Development of a Stable Isotope Dilution LC-MS/MS Method for the Alternaria Toxins Tentoxin, Dihydrotentoxin and Isotentoxin

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Abstract For the Alternaria toxins tentoxin, dihydrotentoxin and isotentoxin, a stable isotope dilution LC-MS/MS method was firstly developed. Triply deuterated internal standards were prepared via total synthesis and introducing the labels in the last step before cyclization. Method validation was carried out by using potato starch, tomato puree and white pepper powder as blank matrices. For the three toxins the limits of detection ranged from 0.10 to 0.99 µg/kg. The inter- /intra-day relative standard deviations of the method were below 8.8 % and the recoveries ranged between 98 and 115 %. Although cyclic peptides are known to show only negligible fragmentation, a low limit of detection was achieved with the optimization of mass spectrometry parameters and cleanup on C18-phenyl SPE columns providing a more selective binding of these phenyl-containing cyclic peptides. The method was applied to 103 food samples including bread, cereals, chips, juice, nuts, oil, sauce, seeds and spices. Of these, 85 % were contaminated with tentoxin and 55 % were contaminated with dihydrotentoxin, whereas isotentoxin was not quantifiable. Maximal concentrations of tentoxin and dihydrotentoxin were 52.4 and 36.3 µg/kg, respectively, and were both detected in paprika powder.

Keywords tentoxin; dihydrotentoxin; isotentoxin; *Alternaria*; mycotoxin; stable isotope dilution assay; LC/MS-MS; synthesis

Introduction

The cyclic tetrapeptide tentoxin (cyclo[*N*-methyl-L-alanyl-L-leucyl-(*Z*)-α,β-dehydro-*N*-methylphenylalanylglycyl], TEN) **1** is a secondary metabolite produced by some Alternaria species, such as *Alternaria alternata (*syn. *Alternaria tenuis)* [1] and *Alternaria citri*.[2] It is considered a phytotoxin and induces species-selective chlorosis. As its mode of action, inhibition of photophosphorylation due to non-specific inhibition of ATPase coupling factor 1 is assumed. [3–5] Metabolism of TEN by P450-3A *in vitro* showed hydroxylated and demethylated metabolites, and during demethylation, isomerization of the dehydrophenylalanin occurred. [6]

Along with TEN, dihydrotentoxin (DHT, **2**) and isotentoxin (isoTEN, **3**) were also isolated as metabolites of Alternaria species. Their structures differ at the unsaturated bond of the N-methyldehydrophenylalanine moiety, which is hydrogenated into a single bond in DHT and is in *E* configuration in isoTEN (**Fig. 1**). Regarding phytotoxicity, isoTEN and DHT showed a much weaker chlorosis effect, which indicated the important role of the double bond. [2, 7] However, considering toxicity to mammals, no data are available. Therefore, the European Food and Safety Authority (EFSA) applied for its preliminary risk assessment the concept of thresholds of toxicological concern (TTC) for TEN, which was set to 1500 ng/kg body weight per day.[8]

For analysis of these metabolites, a LC-UV method for TEN was described by Suemitsu *et al*., 1991. [9] A LC-MS method using external calibration was published by Horiuchi *et al*., 2003 [10] for simultaneous identification of TEN, DHT and isoTEN from the culture liquid. Most recently, a semi-quantitative analysis of TEN in moldy

food samples via LC-MS/MS within their multi-mycotoxin screening without clean-up was developed by Sulyok *et al*., 2007. [11]

Until now, there is very limited analytical data of those toxins in food. Therefore, the purpose of this study was to develop a method to determine the toxins at low concentrations in complicated food matrices. We intended therefore, to establish a stable isotope dilution assay (SIDA) and an efficient sample purification. For other mycotoxins such as the Alternaria toxins alternariol, alternariolmethyl ether, tenuazonic acid and the Fusarium toxins T2 and HT toxin the SIDA approach proved to be a preferable way to obtain accurate results. [12-14]

Materials and Methods

Chemicals and Reagents. L-Glycine, N,N'-dicyclohexylcarbodiimide (DCC), N-[(1,1-dimethylethoxy)carbonyl]-L-leucine (Boc-Leu-OH), 1-hydroxybenzotriazole (HOBt), benzaldehyde, triethylamine (TEA), 4-(dimethylamino)pyridine (DMAP), O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU), trifluoroacetic acid (TFA), thionyl chloride, potassium carbonate, zinc, iodmethane-d_3 (>99.5 atom% D) and tentoxin (>99 %) were obtained from Sigma-Aldrich (Steinheim, Germany); Boc-N-methyl-L-alanine was obtained from Alfa Aesar (Karlsruhe, Germany). The solvents were from Merck (Darmstadt, Germany) and at least of analytical-reagent grade.

Preparative HPLC

Product purification was performed on a LaChrom HPLC system (Merck/Hitachi, Tokyo, Japan).

^{1}H-NMR

The structures of purified compounds were characterized by ^{1}H-NMR on a Bruker AV III system (Bruker Rheinstetten, Germany) operating at a frequency of 500.13 MHz. All compounds were dissolved in $CDCl_3$.

Syntheses

[^{2}H]$_3$-Tentoxin. ***cyclo (N-Methyl-L-alanyl-L-leucyl-N-d$_3$-Methyl- (Z) dehydrophenylalanylglycyl)*** : [^{2}H]$_3$-TEN was prepared from the starting product glycine (**Fig. 2**) and followed a route reported by Loiseau *et al.* [15] with some modifications. The deuterated methyl group at dehydrophenyalanine was introduced before cyclization of the peptide. In contrast to Loiseau *et al.*, [15] the reactions were carried out in a milligram scale, so the solvent volume was adjusted and some reaction times were reduced.

Synthesis of Boc-Leu-Gly-OMe (**6**): L-glycine (**4**) (100 mg) was dissolved in dry methanol (10 mL) and cooled to 0°C. 2.7 mL of thionyl chloride (3 equiv) was added dropwise. The solution was stirred vigorously at 0 °C for 2 h and then at room temperature overnight. Subsequently, the solvent was removed under reduced pressure and the crude product was dissolved in water (10 mL). The solution was alkalized to pH 10 with ammonium hydroxide (25 %) and extracted with diethyl ether (2×10 mL). The extract was then washed with brine and dried over sodium sulfate. After removing the solvent, glycine methyl ester (**5**) (105 mg) was obtained as a colorless oil. For peptide coupling, Boc-Leu-OH (231 mg) was dissolved in dried tetrahydrofuran (4 mL) and cooled at 0°C. Then, HOBt (150 mg) and DCC (206 mg) was added carefully, and the reaction mixture was stirred for 30 min. Thereafter, 90

mg of **5** in dichloromethane (DCM, 2 mL) was added dropwise before bringing the reaction mixture to room temperature and stirring overnight. Then, the suspension was filtered and the filtrate was evaporated. The crude product was dissolved in ethyl acetate (EtOAc, 10 mL), and washed with 5 % sodium hydrocarbonate (10 mL), 5 % citric acid (10 mL) and water (10 mL). After removing the solvent, white solid **6** (150 mg) was obtained.

