Published in:

Journal of Chromatography A, 1289 (2013) 27-36

The final publication is available at Elsevier via https://doi.org/10.1016/j.chroma.2013.03.015

Development of analytical methods for the determination of tenuazonic acid analogues in food commodities

Stefan Asam[1], Martina Lichtenegger[1], Kornelija Muzik[1], Yang Liu[1], Oliver Frank[2], Thomas Hofmann[2,3] and Michael Rychlik[1,3,*]

[1] *Technical University of Munich, Chair of Analytical Food Chemistry, Alte Akademie 10, D-85354 Freising, Germany*

[2] *Technische Universität München, Chair of Food Chemistry and Molecular Sensory Science, Lise-Meitner-Straße 34, D-85354 Freising, Germany*

[3] *Technische Universität München, BIOANALYTIK Weihenstephan, ZIEL Research Center for Nutrition and Food Sciences, Alte Akademie 10, D-85354 Freising, Germany*

* Corresponding Author.
Phone: + 49 8161 71 3153.
Fax: + 49 8161 71 4216.
E-Mail: michael.rychlik@tum.de

Abstract

Analogues of the *Alternaria* mycotoxin tenuazonic acid (TA, biosynthesized by the fungus from the amino acid isoleucine) derived from valine (ValTA), leucine (LeuTA), alanine (AlaTA) and phenylalanine (PheTA) were synthesized and characterized by mass spectrometry (MS) and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. Absolute concentrations of stock solutions were determined by quantitative NMR (qNMR). Two analytical methods based on high performance liquid chromatography (HPLC) and MS detection were developed, one with derivatization with 2,4-dinitrophenylhydrazine (DNPH) and one without derivatization.. Limits of detection (LODs) were between 1–3 µg/kg (with derivatization) and 50–80 µg/kg (without derivatization). Respective LOQs were about three times higher. Beside TA, the analogues LeuTA (about 4 % of TA content) and ValTA (about 10 % of TA content) were found in highly contaminated sorghum infant cereals and sorghum grains. Other analogues were not detected. Quantification of LeuTA and ValTA was performed using [$^{13}C_6$,^{15}N]-TA as internal standard and matrix matched calibration. Recovery was between 95 ± 11 % and 102 ± 10 % for both compounds. Precision (relative standard deviation of triplicate sorghum cereal analyses three times during three weeks) was 7 % for TA (912 ± 60 µg/kg), 17 % for LeuTA (43 ± 8 µg/kg) and 19 % for ValTA (118 ± 22 µg/kg). These results indicate that further toxicological poorly characterized compounds were detected in sorghum based infant food highly contaminated with TA, already.

Introduction

Tenuazonic acid (TA, Fig. 1) is one of the major mycotoxins produced by *Alternaria spp.* (Bottalcio and Logrieco 1998), *Pyricularia oryzae* (Umetsu et al. 1972, Umetsu et al. 1974) and *Phoma sorghina* (Steyn and Rabiet 1976). It exhibits manifold biological activity and has been reported to have antiviral (Miller et al. 1963), antitumor, antibacterial, cytotoxic (Gitterman 1965) and phytotoxic properties (Lebrun et al. 1988) and to be acutely toxic in mammals (Smith et al. 1968). In respect of the oral LD_{50}-values of TA that are 182 or 225 mg kg^{-1} body weight (BW) for male mice (Miller et al. 1968, Smith et al. 1968) and 81 mg kg^{-1} BW for female mice (Miller et al. 1968), TA is regarded as the most toxic *Alternaria* mycotoxin (Bottalcio and Logrieco 1998). Additionally, TA has been made responsible for the outbreak of "onyalai", a human haematologic disorder disease occurring in Africa (Steyn and Rabiet 1976), but further toxicological evidence is lacking.

TA is found regularly in food commodities, especially in cereals (Webley et al. 1997; Li and Yoshizawa 2000; Patriarca et al. 2007; Azcarate et al. 2008; Siegel et al. 2010) and in tomatoes and their respective processing products (Scott and Kanhere 1980; Stack et al. 1985; Mislivec et al. 1987; Da Motta and Soares 2001; Terminello et al. 2006; Asam et al. 2011), beer (Siegel et al. 2010), beverages (Asam et al. 2011), spices (Asam et al. 2012) and even infant food (Asam and Rychlik, submitted).

It has been proved by feeding [^{14}C]-acetate to cultures of *Alternaria tenuis* that biosynthesis of TA proceeds from one molecule L-isoleucine and two molecules of acetate (Stickings and Townsend 1961) via *N*-acetoacetyl-L-isoleucine as intermediate (Gatenbeck and Sierankiewicz 1973 a). Moreover, it has been shown that cultures of *Alternaria* also are able to produce TA analogues derived from other amino acids. After feeding [^{14}C]-L-valine and [^{14}C]-L-leucine, the respective tetramic acid derivatives could be isolated, whereas [^{14}C]-L-Phenylalanin was not utilized by the microorganisms (Gatenbeck and Sierankiewicz 1973 b).

The toxicity of TA analogues was studied with a series of chemically synthesized compounds differing in their substitution at the C-3 position of the tetramic acid nucleus. All TA analogues showed less cytotoxic effects than TA, but the antibacterial activity of the leucine TA analogue (3-acetyl-5-isobutyl-tetramic acid; LeuTA) was identical to TA (3-acetyl-5-*sec*-butyl-tetramic acid) itself (Gittermann 1965). The phytotoxicity of all TA analogues was less than that of TA , but the valine TA analogue (3-acetyl-5-isopropyl-tetramic acid; ValTA) and LeuTA still showed significant phytotoxicity in terms of growth inhibition and leaf browning (Lebrun et al. 1988). Taken together, the TA analogues LeuTA and ValTA seem to have some toxicological relevance, although the available data are even more limited than for TA itself.

However, only the L-valine analogue was identified as a trace compound in fungal extracts (Joshi et al 1984, Lebrun et al. 1990, Shephard et al. 1991) and molded tomatoes (Stack et al. 1985). To the best of our knowledge, no further investigations about the occurrence of TA analogues in food have been made so far, what might result from the difficulty even in analyzing the major compound TA may be a reason for that. The preferred analytical technique for TA analysis is high performance liquid chromatography (HPLC), but as TA is both a strong acid and a metal chelating compound, it shows irreproducible chromatographic behavior unless modifiers like Zn(II)SO_4 are added to the mobile phase (Scott and Kanhere 1980). This approach restricts the detection to less sensitive UV absorption, unfortunately, as modifiers are incompatible with mass spectrometric detection. Only just recently, a method for the analysis of TA after derivatization with 2,4-dinitrophenylhydrazine was described that was fully compatible with LC-MS and showed low limits of detection (Siegel et al.

2009). The development of a stable isotope dilution assay (SIDA), which used [$^{13}C_6$,^{15}N]-TA as internal standard and was based on the derivatization method, allows the sensitive and precise quantification of TA in different matrices (Asam et al. 2011, Asam et al. 2012), finally.

As the derivatization reaction is targeted towards the tetramic acid nucleus (Siegel et al. 2009), it should work with TA analogues in the same manner and allow sensitive detection of these compounds as well. Therefore, it was the aim of the present study to investigate the occurrence of TA analogues in food commodities by using sensitive LC-MS techniques and to quantify identified compounds with [$^{13}C_6$,^{15}N]-TA as internal standard.

Materials and Methods

Chemicals and reagents

Tenuazonic acid, copper(II) salt, L-Leucine, L-Valine, L-Alanine, L-Phenylalanine, 2,2,6-trimethyl-4H-1,3-dioxin-4-one, sodium methylate, 2,4-dinitrophenylhydrazine (phlegmatized with 30 % water), undecylic aldehyde, and Dowex 50 WX80 (100–200 mesh) cation-exchange resin were obtained from Sigma-Aldrich (Steinheim, Germany). All other solvents were obtained from Merck (Darmstadt, Germany) and were of analytical-reagent grade. Water for HPLC was purified by a Milli-Q-system (Millipore GmbH, Schwalbach, Germany). [$^{13}C_6$, ^{15}N]-tenuazonic acid was prepared in our laboratory as published previously (Asam et al. 2011).

