Detection of *N*-acetyl-*S*-[3-(4-methoxyphenyl)allyl]-L-Cys (AMPAC) in Human Urine Samples after Controlled Exposure to Fennel Tea: A New Metabolite of Estragole and *trans*-Anethole

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Supporting Information

Table S1. Mass spectrometric parameters for the detection of the urinary *N*-acetyl-*S*-[3-(4-methoxyphenyl)allyl]-Cys (AMPAC) and the isotope-labeled standard [$^{13}C_3$,^{15}N]*N*-acetyl-*S*-[3-(4-methoxyphenyl)allyl]-Cys ([$^{13}C_3$,^{15}N]AMPAC) by MRM[a]

adduct	transitions		collision energy (V)	cell exit potential (V)
	parent ion	daughter ion		
AMPAC	**308**	**179**	- 20	- 20
	308	163	- 47	- 20
[$^{13}C_3$,^{15}N]AMPAC	**312**	**179**	- 20	- 20
	312	163	- 47	- 20

a Quantifier traces are marked in bold fonts.

In vitro incubation of 1'-acetoxyestragole with *N*-acetyl-L-Cys

A stock solution (A) of 100 mM 1'-acetoxyestragole was prepared from 20.6 mg (100 µmol) 1'-acetoxyestragole and 1 mL dimethyl sulfoxide. A second solution (B) of 100 mM *N*-acetyl-L-Cys was prepared from 16.3 mg (100 µmol) *N*-acetyl-L-Cys and 1 mL dimethyl sulfoxide. Aliquots of 50 µL solution A and 50 µL solution B were diluted in 900 µL 50 mM sodium phosphate buffer (pH 7.4) and gently stirred for 16 h at 37°C. The solution was diluted (1:9) with water and the presence of *N*-acetyl-*S*-[1-(4-methoxyphenyl)allyl]-L-Cys was studied using an Acquity I-Class UPLC system (Waters) coupled with a triple quadrupole hybrid ion trap mass spectrometer QTrap6500 (AB Sciex) via an electrospray ionization source operating in the negative mode. A single-ion recording method was used for the screening of the anticipated mass-to-charge ratio (m/z = 308) and a more sensitive MRM method for the monitoring of the expected constant neutral loss of 129 Da, which is specific for mercapturic acids (m/z = 308 $\rightarrow$ 179, compare Figure 1).

Quantification of estragole in the fennel infusion

A portion of the fennel infusion (20 mL) was mixed with 0.2 mL of a standard solution containing 200 mg/L 1,2,4-trimethoxybenzene in *tert*-butyl methyl ether (tBME) as an internal standard. The sample was diluted with water to a final volume of 100 mL. An aliquot of 10 mL was mixed with 2 g of sodium chloride, extracted with 2 mL tBME for 2 min and centrifuged for 15 min at 3,500 rpm. The organic phase was dried over anhydrous sodium sulfate and analyzed via gas chromatography (GC) coupled with mass spectrometry (MS). The estragole content in the fennel infusion was calculated using eight external calibration samples containing a fixed concentration of the internal standard 1,2,4-trimethoxybenzene (2 mg/L) and varying concentrations of estragole (0 – 6.4 mg/L) in tBME. Exemplary data on the calibration for the calculation of the estragole content are shown in Figure S1.

GC-MS analysis was performed with an Agilent 7890A gas chromatograph connected to an Agilent 5975 C mass spectrometer (Agilent, Böblingen, Germany) operating in electron impact (EI) mode. The MultiPurpose Autosampler (MPS 2; Gerstel, Mülheim, Germany) was controlled by the Maestro software (Gerstel). A RTX-CL pesticides proprietary cross-bond phase column (30 m, 0.25 mm i.d., 0.25 µm film thickness; Restek, Bad Homburg, Germany) was used to separate the analytes. Aliquots of 2 µL of each sample were injected via splitless injection. The injector temperature was set to 220°C. The oven temperature program was as follows: 40°C for 2 min, linear increase from 40°C to 240°C with 6°C per min, 240°C for further 6 min. The interface temperature was set to 260°C. Helium was used as carrier gas (70 kPa). The mass detector was operated in the single ion monitoring mode. Ions used as quantifiers were *m/e* = 148 and *m/e* = 168 for estragole and 1,2,4-trimethoxybenzene, respectively. The ions *m/e* = 147 and *m/e* = 117 were used as qualifiers for estragole and the ion *m/e* = 153 as a qualifier for 1,2,4-trimethoxybenzene.