Synthesis of Boc-Leu-Gly oxazolone (**7**): **6** (150 mg) was deprotected with sodium hydroxide (3 equiv) for 3 h to obtain Boc-Leu-Gly-OH (100 mg) as a colorless oil. The latter was dissolved in DCM (6 mL) and DCC (65 mg) was added. After 20 min the reaction was stopped by filtering the suspension. The filtrate was evaporated to give the target compound **7** as a colorless oil (88 mg).

Synthesis of Boc-Leu-(Δ^Z)Phe oxazolone (**8**): **7** (88 mg) generated in the last step thereafter was immediately dissolved in DCM (2 mL) under a nitrogen atmosphere. Benzaldehyde and TEA (33 µL) were added. The solution was stirred overnight and, after removing the solvent, **8** (53 mg) was obtained as a yellow oil.

Synthesis of Boc-Leu-(Δ^Z)Phe-Gly-OMe (**9**): **8** (53 mg) was first dissolved in DCM (4 mL) and TEA (30 µL, 1.5 equiv), then, glycine methyl ester (15 mg) and a catalytic amount of DMAP were added and the reaction mixture was left overnight. After removing the solvent, the crude product was dissolved in EtOAc (5 mL) and washed. Subsequently, the product was concentrated and purified on a short silica gel column (5 cm) by elution with variable mixtures of EtOAc and n-pentane. A colorless oil was obtained as the product **9** (17 mg).

Synthesis of Boc-MeAla-Leu-(Δ^Z)Phe-Gly-OMe (**10**): After reaction with TFA (500 µL) in DCM (500 µL) for 15 min, the deprotected tripeptide was dissolved in acetonitrile (3 mL). Dried HBTU (18 mg), TEA (15 µL) and Boc-N-methyl-L-alanine (9 mg) were added. After 30 min, the reaction was stopped by evaporating the solvent and after purifying the residue dissolved in EtOAc by elution from a silica gel column (5 cm) with a mixture of ethyl acetate and n-pentane, **10** as a light yellow oil (13 mg) was yielded.

Synthesis of Boc-MeAla-Leu-C[^{2}H]$_3$ (Δ^Z)Phe-Gly-OMe (**11**): **10** (13 mg) was dissolved in dimethylformamide (DMF, 2 mL). Thereafter, potassium carbonate (14 mg, 4 equiv) and iodmethane-d_3 (2 µL) was added. The mixture was stirred for 16 h under nitrogen atmosphere. The product was purified on a silica gel column by elution with EtOAc to give **11** as a red oil (13 mg).

Synthesis of [^{2}H]$_3$-TEN: After deprotection with NaOH and TFA, **11** (13 mg) was dissolved in DMF (6 mL). TEA (10µL, 3 equiv) and dried HBTU (9 mg) were added and the solution was stirred for 30 min. The solvent was then removed and the product was purified first on a silica gel column and then on a Synergi Hydro RP column (250 x 3 mm, 4 µm, 80 Å, Phenomenex, Aschaffenburg, Germany) using acetonitrile-water (3:7, v/v) as the mobile phase. [^{2}H]$_3$-TEN (1 mg, > 99 % pure) was pooled from several runs by detection at 280 nm. Yield of raw and purified [^{2}H]$_3$-TEN was 2 % and 0.24 %, respectively.

MS^2 ions (*m/z*, negative): 416 (deprotonated molecule as precursor), 372, 274, 249, 217, 141; (*m/z*, positive): 418 (protonated molecule as precursor), 361, 333, 315, 305, 220, 199.

UV (MeOH): λ_{max}= 282 nm.

^{1}H-NMR (500 MHz, $CDCl_3$) δ= 0.55 (br. s., 3 H, δ-H, Leu), 0.65 (d, *J*=6.41 Hz, 3 H, δ-H, Leu), 2.87 (br. s., 3 H, N-CH_3, Ala), 7.39 - 7.46 (m, 5 H, C_6H_5, Phe), 7.77 (br. s., 1 H, N-H, Gly).

Isotentoxin. ***cyclo (N-Methyl-L-alanyl-L-leucyl-N-methyl- (E) -dehydrophenylalanylglycyl)***: 250 µL of 1 mg/mL (methanol) TEN was radiated with UV at 254 nm for 2 h.[7] As there was an equilibrium between isoTEN-TEN (1:1), isoTEN could be completely separated from the educt TEN on a Synergi Hydro RP column using an isocratic elution system of acetonitrile-water (3:7, v/v). The purified isoTEN was quantified via quantitative NMR with its signal at 3.27 ppm, using certified glucose as standard for the external calibration. The absolute yield was 23.55 µg (9.4 %).

MS^2 ions (*m/z*, negative): 413 (deprotonated molecule as precursor), 369, 271, 246, 214, 141; (*m/z*, positive): 415 (protonated molecule as precursor), 358, 330, 312, 302, 217, 199.

UV (MeOH): λ_{max}= 280 nm.

^{1}H-NMR (500 MHz, $CDCl_3$) δ= 2.93 (br. s., 3 H, N-CH_3, Ala), 3.27 (br. s., 3 H, N-CH_3, Phe), 7.39 - 7.46 (m, 5 H, C_6H_5, Phe).

Dihydrotentoxin. ***cyclo (N-Methyl-L-alanyl-L-leucyl-N- methyl-phenylalanylglycyl)***: 250 µL of 1 mg/mL (methanol) TEN was dissolved in 0.5 mL water. To this solution, 4 mg of zinc dust moistened with water was added. 0.5 mL formic acid was added and the solution was stirred vigorously. Then, HCl (0.5 mL, 0.5 mol/L) was added dropwise upon stirring. Afterall zinc was dissolved, the reaction mixture was directly

transferred onto an activated 100 mg C18-phenyl SPE column (Phenomenex, Aschaffenburg, Germany), washed with three column volumes of distilled water and eluted with methanol. The product was further purified on a Synergi Hydro RP column with the same conditions as described above and was quantified via q-NMR with its signal at 2.85 ppm. The total yield was 11.01 µg (4.4 %).

MS^2 ions (m/z, negative): 415 (deprotonated molecule as precursor), 371, 273, 248, 216, 141; (m/z, positive): 417 (protonated molecule as precursor), 360, 332, 314, 304, 219, 199.

^{1}H-NMR (500 MHz, $CDCl_3$) δ= 0.79 (br. s., 3 H, δ-H, Leu), 0.81 (br. s., 3 H, δ-H, Leu), 2.85 (br. s., 3 H, N-CH_3, Ala), 2.91 (br. s., 3 H, N-CH_3, Phe).

$[^2H]_3$-isoTEN and $[^2H]_3$-DHT were synthesized from $[^2H]_3$-TEN the same way as unlabeled isoTEN and DHT described above in equivalent yields.