Preparation of standard solutions of tenuazonic acid

Stock solutions of free tenuazonic acid were prepared according to the literature (Siegel et al. 2009, Shepard et al. 1991): Commercial reference substance of tenuazonic acid copper salt (10 mg, $Cu[C_{10}H_{14}NO_3]_2$, M_r = 456 g/mol, 43.9 µmol) was dissolved in the original flask with methylene chloride and the solution was transferred into a 10 mL volumetric flask. The original flask was repeatedly flushed with methylene chloride, which was completely transferred into the 10 mL volumetric flask that was brought up to volume to obtain a stock solution. Dowex ® 50 WX80 (100 – 200 mesh) cation-exchange resin was filled into a 2 mL-plastic syringe without needle (“column”), which was attached to a vacuum manifold (Shephard et al. 1991). The resin was activated by subsequently passing sodium hydroxide (10 mL, 0.5 mol/L), deionised water (10 mL) and hydrochloric acid (10 mL, 0.5 mol/L) through the column. Afterwards the column was washed several times with methylene chloride. An aliquot of the prepared stock solution of tenuazonic acid copper salt (2 mL, 8.78 µmol) was applied to the column and allowed to drain by gravity. The column was flushed with methylene chloride (2 x 2 mL). All eluates were collected and the solvent was evaporated under a gentle stream of nitrogen. The resulting colourless, viscous oil was taken up in methanol, transferred to a 10 mL-volumetric flask and brought to volume with methanol. This solution was diluted with methanol (1+9, v+v) and the absolute amount of tenuazonic acid was determined with UV-spectroscopy using the molar extinction coefficient of 1,298 x 10^4 L mol^{-1} cm^{-1} (Scott and Kanhere 1980, Shephard et al. 1991, Siegel et al. 2009). Further dilutions were made with methanol to obtain working solutions in the range of 1 µg/mL (5 nmol/mL) and 0.01 µg/mL (0.05 nmol/mL).

Synthesis of reference substances

Derivatives of tenuazonic acid (AlaTA, ValTA, LeuTA, PheTA) were synthesized as racemates from the respective amino acids (alanine, valine, leucine, phenylalanine) following a procedure described in the literature (Lebrun, 1988). In short, the amino acids were converted into their methyl esters by thionylchloride in methanol and then acetoacetylated by the diketene releasing reagent 2,2,6-trimethyl-4H-1,3-dioxin-4-one (Asam et al. 2011). The ring closure to obtain the tetramic acid nucleus was accomplished by Dieckmann intramolecular cyclization in the presence of sodium methylate.

Preparative high performance liquid chromatography

Parts of the crude reaction mixtures obtained from synthesis were subjected to preparative high performance liquid chromatography to obtain pure standards. The system consisted of a Merck Hitachi L-7100 quaternary pump, a D-7000 interface, a L-7200 autosampler and a L-7455 diode array

detector (DAD). As stationary phase a Gemini-NX (3 µm, 110 Å, 150 x 4.6 mm; Phenomenex, Aschaffenburg, Germany) was used. The mobile phase consisted of variable mixtures of an ammonium formate solution (5 mmol/L), adjusted to pH 9 with ammonia (A) and acetonitrile (B) following a linear binary gradient as follows: Initial conditions were 90 % A and 10 % B. Three minutes after injection, the content of B was raised to 100 % within four minutes and held on this level for two minutes until the initial conditions were restored 10 minutes after injection. Equilibration time between two runs was 10 minutes and the total flow was 1 mL/min. The effluent from the column was monitored by DAD detection (200–400 nm). All compounds showed a UV-spectrum identical to that of tenuazonic acid with the absorption maximum at 277 nm. Peak collection was done manually, the pooled fractions were neutralized with formic acid and freeze dried.

Quantitative nuclear magnetic resonance (qNMR) spectroscopy

The remainders from freeze drying were extracted with chloroform (1 mL) twice to release the tetramic acids from ammonium formate salt. The combined chloroform extracts were combined and evaporated in a stream of nitrogen. The remaining oils were re-dissolved in 400 µL D_4-methanol (Sigma-Aldrich, Steinheim, Germany) and transferred to NMR-tubes (4 x 180 mm, Bruker BioSpin Corporation, Rheinstetten, Germany) for qualitative and quantitative nuclear magnetic resonance (NMR) spectroscopy measurements. The absolute concentration of the stock solutions were determined by qNMR as published elsewhere (Korn et al. 2011).

ValTA: ^{1}H NMR, δ/ppm (TMS): 3.33 (m, 1H, A), 2.45 (s, 3H, B), 2.18 (m, 1H, C), 1.05 (d, 3H, D-1, signal used for qNMR),0.85 (d, 3H, D-2).

LeuTA: ^{1}H NMR, δ/ppm (TMS): 3.90 (m, 1H, A), 2.43 (s, 3H, B), 1.83 (m, 1H, D), 1.63 (m, 1H, C-1), 1.45 (m, 1H, C-2), 0.95 (m, 6H, E).

PheTA: ^{1}H NMR, δ/ppm (TMS): 7.25 (m, 5H, D), 3.85 (m, 1H, A), 3.15 (m, 1H, C-1), 2.73 (m, 1H, C-2), 2.30 (s, 3H, B).

AlaTA: ^{1}H NMR, δ/ppm (TMS): 3.93 (q, 1H, A), 2.43 (s, 3H, B), 1.33 (d, 3H, C).

Preparation of standard solutions

From the NMR tubes the solutions were transferred to 10 mL-flasks, the D_4-methanol was evaporated under a stream of nitrogen and the flasks were brought to volume with methanol. From these stock solutions respective working standards were obtained by further dilution with methanol. All solutions were stored in the dark at –18 °C to ensure stability (Combina, 1998).

Measurement of native TA analogues

Mass spectrometry of TA analogues

MS and MS/MS spectra were obtained from a hybrid triple-quadrupole/linear ion trap mass spectrometer (API 4000 QTrap; Applied Biosystems Inc., Foster City, CA). The ion source (Turbo Ion Spray) was operated both in the positive and negative electrospray ionization (ESI) mode. The TA analogues (1 µg/mL) were infused into the mass spectrometer at a flow rate of 10 µL/min with a syringe pump. Data acquisition was carried out using Analyst 1.4.2 software (Applied Biosystems INC, Foster City, CA).

Liquid chromatography mass spectrometry (LC-MS/MS)

For LC-MS/MS measurements, the mass spectrometer was operated in the MRM (multiple reaction monitoring) mode. The ion source was operated in the negative ESI mode. Settings were as follows: curtain gas (CUR): 20 psi, collision gas (CAD): medium, ion spray voltage (IS): -4000 V, temperature (TEM): 550 °C, ion source gas 1 (GS1): 60 psi, ion source gas 2 (GS2): 80 psi. The declustering potential was set to -70 V and the entrance potential to -5 V for all compounds. Both quadrupoles were set at unit resolution. The following transitions were monitored (in parentheses, collision energy, CE; collision cell exit potential, CXP):

TA & LeuTA: m/z 196 → 139 (CE -28 V, CXP -1 V), m/z 196 → 112 (CE -36 V, CXP -7 V);

[$^{13}C_6$,^{15}N]-TA: m/z 203 → 142 (CE -28 V, CXP -1 V), m/z 203 → 113 (CE -36 V, CXP -7 V);

ValTA: m/z 182 → 139 (CE -26 V, CXP -1 V), m/z 182 → 112 (CE -34 V, CXP -7 V);

PheTA: m/z 230 → 139 (CE -26 V, CXP -1 V), m/z 230 → 112 (CE -34 V, CXP -5 V);

AlaTA: m/z 154 → 139 (CE -24 V, CXP -1 V), m/z 154 → 112 (CE -28 V, CXP -5 V)

HPLC separation prior to mass spectrometric detection was performed on a Shimadzu LC-20A prominence HPLC system (Shimadzu, Kyoto, Japan). As stationary phase a 150 mm x 4.6 mm i.d., 3 µm, Gemini-NX (Phenomenex, Aschaffenburg, Germany) was used. The mobile phase was mixed from ammonium formate (5 mmol/L) in water, adjusted to pH 9 with ammonia (solvent A) and acetonitrile (solvent B) following a linear binary gradient as follows: Initial conditions were 5 % B and 95 % A. After 3 min isocratic delivery of the solvents, the content of solvent B was linearly raised during the next 5 min to obtain 15 % B and 85 % A. After 8 min the content of B was raised to 100 % during 2 min and these conditions were continued until the end of the run after 14 min. Injection volume was 50 µL, flow rate 0.5 mL/min, and equilibration time between two runs 10 min.