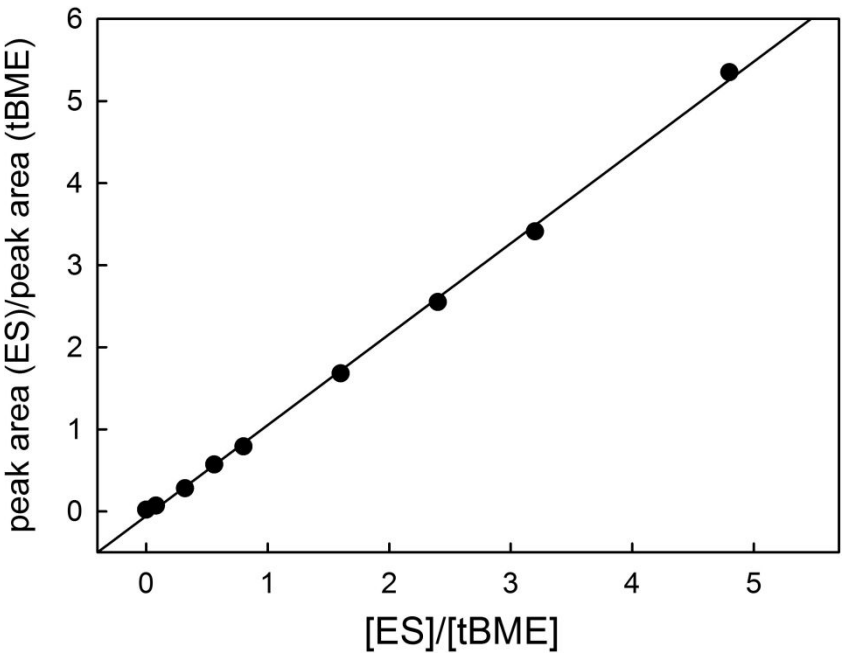


Figure S1. Exemplary data set for the quantification of estragole in fennel infusions applying the method from the German Food and Feed Code (LFGB) (BVL L 47.08-3: 2006-09) [1]. The upper panel shows the external calibration from eight concentrations of estragole (ES) between 0.16 mg/L and 6.4 mg/L and a fixed amount of 2.0 mg/L of the internal standard 1,2,4-trimethoxybenzene (tBME) (r^2 = 0.999). The substances were monitored by GC-MS as described. The estragole concentrations were determined from the ratio of peak area (ES)/peak area (tBME) of the calibration line (upper panel).

Quantification of *trans*-anethole in fennel tea

The content of *trans*-anethole in fennel tea infusion was determined using a standard addition procedure. A tea sample (0.5 mL) was diluted with methanol to a final volume of 25 mL. In separate batches, 0.5 mL of the tea sample was mixed with 0.075 mL, 0.150 mL or 0.225 mL of *trans*-anethole standard solution (1000 mg/L in methanol) and diluted with methanol to a final volume of 25 mL. Standard addition resulted in added concentrations of 3, 6 or 9 mg/L. All samples were prepared in duplicate. Samples were analyzed via a UPLC-UV system (Acquity I-Class UPLC with PDAeλ diode array detector, Waters). Five µL of each sample was injected onto a HSS T3 column (1.8 µm, 2.1 x 100 mm, Waters). The analyte was eluted using water (solvent A) and methanol (solvent B) at a constant flow rate of 0.35 mL/min. A 4-minutes linear gradient from 65 % solvent B to 85 % solvent B was applied. Colum temperature was set to 30°C. The diode array detector was run from 200 nm - 400 nm. Final chromatograms used for quantification of *trans*-anethole were extracted at 257 nm. Data acquisition and handling were performed with Analyst 1.6.2 software (AB Sciex).

No complete validation was carried out as standard addition showed good linearity, the resulting chromatographic areas of the added compound were comparable with similar concentrations in solvent and the signal intensity of the analyte in the tea infusion sample was far above the estimated detection limit. An external standard curve of *trans*-anethole in methanol showed good linearity at least between 0.5 and 48 mg/L.