Quantitative NMR

The method of quantitative NMR for TEN, isoTEN and DHT was similar to that described by Korn *et al*. [16] Briefly, the purified compounds were dissolved in 300 µL of chloroform-d_3 (Euriso-top, Gif sur Yvette Cedex, France) in 3 x 103.5 mm NMR tubes (Norell, ST500-7, Landisville, USA), and soon afterwards analysed on the Bruker AV III system. Saccharose (>99%) from Sigma-Aldrich (Steinheim, Germany) of known concentration was used as external standard. For quantitation, the anomer proton signal at 5.42 ppm (doublet, couplings constant 3.9 Hz) was chosen; for TEN and isoTEN the protons of the methyl group on the nitrogen of the Me(*Δ*)Phe giving signals at 3.21 und 3.27 ppm respectively, were chosen. For DHT the protons of the

methyl group at the nitrogen of MeAla giving a signal at 2.85 ppm were chosen. Peaks were integrated manually.

Preparation of standard solutions

Stock solutions of labeled and unlabeled toxins were prepared in concentrations of 10- 50 µg/mL in methanol. The absolute amounts of unlabeled TEN was determined on a UV spectrometer Specord 50 (Analytik Jena, Jena, Germany) with ε= 20700 at the maximum absorption wavelength of 282 nm, [17, 18] whereas unlabeled isoTEN and DHT were determined by q-NMR as mentioned before. Concentrations of labeled compounds were determined by HPLC-UV at the HPLC conditions detailed for LC-MS/MS but using a UV detector set at 282 nm and using the unlabeled compounds as references. For calibration, stock solutions were further diluted to 1 µg/mL and 0.1 µg/mL.

LC-MS/MS

LC-MS/MS was performed on a Shimadzu LC-20A Prominence system (Shimadzu, Kyoto, Japan) using a Hyperclone BDS C18 column (150 x 3.2 mm, 3 µm, 130 Å, Phenomenex, Aschaffenburg, Germany). The binary gradient system consisted of (A) water and (B) acetonitrile- isopropanol (50: 50, v/v) at a flow rate of 0.2 mL/min. The gradient started at 35 % B and was raised linearly from 35 % B to 80 % B during the first 15 min, then to 100 % B during the next 2 min and was maintained at 100 % B for 2 min. Thereafter, the mobile phase returned to 35 % B within 2 min and the system was equilibrated for 5 min before the next run. The injection volume was 10 µL.

The LC was interfaced to a hybrid triple quadrupole/linear ion trap mass spectrometer (API 4000; Applied Biosystems Inc., Foster City, CA, USA) operated in the negative ESI mode. The ion source parameters were set as follows: curtain gas 15 psi, CAD gas pressure high, ion spray voltage -4500 eV, spray gas 45 psi, dry gas 55 psi, temperature 450°C. MS parameters were optimized by direct infusion of each standard solution (50 ng/mL) into the source. For MS/MS measurements, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode at the conditions detailed in **Tab. 1**. A valve was used to divert the column effluent to the mass spectrometer from 10 to 15 min and to waste for the rest of the run. Data acquisition was carried out using Analyst 1.5 software (Applied Biosystems Inc., Foster City, CA, USA).

Calibration and quantitation

A series of solutions with constant amounts of internal standard (S) and varying amounts of analyte (A) in molar ratios between 0.1 to 10 (1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1) were prepared for the calibration curves of TEN and isoTEN. Peak area ratios [A(A)/A(S)] were then obtained via LC-MS/MS, and a response curve was calculated from molar ratios [n(A)/n(S)] versus [A(A)/A(S)]. Calibration functions were obtained using simple linear regression and revealed a negligible intercept. The slopes of the calibration functions are equivalent to the response factors and were 0.95 and 1.07 for TEN and isoTEN, respectively. The small deviation from the ideal response factor of 1.0 can be attributed to imprecision of the concentration determination by HPLC-UV. For DHT, the adjustment of accurate concentrations of the labeled standard was not possible due to the low yield of the synthesis and the poor UV absorption of DHT. As for TEN and isoTEN the response factors close to 1.0 indicate the absence of

isotope effects and spectral interferences and the structure of DHT being very similar to the other compounds, its response factor was assumed to be 1.0.

Sample preparation

For solid foods, the fine ground samples (0.5 g of spice, 2 g of sauce and 1 g of the other food samples) was spiked with a mixture (40 µl) of $[^2H]_3$-TEN (25 ng/mL) and $[^2H]_3$-DHT (15 ng/mL). $[^2H]_3$-isoTEN (40 µl, 25 ng/mL) was only spiked to apple chip samples. Then, 8 mL of acetonitrile-water (84:16, v/v) was added to the sample and the mixture was shortly vortexed and shaken in a laboratory shaker at 200 rpm for 1.5 h. Subsequently, the sample was centrifuged at 1000 *g* for 10 min

For tomato ketchup and paprika powder, separate cleanup prior to SPE was necessary. To the supernatant of the centrifuged tomato ketchup extract, water (4 mL) and sodium chloride (2g) were added to separate phases and the lower phase was discarded. For paprika powder, n-hexane (8 mL) was added to the supernatant of the centrifugation and the upper hexane phase was discarded after vortexing.

The supernatant or the purified phase was evaporated to dryness under vacuum. The residue was taken up in 100 µL acetonitrile and then transferred onto a 500 mg C18-phenyl SPE column (Phenomenex, Aschaffenburg, Germany), which had been preconditioned successively with 3 mL of acetone, 3 mL of methanol and 3 mL of water. The sample extract was applied on the column under vacuum suction, and thereafter, the SPE column was washed with 3 mL of water and 3 mL of methanol-water (30:70, v/v). After being rapidly dried by aspirating air, the analytes were eluted with 1.5 mL of methanol. The eluate was evaporated to dryness, dissolved in 200 µL methanol and filtered through a 0.22 µm membrane filter prior to LC-MS/MS.

For liquid samples: 5 g of oil or 20 g of juice was sampled and spiked with a mixture (40 µl) of $[^2H]_3$-TEN (25 ng/mL) and $[^2H]_3$-DHT (15 ng/mL). Then, the same volume of ethyl acetate was added, and the mixture was shortly vortexed and shaken in a laboratory shaker at 200 rpm for 1.5 h. The organic upper phase was then separated and evaporated to dryness under vacuum. Further processing was the same as for solid samples.

Method Validation

Limits of Detection (LODs) and Quantitation (LOQs). Potato starch, self-made tomato puree and white pepper powder were chosen as blank matrices and spiked with unlabeled compounds at 0.8, 1.6, 3.2 and 6.4 µg/kg (each in triplicate). Analysis was performed as detailed above. Then, the data obtained from stable isotope dilution assays and spiked amounts were correlated. A subsequent regression calculation provided the calibration line and the confidence interval, which was used to compute the LODs and LOQs .[19]

Precision. Naturally contaminated tomato paste and paprika powder with TEN concentrations at 3 and 26 µg/kg, respectively, were used for the intra-day (n= 6) and inter-day (n= 3 within 3 weeks) precision measurement.