Sample preparation

About 8 g of the sample was weighed in a 50 mL centrifugation vial (Sarstedt AG & Co., Nümbrecht, Germany) and spiked with labeled standard (concentration 1 µg/mL, added amount 100 µL ≡ 12.5 µg/kg). Afterwards, the extraction solvent (methanol/acetonitrile/water, 30/45/45, v/v/v, adjusted to pH 3 with concentrated hydrochloric acid) was added (15 mL), followed by 15 min ultrasonication and 60 min vigorously shaking. The vial was centrifuged (5 min, 4,600 rpm ≡ 4,000 g, 25° C) by means of a Heraeus Multifuge 3 L-R (Thermo Fisher Scientific Inc., Waltham, MA) and the supernatant was diluted with the threefold amount of water (≡ 1:4).

A 6-mL C_{18}-SPE column (500 mg, 55 µm, 140 Å, Strata C18-T; Phenomenex, Aschaffenburg, Germany) was attached to a vacuum manifold and preconditioned successively with 4 mL of acetone, 4 mL of methanol, and 4 mL of water, at a flow rate of about 1 drop/s by gentle vacuum. The sample extract was applied to the column at the same flow rate. Afterwards, the column was washed with 10 mL of water and rapidly dried by aspirating air after the last washing step. Elution of the target compounds was carried out with 2 x 5 mL of acetonitrile. The solvent was removed by means of a rotary evaporator and the residue taken up in 300 µL of acetonitrile/ammonium formate (5 mmol/L, pH 9) (5/95; v/v). The extract was membrane filtered (0.22 µm; regenerated cellulose; Whatman, Maidstone, UK) before LC-MS/MS analysis.

Measurement of TA analogues as dinitrophenylhydrazine derivatives

Preparation of derivatization and quenching reagent

Following a modified procedure from literature (Brady and Elsmie 1926, Siegel et al. 2009), the derivatization reagent was prepared from 2,4-dinitrophenylhydrazine (150 mg, 0.5 mmol) in hydrochloric acid (2 mol/L, 65 mL) to give a stock solution (7.7 mmol/L). It was used either directly or after dilution (1:10) with hydrochloric acid (2 mol/L). Undecylic aldehyde (0.05 % in ethyl acetate, 2.4 mmol/L) was used as quenching reagent in order to destroy excess derivatization reagent after the derivatization step (Siegel et al. 2009).

Derivatization of TA analogues

From the respective stock solution, an aliquot (1 eq, 90 nmol, 14 – 21 µg, depending on the substance) was transferred to an 1.5 mL-vial and treated with derivatization reagent (10 eq, 900 nmol, 120 µL x 7.7 mmol/L) for 10 minutes in an ultrasonic bath. Afterwards, quenching reagent (10 eq, 900 nmol, 375 µL x 2.4 mmol/L) was added, followed by another 10 minutes of ultrasonication. The solution was brought to dryness in a stream of nitrogen and redissolved in methanol/water (50/50, v/v, 2 mL) to yield stock solutions of derivatized TA analogues (5 – 10 µg/mL).

Mass spectrometry of dinitrophenylhydrazones of TA analogues

The derivatized TA analogues (1 µg/mL) were infused into the mass spectrometer in the same manner as described above to obtain MS and MS/MS spectra.

Liquid chromatography mass spectrometry (LC-MS/MS)

The same LC-MS/MS equipment was used as described above and the settings of the ion source were identical, also. The following transitions were monitored (in parentheses, collision energy, CE; collision cell exit potential, CXP):

TA-DNPH & LeuTA-DNPH:	m/z 376 → 182 (CE -34 V, CXP -9 V), m/z 376 → 122 (CE -64 V, CXP -7 V), m/z 376 → 301 (CE -30, CXP -7), m/z 376 → 287 (CE -32, CXP -7);
[$^{13}C_6$,^{15}N]-TA-DNPH:	m/z 383 → 182 (CE -34 V, CXP -9 V), m/z 383 → 122 (CE -64 V, CXP -7 V), m/z 383 → 306 (CE -30, CXP -7);
ValTA-DNPH:	m/z 362 → 182 (CE -34 V, CXP -7 V), m/z 362 → 122 (CE -64 V, CXP -7 V), m/z 362 → 301 (CE -34, CXP -7)
PheTA-DNPH:	m/z 410 → 182 (CE -34 V, CXP -7 V), m/z 410 → 122 (CE -64 V, CXP -7 V), m/z 410 → 363 (CE -26, CXP -1)
AlaTA-DNPH:	m/z 334 → 182 (CE -28 V, CXP -5 V), m/z 334 → 122 (CE -50 V, CXP -5 V), m/z 334 → 287 (CE -26, CXP -7)

As stationary phase a 150 mm x 2 mm i.d., 4 µm, Synergi Polar RP (Phenomenex, Aschaffenburg, Germany) was used. The mobile phase was mixed from water (solvent A) and methanol (solvent B) following a linear binary gradient as follows: Initial conditions were 50 % B and 50 % A. After 2 min isocratic delivery of the solvents, the content of solvent B was linearly raised during the next 3 min to

obtain 100 % B 5 min after injection. These conditions were continued until the end of the run after 13 min. Injection volume was 10 µL, flow rate 0.2 mL/min, and equilibration time between two runs 10 min.

Correction for spectral interferences of the isobaric compounds TA-DNPH and LeuTA-DNPH

Ion intensity ratios of the mass transitions m/z 376 → 301 and m/z 376 → 287 against the sum of both transitions were calculated from solutions of TA and LeuTA in five concentration levels (0.1, 0.5, 1, 5, 10 µg/mL; ten injections each) after derivatization. For pure compounds, constant ion intensity ratios were obtained as follows:

TA-DNPH: $\frac{A^{TA}_{\mathrm{m/z\ 301}}}{A^{TA}_{\sum \mathrm{m/z\ 301+287}}} =$ 96.9 ± 1.5 % (c)

$\frac{A^{TA}_{\mathrm{m/z\ 287}}}{A^{TA}_{\sum \mathrm{m/z\ 301+287}}} =$ 1.5 ± 0.3 % (d)

LeuTA-DNPH: $\frac{A^{LeuTA}_{m/z\ 301}}{A^{LeuTA}_{\sum \mathrm{m/z\ 301+287}}} =$ 13.8 ± 0.2 % (e)

$\frac{A^{LeuTA}_{\mathrm{m/z\ 287}}}{A^{LeuTA}_{\sum \mathrm{m/z\ 301+287}}} =$ 86.2 ± 0.2 % (f)

If both compounds were present in a mixture, spectral interferences will occur and the ratio TA/LeuTA could be calculated according to following formulas that are similar to the calculation of the ratio of lipids from a mixture by analyzing the fatty acid methyl esters according to DIN EN standard 5508.