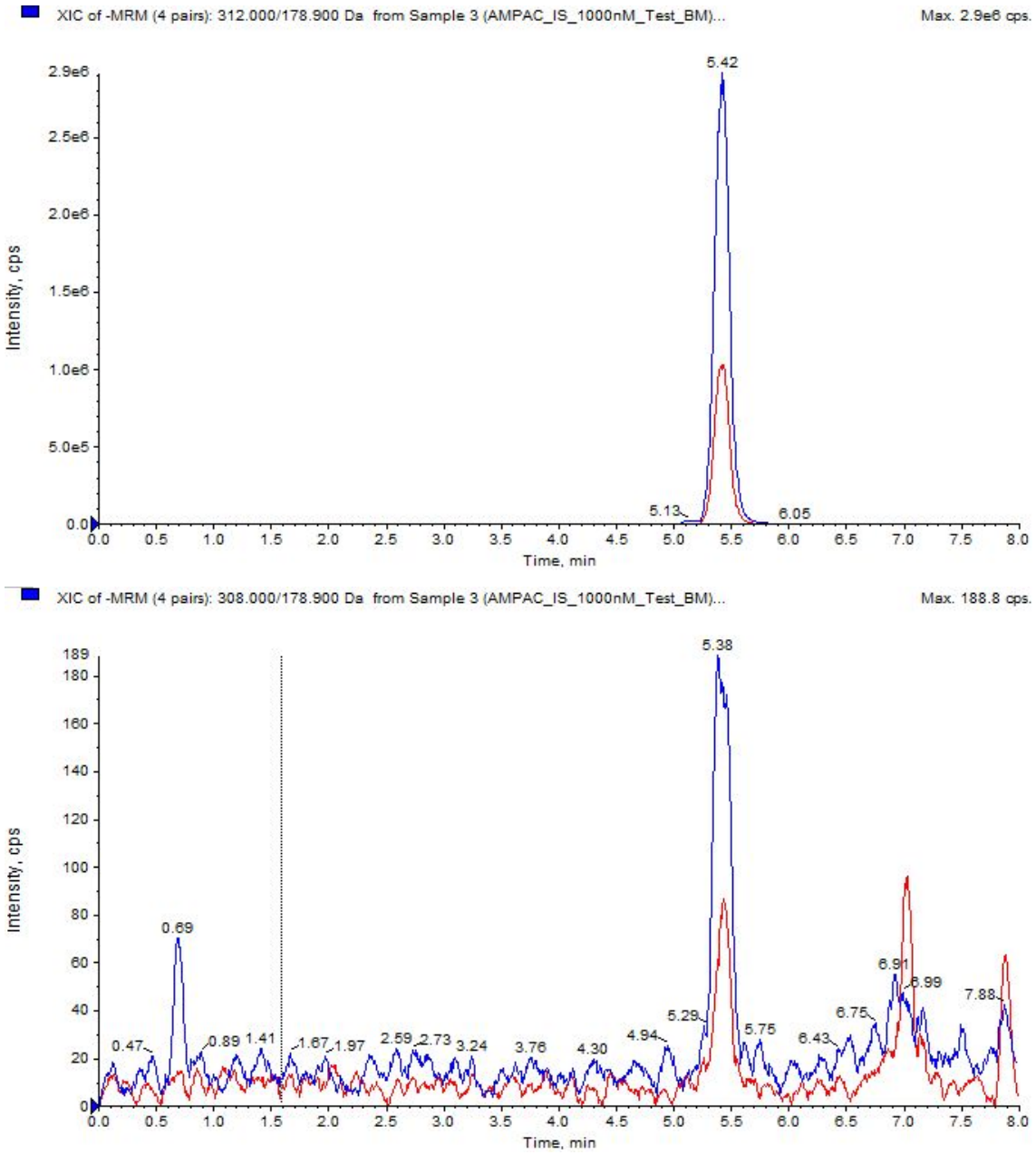


Figure S2. UPLC-MS/MS chromatograms resulting from injection of 8 pmol [$^{13}C_3$,^{15}N]AMPAC (upper panel: *mz* = 312.0 → 178.9, blue trace) without urine biomatrix. The recording of the quantifier of the analyte with *mz* = 308.0 → 178.9 showed the impurity of AMPAC in the sample (lower panel, blue trace). The calculation of the ratio of peak areas AMPAC/[$^{13}C_3{}^{15}N$]AMPAC ($1.95 \cdot 10^3 / 2.68 \cdot 10^7 = 72.6 \cdot 10^{-6}$) allowed estimating that the injection was contaminated with 0.58 fmol AMPAC. The presence of this impurity in the internal standard did not interfere with the quantification of AMPAC in urine samples.