Recovery of SIDA. Relative recovery was determined by addition of the analytes at levels of 2 µg/kg to tomato paste, 3 µg/kg to potato starch and 10 µg/kg to white pepper powder (each in triplicate) and calculating the ratio between detected and spiked amount.

Comparison with external calibration

Tomato puree and white pepper powder were spiked with 2 µg/kg and 30 µg/kg TEN and DHT, respectively. The absolute recovery was then calculated using external calibration.

Quantitation by standard addition

Contaminated paprika powder (26 µg/kg of TEN; 25 µg/kg of DHT determined by SIDA) was spiked with 0, 40, 60 and 80 µg/kg of both toxins. After LC-MS/MS analysis, a standard addition curve was constructed from the spike levels using linear regression and the amount of the analytes in the sample was calculated from the x-intercept of the curve.

Results and Discussion

Synthesis of labeled tentoxin

As a suitable precursor for introducing stable isotopes into the TEN molecule is neither reported nor commercially available, a total synthesis of TEN was necessary. The first chemical synthesis of TEN was reported by Rich *et al.*.[17] After that, numerous articles were published to improve the overall yield. The main barriers were introducing the dehydro residue with cis configuration and achieving the

316 cyclization.[18] Regarding the effective introduction of the label, those routes including
317 labeling at the last steps of the route should be preferred. Therefore, we chose the
318 reaction sequence reported by Loiseau *et al.*,[15] which offered the perspective to
319 attach a trideuteromethyl group at dehydrophenylalanine prior to the final cyclisation
320 of the tetrapeptide (**Fig. 2**). Following the route of the latter authors, we had to adjust
321 the amounts of reactants and solvent as we performed the sequence in a milligram
322 scale. As micro-scale syntheses generally involve more losses due to more
323 complicated adjustment of conditions and more difficult cleanup steps, we obtained
324 only 1 mg of the targeted $[^2H]_3$-TEN.

325 The synthesis of $[^2H]_3$-TEN yielded two other diastereomers, which revealed the
326 same masses and similar fragments but different fragment ratios in LC-MS/MS. All
327 TEN diastereomers were resolved in HPLC and we assume that the two further
328 configurations contain N-methyl-D-alanine, the latter of which is formed by
329 racemization during the final cyclisation step. The latter diastereomers were reported
330 by Rich *et al.*[18] but they were not structurally assigned in our study. Moreover, the
331 analytical results showed that none of them was present in any of the food samples.

332 **DHT synthesis**

333 Hydrogenation of TEN yields theoretically two DHT diastereomers, but only one of
334 them is found in nature.[2] In the study of Meyer *et al.,*[20] reduction of TEN over 5 %
335 Pd-C yielded 100 % of the not naturally occurring DHT diastereomer.

336 In contrast to this, both DHT diastereomers were obtained in our study via mild
337 hydrogenation of TEN with Zn-HCl/formic acid mixture at room temperature. Both of
338 them revealed similar MS^2 fragmentation but rather different retention time on HPLC.

The natural diastereomer was collected from preparative HPLC by monitoring its unspecific peptide bond absorption below 220 nm.

Proposed MS^2 fragmentation

In positive ESI mode, the fragmentations of TEN are in agreement with the results published by Eckart *et al.*[21] using fast atom bombardment combined with tandem mass spectrometry. All cleavages of TEN and its derivatives occur at peptide bonds (**Tab.2**).

In negative ESI mode, TEN, DHT and isoTEN revealed similar MS/MS fragments but different fragment ratio, which allows to distinguish them (**Fig.3**). The only exception is DHT that has a distinct transition of m/z 415 → 228, which can be explained by the reduced double bond of Phe. The fragmentation of deprotonated analytes in negative ESI mode is assigned in **Tab.3** and displayed for TEN as an example in **Fig.4**.

In negative ESI mode there are two possible forms of deprotonated TEN due to the two unsubstituted amides. They yield fragments (a) and (b) upon simultaneous fragmentation at the Leu-MeAla and the Me(Δ^Z)Phe-Gly peptide bond. Fragment (c) is proposed to be generated upon split at the Leu-Me(Δ^Z)Phe peptide bond and the bond between the nitrogen and C_α of MeAla. The trans configuration of dehydrophenylalanine stereochemically favors the cleavage of the Leu-Me(Δ^E)Phe peptide bond, which explains the remarkably high ratio of fragment (c) for isoTEN. The elimination of the isopropyl rest of Leu results in fragment (d).

Due to the incorporation of three deuteriums in the N-methylalanine moiety, labeled and unlabeled TENs differ in 3 Da for fragments (b), (c) and (d). In contrast to this, all

compounds reveal the fragment *m/z* 413→141 (a) due to the loss of the labeled methyl group.

Separation of toxins

As the isotope abundances of natural TEN and isoTEN are 414 (100 %), 415 (26.4 %), 416 (4.1 %) and 417 (0.4 %) Da and DHT has a mass of 416 Da, a significant spectral overlap of these metabolites occurred. Therefore, along with resolution of TEN and isoTEN, a complete chromatographic separation between TEN isomers and DHT is also necessary. Among the tested RP-C18 HPLC columns, Hyperclone® and YMC® were best suited to meet these requirements. On a pure acetonitrile-water mobile phase, a YMCPack Pro C18 column (150 x 3 mm, 3 µm, 100 Å, YMC, Dinslaken, Germany) presented the best separation between TEN and DHT, because the peaks were rather sharp. However, with this mobile phase, no column allows a complete separation from isoTEN. By adding 50 % of isopropanol to acetonitrile, TEN and isoTEN were completely separated on Hyperclone® and the three toxins were eluted within the shortest time due to base deactivation of the latter stationary phase (**Fig.5**).

Linearity

The linearity of relative responses between analyte and internal standard was good, with the coefficient of determination exceeding 0.998 for each of the three toxins via linear regression. Linearity of the response was checked by analysis of the residuals (homogeneity and normal distribution) after linear regression and was found to be linear for the chosen molar ratios (0.1 – 10).

Method Validation

LODs and LOQs. Depending on the matrix used, LODs ranged from 0.10to 0.99 µg/kg and LOQs ranged from 0.33 to 2.94 µg/kg (**Tab**. **4**). White pepper powder as the matrix is more demanding than the other two matrices potato starch and tomato paste are, hence the LODs and LOQs in it were higher. To the best of our knowledge, LOD for TEN in potato starch was the lowest so far reported. Sulyok *et al.* [11] published the semi-quantitative determination of 87 mycotoxins in moldy foods with a LOD of 0.5 µg/kg for TEN, estimated at the lowest evaluable concentration levels both of spiked samples as well as of liquid standards corresponding to a signal-to-noise ratio of 3:1. For DHT and isoTEN, no validation data have been published so far.