(I) $\frac{A^{TA+LeuTA}_{\mathrm{m/z\ 301}}}{A^{TA+LeuTA}_{\sum \mathrm{m/z\ 301+287}}} = TA\ [\%] \cdot \frac{A^{TA}_{\mathrm{m/z\ 301}}}{A^{TA}_{\sum \mathrm{m/z\ 301+287}}} + LeuTA\ [\%] \cdot \frac{A^{LeuTA}_{\mathrm{m/z\ 301}}}{A^{LeuTA}_{\sum \mathrm{m/z\ 301+287}}}$, or reduced:

(Ia) $a = x \cdot c + y \cdot e$

(II) $\frac{A^{TA+LeuTA}_{\mathrm{m/z\ 287}}}{A^{TA+LeuTA}_{\sum \mathrm{m/z\ 301+287}}} = TA\ [\%] \cdot \frac{A^{TA}_{\mathrm{m/z\ 287}}}{A^{TA}_{\sum \mathrm{m/z\ 301+287}}} + LeuTA\ [\%] \cdot \frac{A^{LeuTA}_{\mathrm{m/z\ 287}}}{A^{LeuTA}_{\sum \mathrm{m/z\ 301+287}}}$, or reduced:

(IIa) $b = x \cdot d + y \cdot f$

These equations could be solved for x (TA [%]) and y (LeuTA [%]):

(III) $x = \frac{\frac{a}{e} - \frac{b}{f}}{\frac{c}{e} - \frac{d}{f}}$

(IV) $y = \frac{\frac{a}{c} - \frac{b}{d}}{\frac{e}{c} - \frac{f}{d}}$

In samples containing a mixture of TA and LeuTA, the obtained areas from LC-MS/MS measurements had to be corrected as follows:

(V) $A^{TA\ (sample)}_{m/z\ 301} = \frac{A^{TA+LeuTA\ (sample)}_{\mathrm{m/z\ 301}}}{A^{TA+LeuTA\ (sample)}_{\sum \mathrm{m/z\ 301+287}}} \cdot TA_{sample}\ [\%] \cdot \frac{A^{TA}_{\mathrm{m/z\ 301}}}{A^{TA}_{\sum \mathrm{m/z\ 301+287}}} = a_{sample} \cdot x_{sample} \cdot c$

(VI) $$A_{m/z\ 287}^{LeuTA\ (sample)} = \frac{A_{\mathrm{m/z\ 301}}^{TA+LeuTA\ (sample)}}{A_{\sum \mathrm{m/z\ 301+287}}^{TA+LeuTA\ (sample)}} \cdot LeuTA_{sample}\ [\%] \cdot \frac{A_{\mathrm{m/z\ 287}}^{LeuTA}}{A_{\sum \mathrm{m/z\ 301+287}}^{LeuTA}} = a_{sample} \cdot y_{sample} \cdot f$$

From the corrected areas the amount of TA and LeuTA could be calculated with the respective response functions as usual (see below).

Mixtures of TA and LeuTA were prepared in different ratios from 1:10 – 10:1 by adding different amounts of LeuTA (3–300 ng) to constant amount of TA (30 ng). After derivatization, these solutions were analyzed by LC-MS/MS and the ratio of TA/LeuTA was calculated from the results with the formulas shown above. The absolute content of both compounds was calculated with the respective response functions (see below) and the aberration between the real and calculated amount was calculated thereof.

Sample preparation

The sample preparation is based on methods for the analysis of TA described in literature (Siegel et al. 2009; Asam et al. 2011). About 2 g of the sample was weighed into a 50-mL centrifugation tube (Sarstedt, Nümbrecht, Germany) and spiked with labeled standard (concentration: 1 µg/mL; added volume: 30 µL ≡ 15 µg/kg). Thereafter, the derivatization reagent (15 mL) was added, followed by 10 min ultrasonication and 20 min vigorously shaking. After adding the quenching reagent (10 mL), shaking was continued for another 10 min. The centrifugation tube was centrifuged (5 min, 4,600 rpm ≡ 4,000 g, 25°C) by means of a Heraeus Multifuge 3 L-R (Thermo Fisher Scientific, Waltham, MA, USA), and the organic phase was transferred into a 25-mL pear-shaped flask. The watery phase was further extracted with another portion of ethyl acetate (10 mL) for 10 min by shaking followed by centrifugation. The organic phases were combined and brought to dryness by a rotary evaporator. The remainder was taken up in acetonitrile (1 mL) and transferred to a 10-mL centrifugation tube (Sarstedt, Nümbrecht, Germany). Water (3 mL) was added followed by centrifugation (5 min, 4,600 rpm ≡ 4,000g, 25°C). The supernatant was used for C_{18} solid phase extraction as described above, with a modification of the washing step that was carried out with 5 mL of water and 3 mL of acetonitrile/water (30/70; v/v). After evaporation of the purified extract, the residue was taken up in 500 µL of acetonitrile/water (30/70; v/v) for LC-MS/MS measurements.

Method validation

Limits of detection (LOD) and limits of determination (LOQ)

Limits of detection (LOD) and limits of determination (LOQ) were determined according to a procedure described in literature (Vogelgesang and Hädrich, 1998) that is a modification of DIN EN standard 32645. Accordingly, a sorghum grain sample that contained 9 µg/kg of TA, only, was spiked with the analytes at four concentration levels in a range of 50 – 500 µg/kg (method without derivatization) and 1.5 – 15 µg/kg (method with derivatization). However, as no sample was available that contained TA in levels below the LOD of the derivatization method, starch powder was used as blank matrix for TA, therefore. After addition of [$^{13}C_6$,^{15}N]-TA in the same amount as the respective amount of analyte, all samples underwent sample preparation and cleanup as described above and were finally analyzed by LC-MS/MS. LODs and LOQs were derived statistically from the data according to the literature (Vogelgesang and Hädrich 1998).

Calibration and quantification (only method with derivatization)

Response solutions were prepared by mixing analytes (A) and labeled standard (S) in 9 molar ratios n(A)/n(S) from 0.01 to 100. In detail, mixtures of n(A)/n(S) of 1:100, 1:50, 1:10, 1:5, 1:1, 5:1, 10:1, 50:1 and 100:1 were prepared by adding constant amounts of standard (30 µL, 0.1 µg/mL) to varying amounts of analyte (30–150 µL; 0.001–10 µg/mL). After addition of the derivatization reagent (50 µL; 0.77 mmol/L) and ultrasonication (10 min), the quenching reagent was added (30 µL; 2.4 mmol/L) followed by further ultrasonication. The solvent was then removed in a stream of nitrogen, and the residue was taken up in methanol/water (100 µL, 50/50, v/v).

Matrix-matched calibration was performed for Leu-TA and Val-TA with two blank matrices (sorghum grains and tomato puree). Accordingly, respective samples (2 g) were spiked in duplicate with analytes (A) and labeled standard (S; 30 µg/kg) in 5 molar ratios n(A)/n(S) from 0.1 (3 µg/kg) to 10 (300 µg/kg) (1:10, 1:5, 1:1, 5:1, 10:1). The samples were subjected to further sample preparation as described above.

After measuring these solutions with LC-MS/MS, response curves were constructed from the obtained signal area ratios A(A)/A(S) (≡ x) against the respective molar ratios n(A)/n(S) (≡ y) using weighted linear regression (weighing factor $1/y^2$). Mandel's fitting test was performed for checking linearity.

The content n(A) of analyte in samples was calculated from the recorded signal area ratios A(A)/A(S), the equation of the respective response curve and the amount of labeled standard n(S) added to the respective sample. All data of food samples were based on duplicate analyses.

Recovery (only method with derivatization)

Recovery was determined by spiking the analytes (TA, LeuTA and ValTA) to respective blank matrices (tomato puree and sorghum cereals) or surrogates (starch powder) (2 g each) in three contamination levels (15 µg/kg, 75 µg/kg and 150 µg/kg; each in triplicate). After addition of labeled standard (30 µL, 1 µg/mL ≡ 15 µg/kg), sample preparation, clean-up and LC-MS/MS measurement, the recovery was calculated as the mean of the spiking experiments using the respective matrix matched calibrations.

Precision (only method with derivatization)

Interassay precision was determined by analyzing a naturally contaminated matrix (whole meal sorghum cereals; 900 µg/kg TA, 120 µg/kg ValTA, 40 µg/kg LeuTA) three times in triplicate during three weeks.

Results and discussion

Method development

Mass spectrometry of tenuazonic acid analogues

Due to their acidity all tenuazonic acid analogues can be readily deprotonated and are most sensitively detectable as anions $[M–H]^-$ using mass spectrometry with negative electrospray ionization (ESI). When tandem mass spectrometry (MS/MS) was applied using collision induced dissociation (CID), all tenuazonic acid analogues showed the same fragment ions at m/z 139 and m/z 112 from the respective precursor ion $[M–H]^-$. The fragmentation behavior of tenuazonic acid itself in the negative ESI mode has been described previously (Asam et al. 2011). The proposed fragmentation route involves the cleavage of the side chain to yield the tetramic acid nucleus (m/z 139) that undergoes further fragmentation (m/z 112). No differentiation between the isobaric tenuazonic acids derived from isoleucine (TA) and leucine (LeuTA) could be performed by mass spectrometry under these conditions, therefore.

