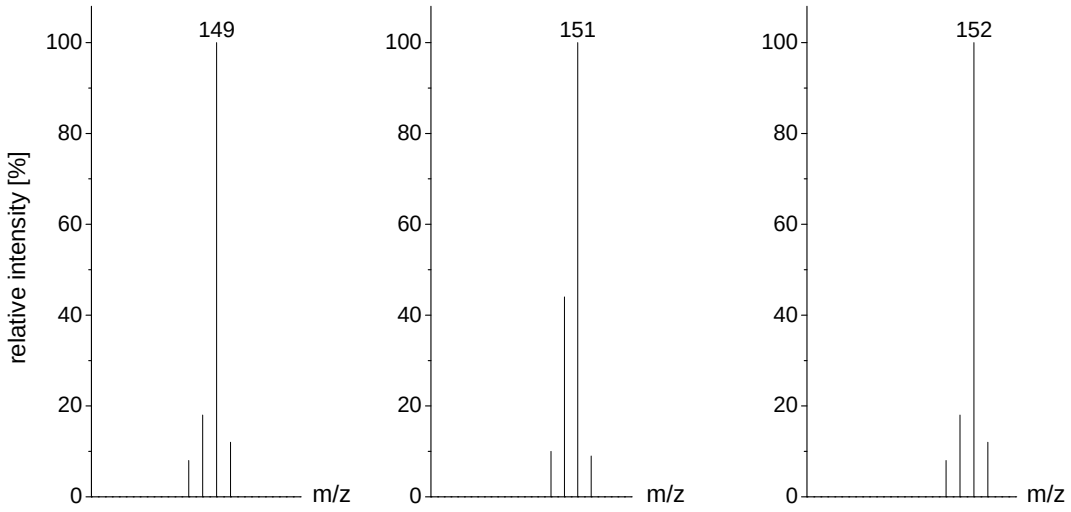

Figure 2

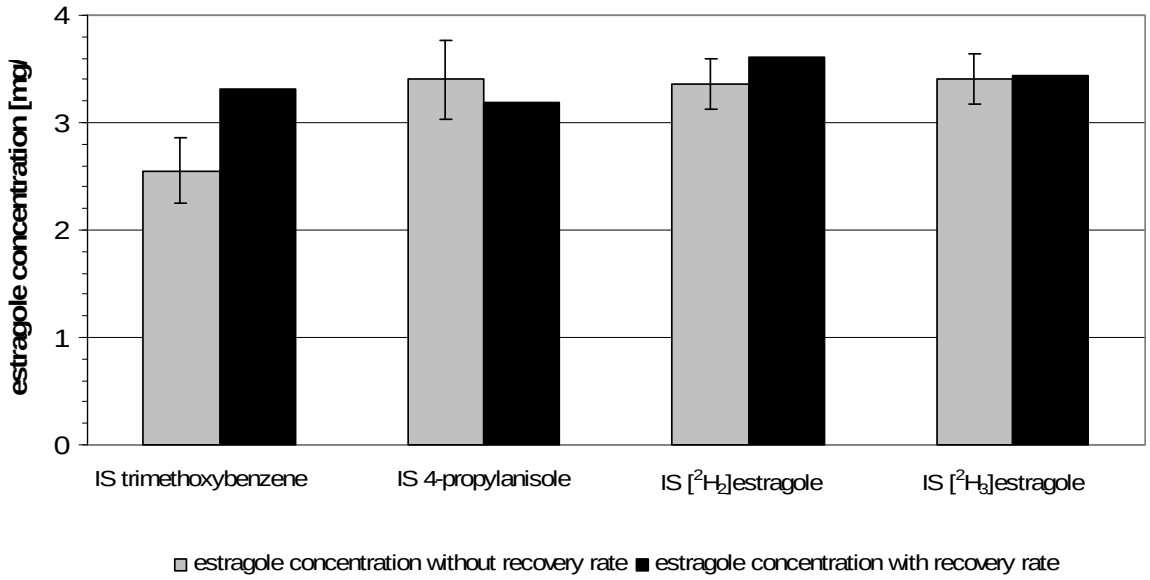
estragole concentration [mg]
4
3
2
1
0
IS trimethoxybenzene
IS 4-propylanisole
IS [2H2]estragole
IS [2H3]estragole
estragole concentration without recovery rate
estragole concentration with recovery rate

Figure 3

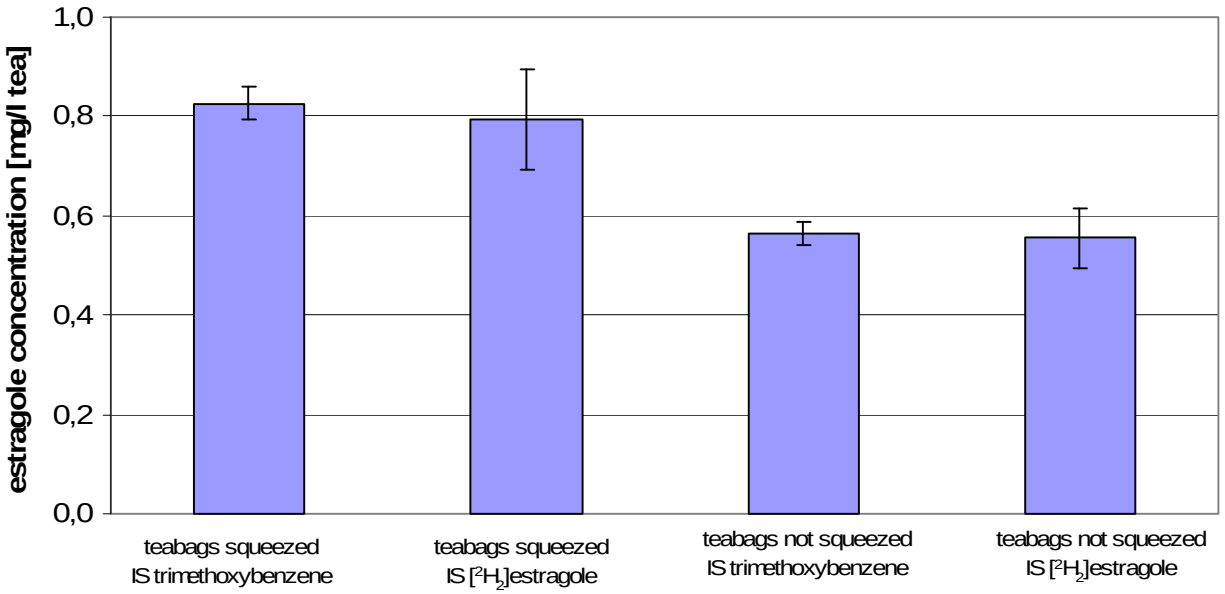
estragole concentration [mg/l tea]
1,0
0,8
0,6
0,4
0,2
0,0
teabags squeezed IS trimethoxybenzene
teabags squeezed IS [2H2]estragole
teabags not squeezed IS trimethoxybenzene
teabags not squeezed IS [2H2]estragole

Figure 4