For juices, the MS background noise was much less and signal intensity of the standards was decisively higher than in solid foods. As the sample size for liquids (20 g) 20 times exceeded that of the solid food samples (1g), the LODs and LOQs of liquid samples were estimated to be the twentieth part of those of the solid samples.

Precision. The intra-day and inter-day coefficients of variation of TEN and DHT ranged between 5.7 and 8.8 % (**Tab.4**).

Recovery of SIDA. SIDA recoveries ranged between 98 and 115 %, with maximal relative standards deviation (RSD) of 5.9 % (**Tab.4**). This is a typical recovery level for SIDAs, in which instrument variations, losses during sample preparation, as well as matrix suppression are corrected.

Absolute recoveries obtained from external calibration

Absolute recovery of TEN and DHT in tomato puree was 50 and 31 %, while in white pepper powder it was 32 and 43 %, respectively. The average recovery of TEN and DHT in spiked paprika samples were 20 and 25 %, respectively, with RSDs exceeding 20 %. Thus the recovery differed for each matrix and even for the same matrix at different concentrations; much more measurements would be needed to ensure the accuracy and precision of the results if external calibration was used. These data are similar or lower compared to those reported for tenuazonic acid in tomato products, for which an absolute recovery of 56 % was found for LC-MS/MS with external calibration. [14] As SIDA yields accurate and more precise data for all matrices analyzed, its superiority over external calibration was again demonstrated.

Quantitation by standard addition

When applying the standard addition procedure, TEN and DHT concentration in the paprika powder sample was found to be 23 µg/kg and 21 µg/kg in comparison to 26 and 25 µg/kg found by SIDA, respectively. Hence, the deviations from SIDA results were 12 % and 16 % and exceed the bias of 7.3 % found for tenuazonic acid. [14]

Occurrence in foods

In a total of 103 analyzed commercial food samples, 88 were contaminated with TEN and 57 were contaminated with DHT as listed in **Tab.5**.

All bread samples contained TEN with an average concentration of 3.35 µg/kg, which is comparable with the median of 1.8 µg/kg reported by Sulyok *et al.*[22] DHT was detected in most of the samples. Pumpernickel bread samples showed obviously higher amounts of both toxins. DHT/ TEN ratios varied from 30 % to 49 % in all samples.

The heaviest contamination in rice samples was detected in parboiled rice. Six parboiled rice samples were contaminated with a TEN concentration ranging from 1.42 to 9.56 µg/kg. DHT was only detectable in 3 samples, among which the highest amount was found in one parboiled rice sample also showing the highest amount of TEN. The contamination of these samples appears plausible from different effects of the parboiling process: on the one hand, the process involves a soaking procedure, which favors the mycotoxin transport into the kernels due to water diffusion; on the other hand, the endosperm expands and enables the contact with the outer hull. This also increases the diffusion of the mycotoxins, which generally are more abundant on the hull.

All juices from berries and cherries contained little TEN and DHT in contrast to apple and pear juice, in which no contamination was detected.

The measured nut samples showed very little or no contamination with the toxins.

In all sauces the toxins were detected with an average TEN concentration of 0.99 µg/kg. The detectable DHT amount was mostly between LOD and LOQ.

TEN and DHT were also detected in oil seeds, and the highest level was found in hemp seeds with 17.4 µg/kg of TEN. Pumpkin seeds showed no contamination of the three toxins.

TEN was detected in all the 6 sunflower oil samples with the highest amounts found in organic first cold-pressed sunflower oil samples. In contrast to this, rapeseed and thistle oil showed very little or no contamination. The highest levels of TEN and DHT concentrations with an average of 34.6 and 30.1 µg/kg, respectively, were obtained from paprika powder samples. The other kinds of spices analyzed in the present

study did not show high amount of both toxins. The average TEN and DHT concentrations were 6.22 and 2.81 µg/kg, respectively. A phytotoxic effect of TEN on plants for food use cannot be confirmed in the observed concentration as in studies involving seedlings or chloroplasts only concentrations in the µg/g level were applied. [3-5]

IsoTEN, which is considered a photo degradation product of TEN, occurred in concentrations of below 10 % of those of TEN in all positive samples. However, in two apple chips samples the amount of isoTEN (0.57 µg/kg) was almost as much as that of TEN. The abundant amount of isoTEN in these chips samples was probably due to the microwave vacuum drying process, which was reported as a new trend in chips manufacturing reserving the original color of apples due to a reduced thermal and oxidative burden. [23] In contrast to this, the conventionally processed apple chip sample, which can be identified by its brown color, contained mainly TEN (1.47 µg/kg). Recently, the microwave-induced trans/cis isomerization of conjugated carbon-carbon double bonds and stereoselective β-lactam using microwave irradiation have been reported. [24, 25] Although non-thermal effects of microwave are still controversially discussed, Wasielewski *et al.* [26] reported that the duration of photosynthetic charge separation can be controlled with microwave radiation. As TEN is known to be readily converted into isoTEN under UV irradiation, this could be explained by the microwave vacuum process.

Regarding the risk posed by TEN to the consumer, EFSA classified TEN as a non-genotoxic compound in Cramer structural class III according to the concept of TTC. Therefore, the TTC was set to 1500 ng/kg body weight per day in equivalence to the further Alternaria toxin tenuazonic acid. [27] Whereas this threshold is likely to be

exceeded for tenuazonic acid in baby food [28] the TEN contents in foods found in our study cannot be supposed to impose a risk unless substantial toxicity data are available.

Acknowledgments

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Tables

Table 1. Compound-dependent parameters for MRM mode in LC-MS/MS.

Compound name	MRM (*m/z*, negative)	Retention Time (min)	Declustering Potential (V)	Collision Energy (V)	Collision cell exit Potencial (V)
isoTEN	413→246 [a]	11.66	-80	-20	-5
	413→271 [b]	11.66	-80	-30	-5
$[^2H]_3$-isoTEN	416→249 [a]	11.62	-80	-20	-5
	416→274 [b]	11.62	-80	-30	-5
TEN	413→141 [a]	12.50	-80	-30	-5
	413→271 [b]	12.50	-80	-30	-5
$[^2H]_3$-TEN	416→141 [a]	12.45	-80	-30	-5
	416→274 [b]	12.45	-80	-30	-5
DHT	415→273 [a]	12.86	-80	-35	-5
	415→141 [b]	12.86	-80	-35	-5
$[^2H]_3$-DHT	418→276 [a]	12.80	-80	-35	-5
	418→141 [b]	12.80	-80	-35	-5

[a] MRM used as the quantifier transition

[b] MRM used as a qualifier transition

Table 2. Fragments of protonated analytes in ESI(+)-MS/MS

Fragment (*m/z*)						Assignment
TEN	**$[^2H]_3$-TEN**	**isoTEN**	**$[^2H]_3$-isoTEN**	**DHT**	**$[^2H]_3$-DHT**	
358	361	358	361	360	363	$[M+H]^+$-Gly
330	333	330	333	332	335	$[M+H]^+$-MeAla
312	315	312	315	314	317	$[M+H]^+$-H_2O-MeAla
302	305	302	305	304	307	$[M+H]^+$-Leu
217	220	217	220	219	222	$[M+H]^+$-MeAla-Leu
199	199	199	199	199	199	$[M+H]^+$-Gly-Me(*Δ*)Phe/MePhe