Mass spectrometry of dinitrophenylhydrazones of tenuazonic acid analogues

After derivatization of tenuazonic acid with 2,4-dinitrophenylhydrazine (DNPH) the mass spectrometric measurement of the resulting dinitrophenylhydrazone was reported both in the positive (Siegel et al. 2009) and in the negative (Asam et al. 2011) ESI mode. The measurement of dinitrophenylhydrazones of tenuazonic acid analogues was also possible at both polarities (Fig. 2), but the ESI negative mode was much more intense for all compounds with our instrument.

In the positive mode, MS/MS fragmentation of $[M+H]^+$ nearly exclusively yielded a fragment ion of m/z 140 (TA, LeuTA, ValTA) or m/z 139 (AlaTA, PheTA). Thus, MS/MS measurements in the multiple reaction monitoring (MRM) mode were highly sensitive using these transitions, but further diagnostic ions for identification could hardly be measured in low concentrations with our instrument. Besides this drawback concerning sensitivity, no differentiation between the isobaric compounds TA and LeuTA was possible in the positive ESI mode, as the MS/MS spectra of both compounds were identical even in the minor fragment ions. In respect of the proposed structures of the fragment ions of TA-DNPH in the positive mode (Siegel et al., 2009) this finding seems reasonable, as the side chain of TA is not involved in fragmentation routes.

In the negative ESI mode, the MS/MS spectra of tenuazonic acid dinitrophenylhydrazone can be separated in two series of signals (Fig. 2). One set of signals (m/z 330, 329, 301) include the tenuazonic acid nucleus and, thus, appear with a distinctive mass shift in the spectrum of the labeled standard (m/z 337, 336, 306). Another set of signals (m/z 122, 152, 182) revealed fragments of the DNPH moiety (Asam et al. 2011). These signals appear in the spectrum of the labeled standard as well, and are not characteristic for the analyte, therefore. The proposed fragmentation route of TA-DNPH (Asam et al., 2011) indicates that fragmentation of the side chain of TA is required to obtain the diagnostic ions. Sometimes it is possible to differentiate between two closely related isobaric substances on the basis of different fragment ion formation in MS/MS experiments, as it has been shown for 3-acetyl- and 15-acetyl-deoxynivalenol (Berthiller et al., 2005). Indeed, LeuTA-DNPH yielded the same fragmentation ions as TA-DNPH, but different intensity was observed for the respective product ions at m/z 301 (predominant with TA) and m/z 287 (predominant with LeuTA).

As both compounds could not be separated chromatographically (see below), the differentiation had to be performed by mass spectrometry.

Therefore, the ion intensity ratios between m/z 301 and m/z 287 related to the sum of both transitions, respectively, were calculated and found to be constant and characteristic for TA and LeuTA. Thus, it was possible not only to detect a simultaneous occurrence of TA and LeuTA, but also to calculate their ratio by monitoring the respective ion intensity ratios. The formulas used for this calculation were similar to the calculation of the ratio of lipids from a mixture by analyzing the fatty acid methyl esters as described in DIN EN standard 5508. To prove this calculation, mixtures of TA and LeuTA were prepared and analyzed. Compared to the real value, about 104 ± 8 % of TA and 102 ± 7 % of LeuTA were calculated with the formulas from the ion intensity ratios. Thus, it was possible to compensate for the spectral interference of TA and LeuTA with this procedure.

High performance liquid chromatography without derivatization

The chromatographic separation of tenuazonic acid without using special modifiers that impede LC-MS detection has been presented once (Kocher et al. 2008), but never been published in detail. Although stationary phases nowadays are available that allow retention and reasonable peak shape of tenuazonic acid with as little as 0.05 % trifluoroacetic acid (TFA) in the mobile phase (Siegel et al. 2009), even this concentration of modifier can lead to severe ion suppression in the ion-source of the mass spectrometer so that this approach should be avoided. However, by the use of a pH-stable stationary phase and the mobile phase buffered with ammonium formate and set at pH 9, all tenuazonic acid analogues successfully could be separated as anions (Fig. 3). Differentiation between the isobaric compounds TA and LeuTA was easily possible due to different retention time.

High performance liquid chromatography of tenuazonic acid analogues dinitrophenylhydrazones

It has been shown that the derivatization of tenuazonic acid with 2,4-dinitrophenylhydrazine leads to a stable compound that can be separated on standard C_{18} reversed phase columns with good peak shape using mobile phases that are compliable with MS detection (Siegel et al. 2009). The tenuazonic acid analogues dinitrophenylhydrazones showed identical behavior, but chromatographic separation between the single compounds was only marginal (Fig. 4). The isobaric compounds TA and LeuTA had to be differentiated by mass spectrometric means as described above, therefore. However, as all compounds were eluted rather close to the internal standard [$^{13}C_6$,^{15}N]-TA, the compensation of interfering effects ion suppression and ion enhancement was expected to be counterbalanced as good as possible.

Method validation

Limits of detection and quantitation

Limits of detection (LOD) and limits of quantitation (LOQ) were determined according to a procedure described in literature (Vogelgesang and Hädrich 1998) that is comparable to DIN EN standard 32645 after spiking of a blank matrix (sorghum grain) with tenuazonic acid and its analogues (Tab. 1). The LODs and LOQs of the method without derivatization were about 20–30 times higher than those after derivatization with 2,4-dinitrophenylhydrazine. For an instrument different to ours LODs of 10 µg/kg (with derivatization) and 2000 µg/kg (without derivatization and trifluoroacetic acid (TFA) as additive in the mobile phase) were reported for the determination of tenuazonic acid with LC-MS/MS (Siegel et al. 2009). However, even without the use of TFA, no LODs below 50 µg/kg could be

achieved without derivatization with our instrument. Within the respective methods the values for the LODs were rather similar for all tenuazonic acid analogues (Tab. 1), ranging from 50–80 µg/kg (without derivatization) and 1–3 µg/kg (with derivatization). The LOQs were about three times higher. Thus, the method without derivatization was not sensitive enough to determine tenuazonic acid analogues that are present only in traces, generally, and could be only used for determination of tenuazonic acid in highly contaminated samples or for confirmation purposes. Further method validation that dealt with quantification of the analytes was only performed for the method with derivatization, therefore.

Calibration and quantification

Response curves were recorded by analyzing mixtures of tenuazonic acid analogues with the internal standard [$^{13}C_6$,^{15}N]-TA in different molar ratios either in pure solvent or after spiking to respective blank matrices (matrix matched calibration). Linearity was observed between molar ratios of analyte (A) and labeled standard (S) of n(A)/n(S) = 0.02–100 (pure solvents) and 0.1–10 (matrix matched calibration). However, heteroscedascity was obvious due to the wide working range and, thus, respective response factors of tenuazonic acid analogues relative to the internal standard [$^{13}C_6$,^{15}N]-TA were calculated after weighted linear regression (Tab. 2) and used for quantification of samples.

Recovery

Recovery was determined by spiking tenuazonic acid (TA), LeuTA and ValTA to respective blank matrices or surrogate matrices (Tab. 3). Tenuazonic acid was determined with a stable isotope dilution assay and the recovery was 100 %, therefore, as any losses of the analyte were perfectly counterbalanced by the isotopically labeled internal standard. LeuTA and ValTA were quantified with [$^{13}C_6$,^{15}N]-TA as internal standard and respective matrix matched calibration. The recoveries of these two compounds were also around 100 %, but with larger imprecision compared to TA due to the less precise analytical technique. No correction for recovery was applied for the calculation of the analyte content of samples, therefore. Without any internal standard the recovery of TA has been reported to be 79 ± 11 % (Siegel et al. 2009). Thus, the use of an internal standard clearly improves the quality of the measurement.