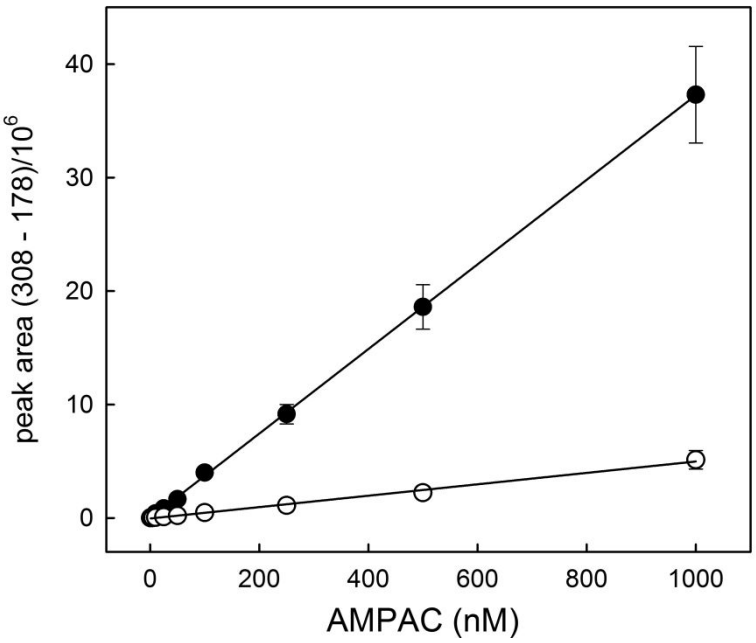
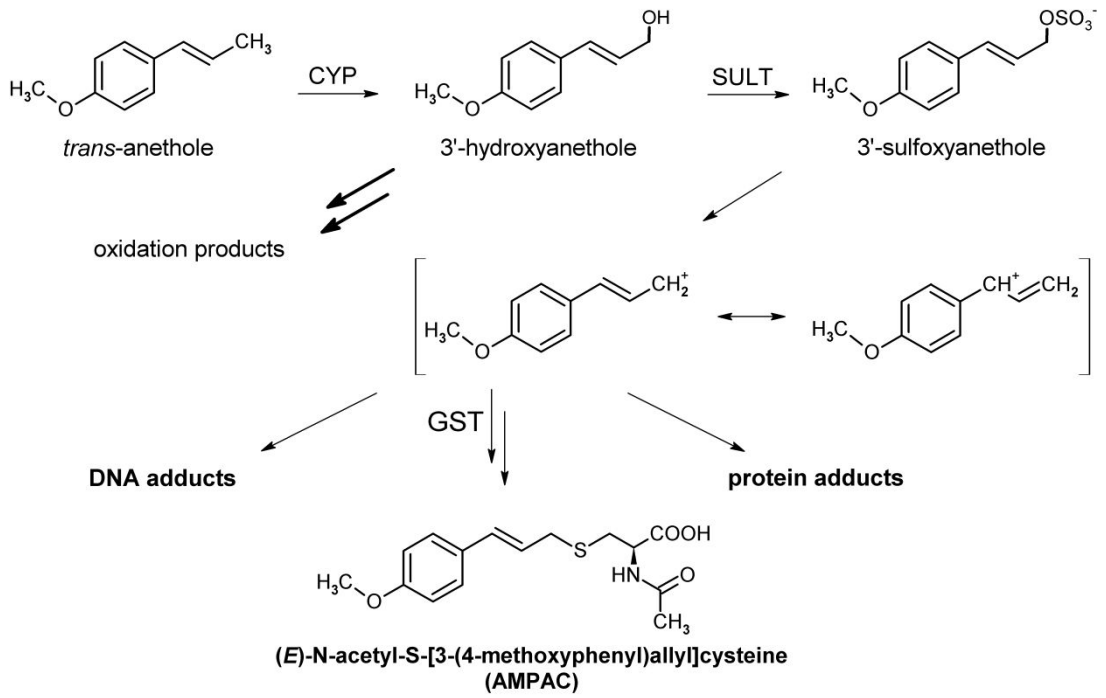


Figure S3. Peak areas (*m/z* = 308 → 179) recorded by direct injection of 16 samples of AMPAC with concentrations in the range of 0.01 nM and 1000 nM (black dots). The mean of four dilutions (± SD) was fitted with a trend line by least-squares linear regression (r^2 = 0.999). AMPAC was also measured in the same solutions prepared in the presence of processed ´blank` urine sample (pooled urine of six individuals; white dots). The mean (n = 4 ± SD) was also fitted by linear regression (r^2 of > 0.999). The comparison of the two slopes allowed estimating the matrix-dependent reduction of the mass spectrometric signal (86 %).



Scheme S1. Hypothetical bioactivation of *trans*-anethole via 3'-hydroxyanethole to 3'-sulfoxyanethole. Release of the sulfate ion leads to a reactive carbocation with the same structure as that formed from bioactivation of estragole (compare Scheme 1). Thus, the shown bioactivation pathway may also lead to the formation of *N*-acetyl-*S*-[3-(4-methoxyphenyl)allyl]-L-Cys (AMPAC) or other conjugates and adducts possibly formed by estragole.

Reference

(1) Federal Office of Consumer Protection and Food Safety (BVL) (2006) L 47.08-3: Bestimmung von Estragol in Aufgüssen aus Fenchel und anderen teeähnlichen Erzeugnissen, GC–MS-Verfahren, In *Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB*, Beuth Verlag GmbH, Berlin (Germany).