Table 3. Fragments of deprotonated analytes in ESI(-)-MS/MS

Fragment (*m/z*)						Assignment
TEN	**$[^2H]_3$-TEN**	**isoTEN**	**$[^2H]_3$-isoTEN**	**DHT**	**$[^2H]_3$-DHT**	
369	372	369	372	371	374	$[M-H]^-$-C_3H_8
271	274	271	271	273	276	$[M-H]^-$-Gly-MeAla
246	249	246	249	248	251	$[M-H]^-$-Leu-CO-C_2H_3
141	141	141	141	141	141	$[M-H]^-$-Leu-Me(*Δ*)Phe/MePhe

Table 4. Results of the method validation

Analyte	**LOD (µg/kg)**			**LOQ (µg/kg)**			**Precision (RSD, %)**		**Recovery (%)**		
							Intra-day (n=3)	Inter-day (n=3)	Spike: 2µg/kg	Spike: 3µg/kg	Spike: 10µg/kg
	potato starch	tomato puree	white pepper powder	potato starch	tomato puree	white pepper powder	paprika powder	paprika powder	tomato puree	potato starch	white pepper powder
TEN	0.18	0.39	0.99	0.54	1.17	2.94	5.8	5.7	109 ± 2.7	98 ± 2.6	112 ± 3.4
DHT	0.35	0.19	0.30	1.05	0.62	0.88	8.8	8.1	102 ± 3.7	110 ± 2.5	115 ± 5.1
isoTEN	0.19	0.10	0.45	0.57	0.33	1.32	-	-	100 ± 5.3	102 ± 2.4	103 ± 5.9

Table 5. TEN and DHT in food samples (µg/kg)

sample name	TEN			DHT		
	positive samples	max.	mean/single value	positive samples	max.	mean/single value
cereals (flour)						
millet	3	4.08	3.19	3	2.02	1.73
rye flour	2	6.59	4.83	2	2.71	2.03
whole grain wheat	1		3.16	1		n.q.
cereals (rice)						
puffed rice	1		1.35	1		n.q.
basmati rice	1		n.q.	-		-
long grain rice	1		n.q.	-		-
parboiled rice	6	9.56	3.73	1	6.97	1.16
rice mixture	1		4.41	1		3.93
thai black rice	1		n.q.	-		-
white glutinous rice	-		-	-		-
cereal product						
crisp wheat bread	1		5.56	1		2.47
potato bread	1		2.48	1		n.q.
pumpernickel	7	6.74	4.24	7	2.90	1.82
rye bread	2	2.28	1.77	2	n.q.	n.q.
wheat bread	5	6.58	3.15	4	1.16	n.q.
wheat toast	2	2.47	1.78	-		-
chips						
apple chips	2	0.65	0.60	-		-
apple chips (conventional)	1		1.47	-		-
banana chips	-		-	-		-
potato chips	1		n.q.	-		-
juices						
apple cherry juice	1		0.07	1		n.q.
apple juice (2)	-		-	-		-
black ribes juice	1		0.18	1		n.q.
carrot juice	-		-	-		-
cherry juice	1		n.q.	-		-
passion fruit juice	-		-	-		-
pear juice	-		-	-		-
red raspberry juice	1		0.10	1		n.q.
rhubarb juice	-		-	-		-
sour cherry juice	1		0.06	-		-
tomato juice	1		0.16	1		0.18

nuts						
peanuts	-		-	-		-
almond	1		0.83	-		-
wal nuts	-		-	-		-
oils						
pumpkin seed oil	-		-	-		-
rapeseed oil	-		-	-		-
rapeseed oil (first cold-pressed)	1		0.64	1		n.q.
sunflower oil	3	3.95	1.83	-		-
sunflower oil (first cold-pressed)	3	6.73	4.83	3	4.48	2.54
thistle oil	1		n.q.	-		-
sauces						
mustard	2	2.76	2.65	2	1.33	1.12
paprika paste	2	2.17	2.12	2	1.13	n.q.
sieved tomato	1		n.q.	1		n.q.
tomato ketchup	4	n.q.	n.q.	3	n.q.	n.q.
tomato paste	3	3.31	1.30	1	1.87	n.q.
tomato sauce	4	n.q.	n.q.	2	n.q.	n.q.
seeds						
hemp seed	1		17.4	1		11.8
pumpkin seed	-		-	-		-
pine seed	1		1.87	1		1.08
sunflower seed	2	6.30	3.87	2	1.37	n.q.
spices						
caraway	3	8.07	5.16	2	6.99	4.02
curry	1		13.2	1		10.4
garlic powder	1		1.05	-		-
herbs	1		12.5	-		-
paprika powder	4	52.4	34.6	4	36.3	30.1
parsley	-		-	-		-
pepper powder (black)	2	7.07	7.43	1		n.q.
pepper powder (white)	-			-		-
spices mixture	2	14.9	8.82	1		5.04

-, not detected; n.q., not quantifiable

Legend to the Figures

Fig. 1. Structures of TEN, DHT and isoTEN

Fig. 2. Synthesis pathway of $[^2H]_3$-TEN

Fig. 3. MS/MS spectra in negative ESI mode using the same parameters: DP= -80 V, CE= -25 V, EP= -10 V and CXP= -5 V.

Fig. 4. Proposed fragmentation of TEN, * : $[^2H]_3$-labeled methyl group

Fig. 5. LC-MS/MS chromatograms of a tomato paste sample

Figures

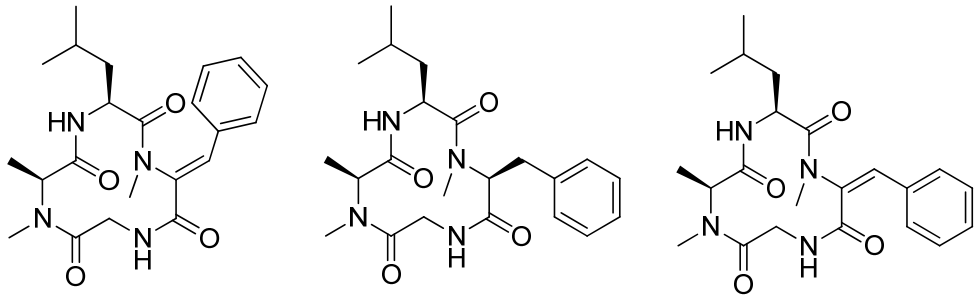

Figure 1.