Precision

Precision was determined by analyzing sorghum infant cereals that were naturally contaminated with TA (912 ± 60 µg/kg), LeuTA (43 ± 8 µg/kg) and ValTA (118 ± 22 µg/kg) three times in triplicate during three weeks. The precision (relative standard deviation) was 7 % for TA, 17 % for LeuTA and 19 % for ValTA. TA was quantified with a stable isotope dilution assay and the high precision of 7 % is attributable to this technique. However, the precision of the determination of LeuTA and ValTA using matrix matched calibration and [$^{13}C_6$,^{15}N]-TA as internal standard was still acceptable.

Determination of tenuazonic acid analogues in food commodities

A series of food commodities recently was analyzed for tenuazonic acid in our laboratory including tomato products (Asam et al. 2011), cereals, fruit juices, spices (Asam et al. 2012) and also infant food and sorghum cereals (Asam et al. submitted). Tenuazonic acid analogues were detected only in samples that exhibited a high content of tenuazonic acid, already, and in matrices that allowed low limits of detection at the same time (i.e. all except spices). Thus, no tenuazonic acid analogues were determined in tomato products, cereals, fruit juices and spices, but in sorghum based infant cereals,

sorghum grain and some other samples, LeuTA and ValTA were readily detected (Tab. 4). In a few highly contaminated samples the detection of those two tenuazonic acid analogues was even possible both with and without derivatization (Fig. 5 and 6). The other tenuazonic acid analogues AlaTA and PheTA were not detected, as it could have been expected from the literature (Gatenbeck and Sierankiewicz 1973 b). In comparison to the amount of tenuazonic acid detected in those products, the mean ratio was 4 % (of the TA value) for LeuTA and 10 % (of the TA value) for ValTA. In other samples like fennel tea and goji berries, the ratios were lower, although these samples were also highly contaminated with tenuazonic acid. However, more samples have to be analyzed to elucidate if there is a general tendency related to sorghum. Nevertheless, the occurrence of the toxicological poorly characterized tenuazonic acid analogues LeuTA and ValTA in sorghum based infant food raises further concerns about these products the safety of which already has been called to question due to the high contamination with tenuazonic acid itself.

Acknowledgments

The authors thank Christopher Zeck, BIOANALYTIK Weihenstephan, Technische Universität München, for excellent technical assistance.

References

Tables

Table 1 Limits of detection (LOD) and limits of determination (LOQ) of tenuazonic acid (TA) and its analogues derived from valine (ValTA), leucine (LeuTA), phenylalanine (PheTA) and alanine (AlaTA) determined according to DIN EN standard 32645 for the two developed methods with and without derivatization with 2,4-dinitrophenylhydrazine.

	Without derivatization		With derivatization	
	LOD	LOQ	LOD	LOQ
TA	60	180	1	3
ValTA	55	165	2	6
LeuTA	60	180	3	9
PheTA	50	150	3	9
AlaTA	80	240	2.5	7

Table 2 Response factors of tenuazonic acid (TA) and its analogues derived from valine (ValTA), leucine (LeuTA), phenylalanine (PheTA) and alanine (AlaTA) relative to the internal standard [$^{13}C_6$,^{15}N]-TA for different matrices (matrix matched calibration) (method with derivatization).

Matrix	TA	LeuTA	ValTA	PheTA	AlaTA
Pure solvent	0.92	1.08	1.37	0.84	1.26
Tomato puree	n. d.[1]	1.30	1.73	0.93	1.94
Sorghum cereals	n. d.[1]	1.49	1.41	0.86	1.89

[1] n. d. = not determined (no blank matrix available)

508 Table 3 Recovery of tenuazonic acid (TA) and its analogues derived from valine (ValTA) and
509 leucine (LeuTA) from different blank matrices calculated with the respective matrix
510 matched calibration (method with derivatization).

Matrix	TA	LeuTA	ValTA
Tomato puree	105 ± 2 %[1]	95 ± 11 %[2]	96 ± 15 %[2]
Sorghum cereals	100 ± 1 %[1, 3]	101 ± 4 %[2]	102 ± 10 %[2]

[1] Stable isotope dilution assay

[2] Calculated with [$^{13}C_6$,^{15}N]-TA as internal standard and matrix matched calibration

[3] Starch powder as surrogate matrix

511

512

513 Table 4 Content of tenuazonic acid (TA) and its analogues derived from valine (ValTA) and
514 leucine (LeuTA) in different food commodities (method with derivatization).

	TA [µg/kg]	LeuTA [µg/kg]	% of TA value	ValTA [µg/kg]	% of TA value
Fennel Tea (drug)	1500 ± 275	21 ± 14	1 %	168 ± 3	11 %
Fennel Tea (drug)	860 ± 75	11 ± 3	1 %	51 ± 1	6 %
Goji berries	1040 ± 100	– [1]	– [1]	70 ± 2	7 %
Goji berries	760 ± 70	(4 ± 0.5)[2]	(0.5)[2]	7 ± 0.7	1 %
Sorghum infant cereals	1200 ± 100	74 ± 8	6 %	150 ± 22	12 %
	930 ± 20	31 ± 2	3 %	68 ± 3	7 %
	900 ± 40	31 ± 3	3 %	46 ± 5	5 %
	900 ± 70	50 ± 7	6 %	112 ± 5	12 %
	620 ± 60	31 ± 2	5 %	78 ± 17	13 %
	380 ± 30	12 ± 1	3 %	22 ± 5	6 %
	370 ± 20	24 ± 1	6 %	51 ± 5	14 %
	130 ± 5	(6 ± 0.5)[2]	5 %	20 ± 1	16 %
Sorghum grains	670 ± 20	(3 ± 0.4)[2]	(1 %)[2]	44 ± 5	7 %
	460 ± 10	23 ± 2	5 %	40 ± 4	9 %
	230 ± 10	(8 ± 0.7)[2]	(3 %)[2]	20 ± 2	9 %
	105 ± 10	(3 ± 0.3)[2]	(3 %)[2]	8 ± 0.9	8 %
Sorghum sweets	1200 ± 120	64 ± 5	5 %	172 ± 15	14 %

[1] Below limit of detection

[2] Between limit of detection and limit of quantification

515

Legends to the figures

Figure 1 Structure of tenuazonic acid and related analogues (only one isomer shown, however the synthesis yielded racemates). Relevant hydrogen atoms for nuclear resonance spectroscopy (NMR) are enumerated with *A* – *E*, respectively. *(1)* tenuazonic acid, *(2)* [$^{13}C_6$,^{15}N]-tenuazonic acid, *(3)* leucine tenuazonic acid, *(4)* alanine tenuazonic acid, *(5)* valine tenuazonic acid, *(6)* phenylalanine tenuazonic acid.

Figure 2 MS/MS spectra of the dinitrophenylhydrazones of tenuazonic acid analogues in the positive and the negative ionization mode with proposed structures of fragment ions according to the literature (Siegel et al., 2009; Asam et al., 2011).

Figure 3 LC-MS/MS run of tenuazonic acid analogues (AlaTA, ValTA, TA, LeuTA, PheTA) (only quantifier traces shown).

Figure 4 LC-MS/MS run of tenuazonic acid analogues dinitrophenylhydrazones (AlaTA-DNPH, ValTA-DNPH, TA-DNPH, LeuTA-DNPH, PheTA-DNPH) (only quantifier traces shown).

Figure 5 LC-MS/MS run (method without derivatization) of a sorghum sweet sample containing TA (7.9 min; 1200 µg/kg), LeuTA (9.6 min; 64 µg/kg) and ValTA (5.1 min; 172 µg/kg) together with the internal standard [$^{13}C_6$,^{15}N]-TA (7.9 min) (only quantifier traces shown).

Figure 6 LC-MS/MS run (method with derivatization) of a sorghum infant cereal sample containing TA-DNPH (9.0 min; 900 µg/kg), LeuTA-DNPH (9.0 min; 50 µg/kg) and ValTA-DNPH (8.7 min; 112 µg/kg) together with the internal standard [$^{13}C_6$,^{15}N]-TA (9.0 min) (only quantifier traces shown).