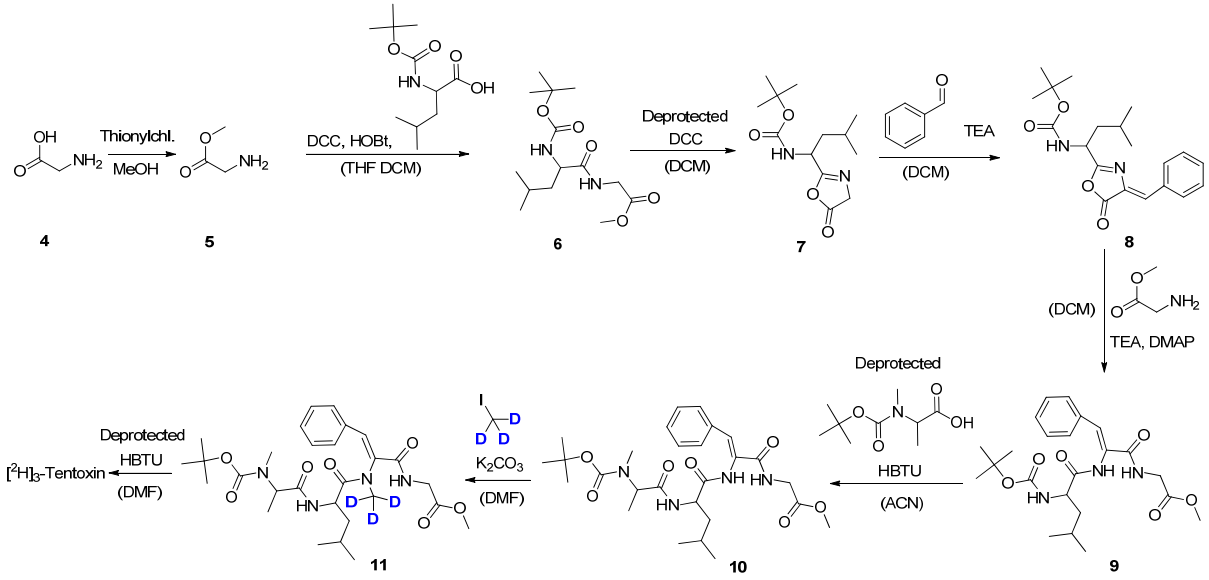

Fig. 2.

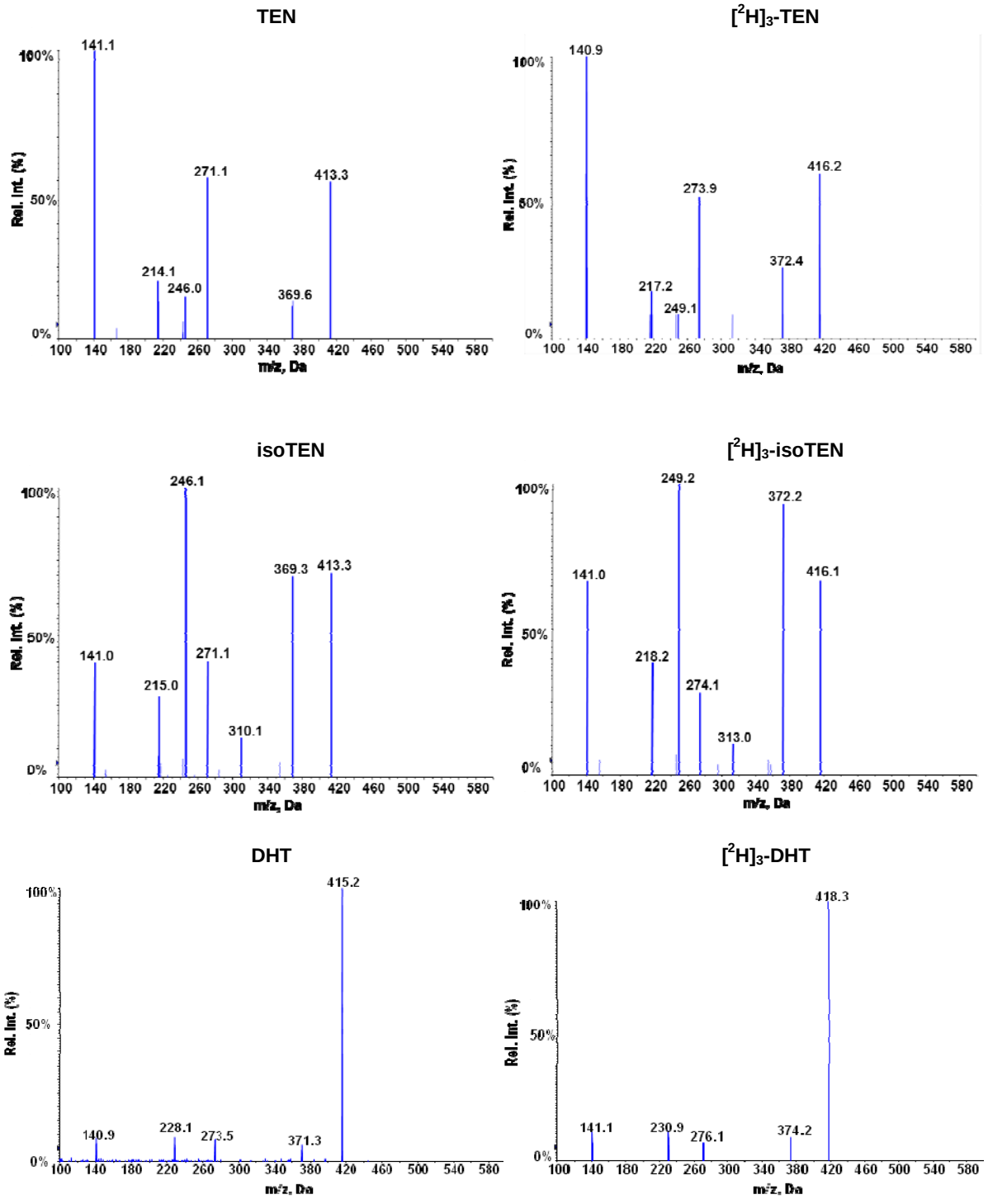
TEN
141.1
271.1
413.3
214.1
246.0
369.6
[2H]3-TEN
140.9
416.2
273.9
372.4
217.2
249.1
isoTEN
246.1
369.3
413.3
141.0
271.1
215.0
310.1
[2H]3-isoTEN
249.2
372.2
141.0
416.1
218.2
274.1
313.0
DHT
415.2
140.9
228.1
273.5
371.3
[2H]3-DHT
418.3
141.1
230.9
276.1
374.2
Rel. Int. (%)
m/z, Da

Fig. 3.

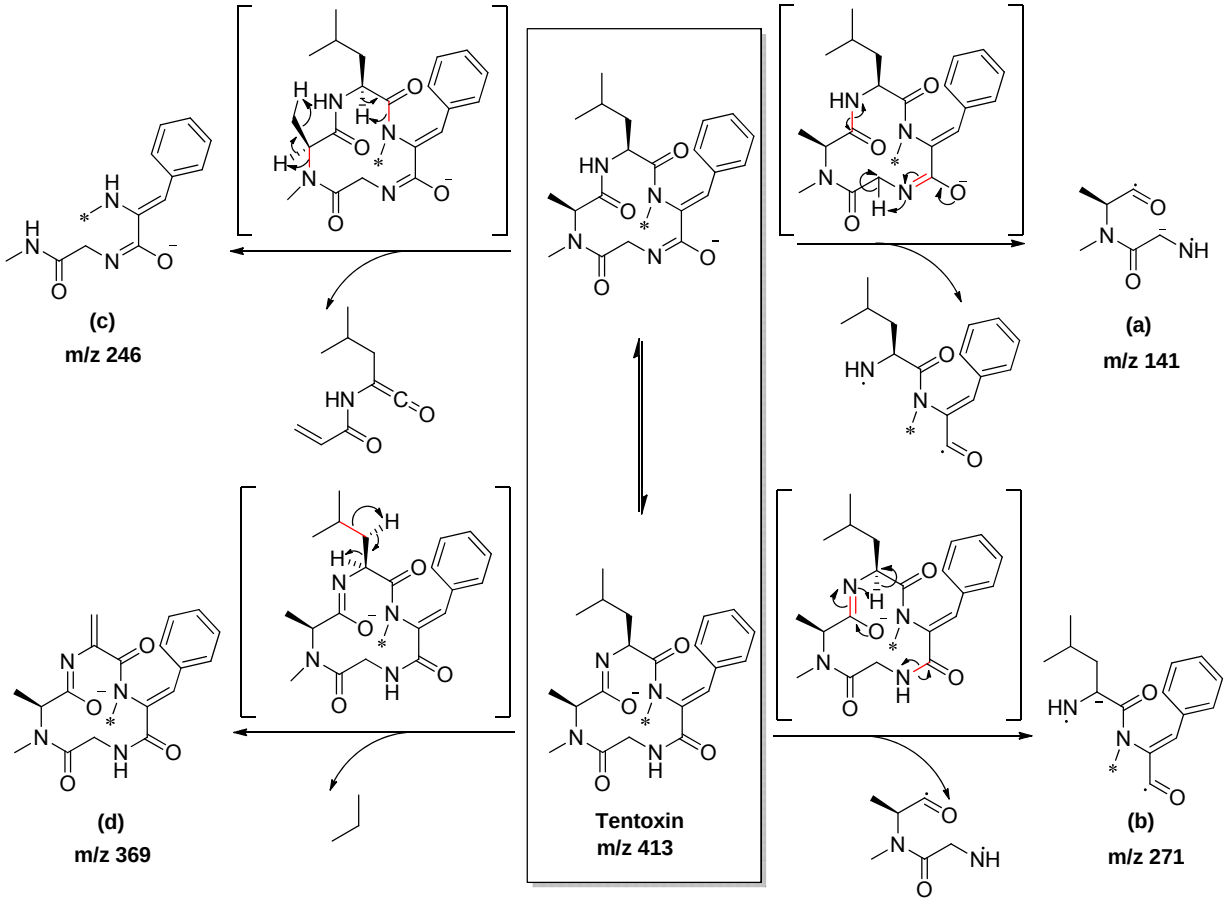

Fig. 4.

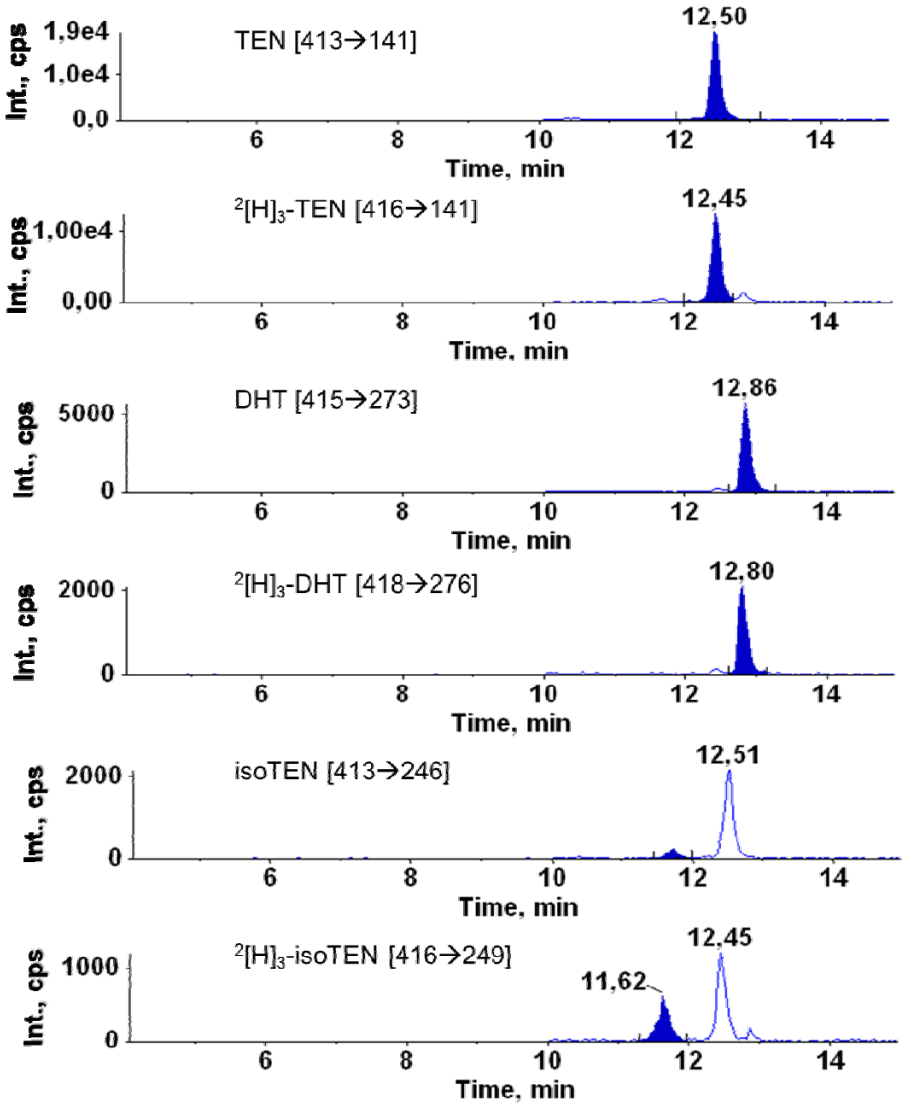
TEN [413→141]
12,50
Int., cps
1,9e4
1,0e4
0,0
6
8
10
12
14
Time, min
$^2[H]_3$-TEN [416→141]
12,45
1,00e4
0,00
DHT [415→273]
12,86
5000
0
$^2[H]_3$-DHT [418→276]
12,80
2000
isoTEN [413→246]
12,51
$^2[H]_3$-isoTEN [416→249]
12,45
11,62
1000

Fig. 5.

TOC Graphic