Figures

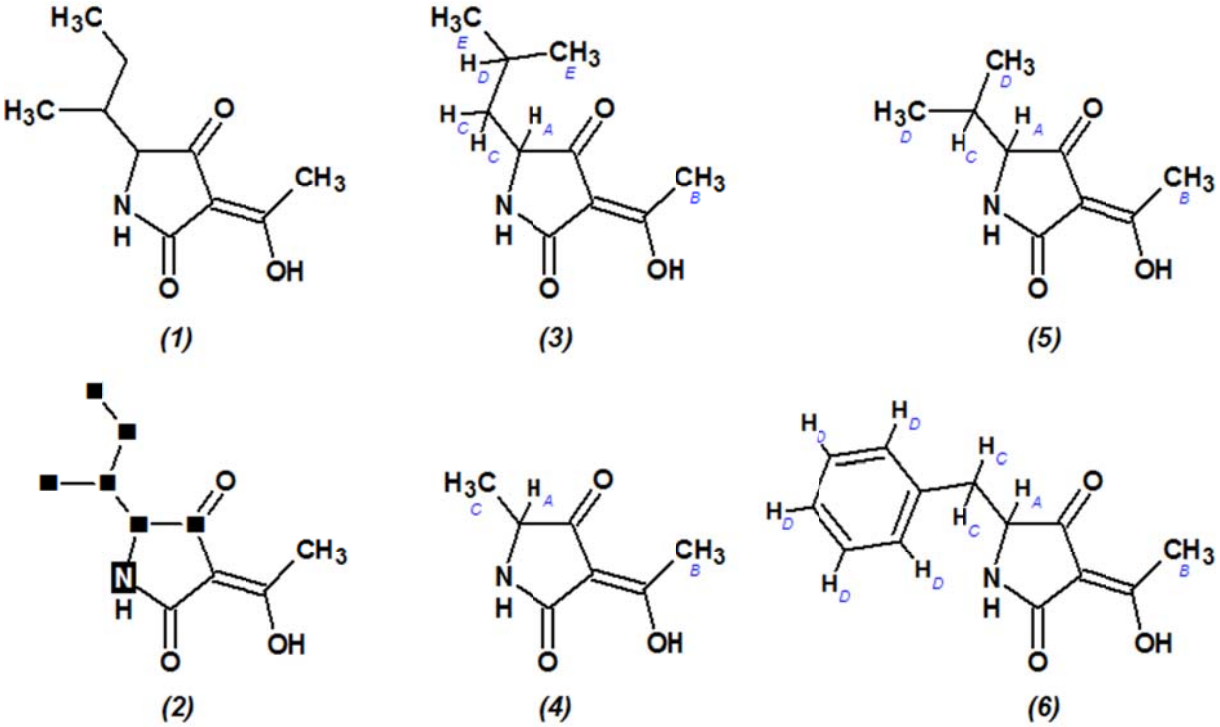
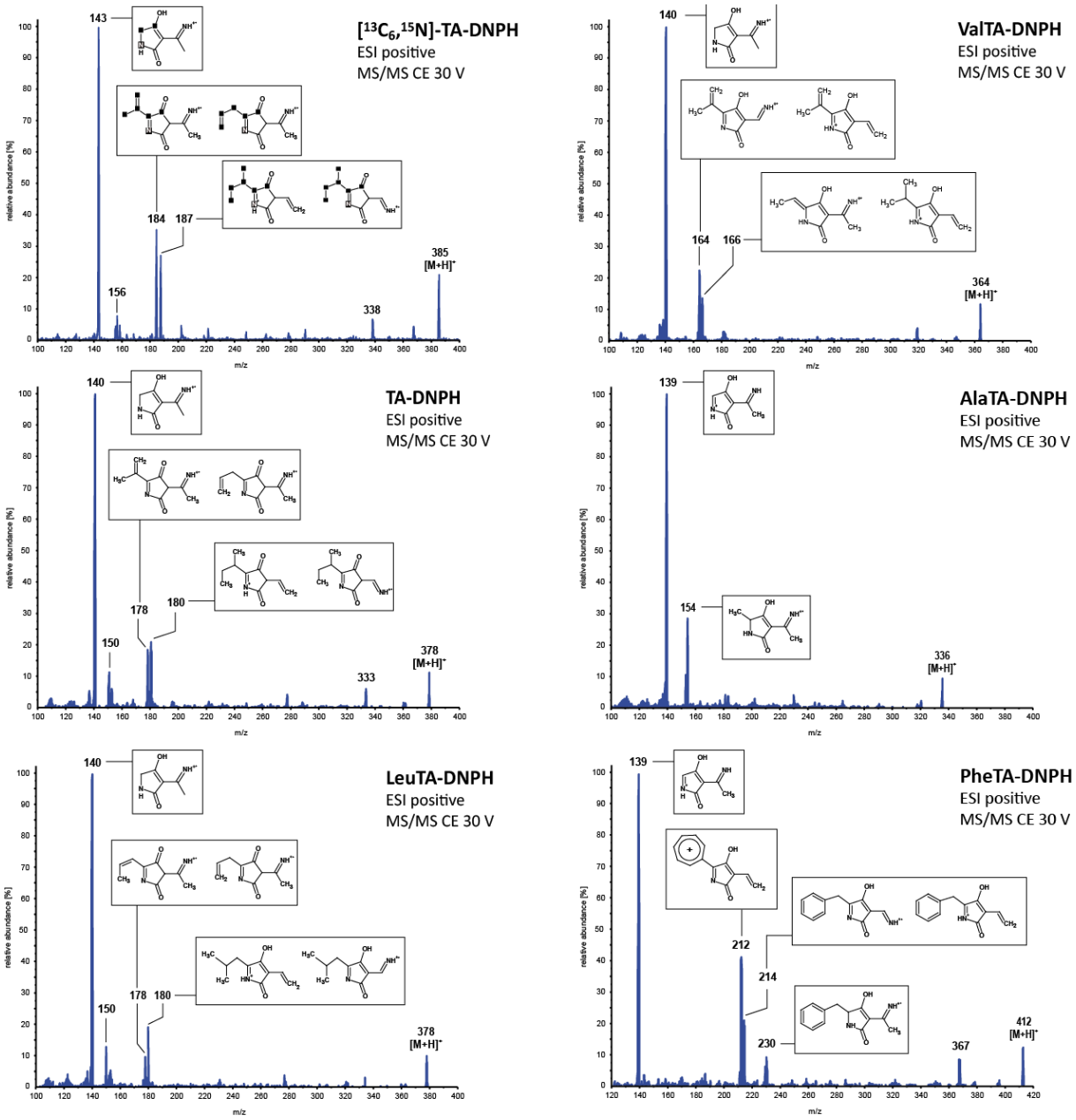


Figure 1



545

546 Figure 2

547

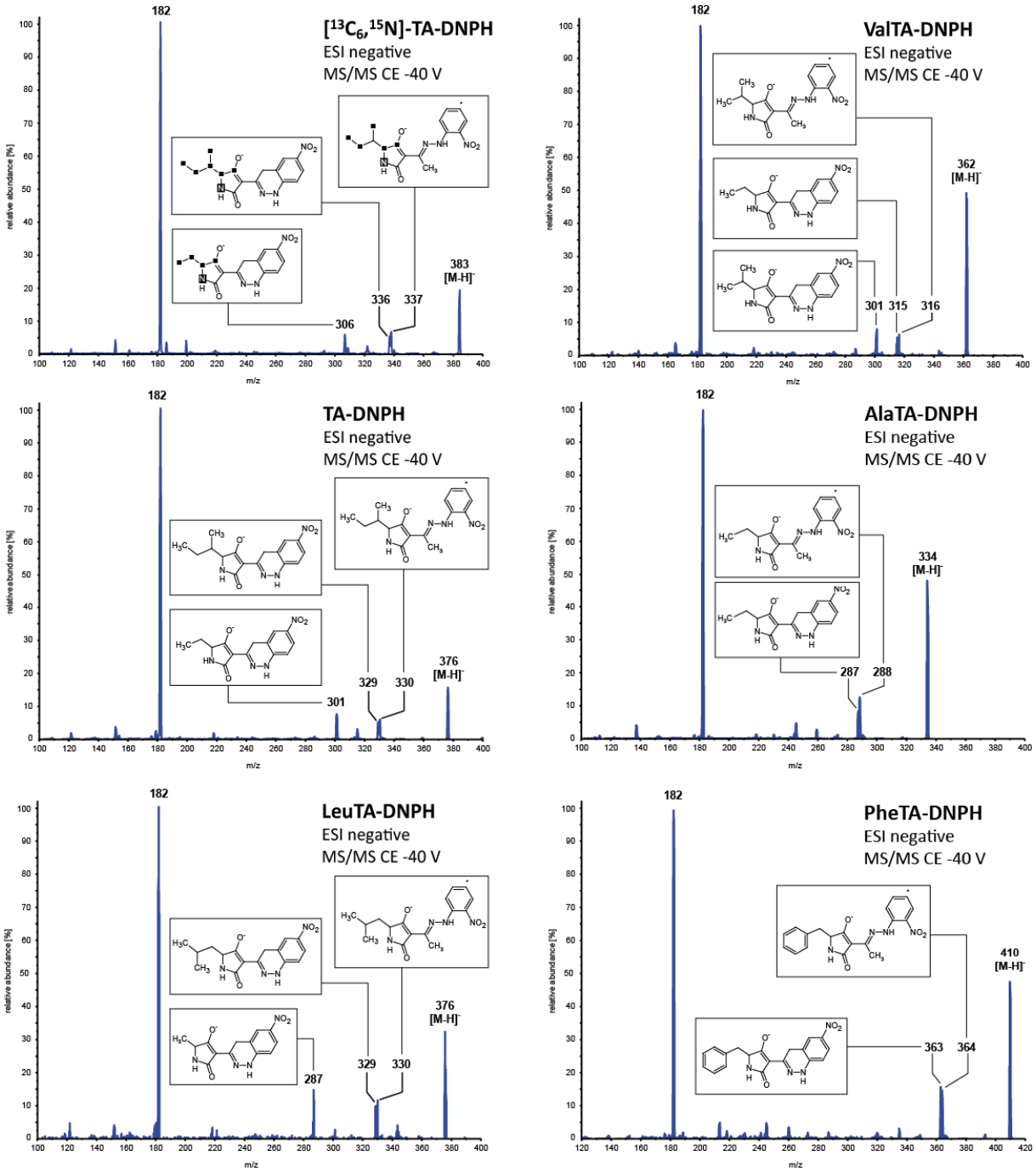
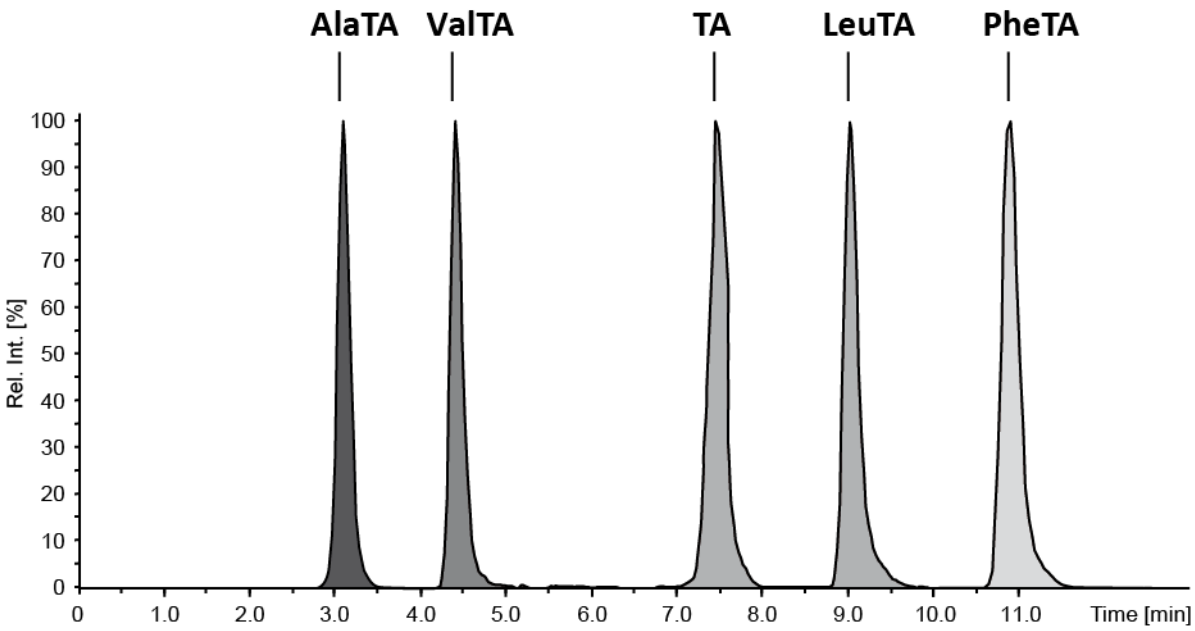


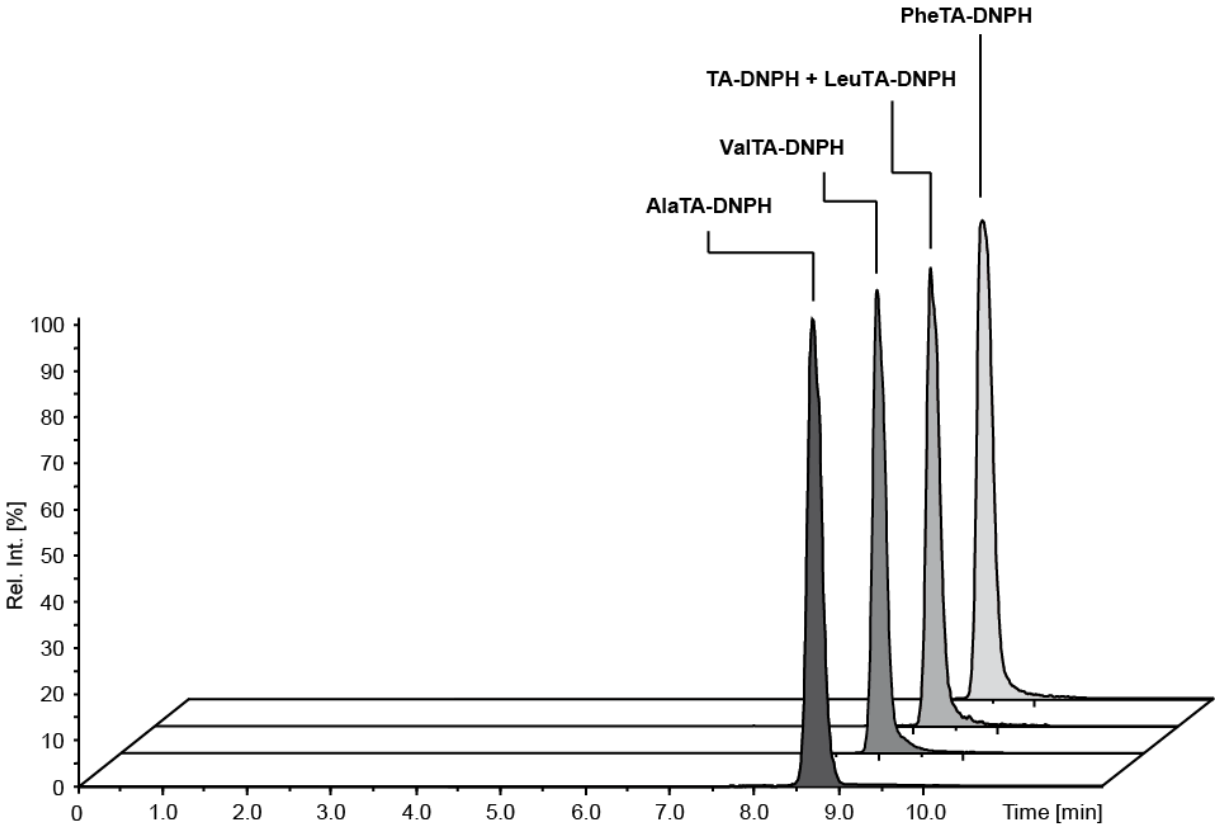
Figure 2 (continued)



554

555 Figure 3

556



557

558 Figure 4

559

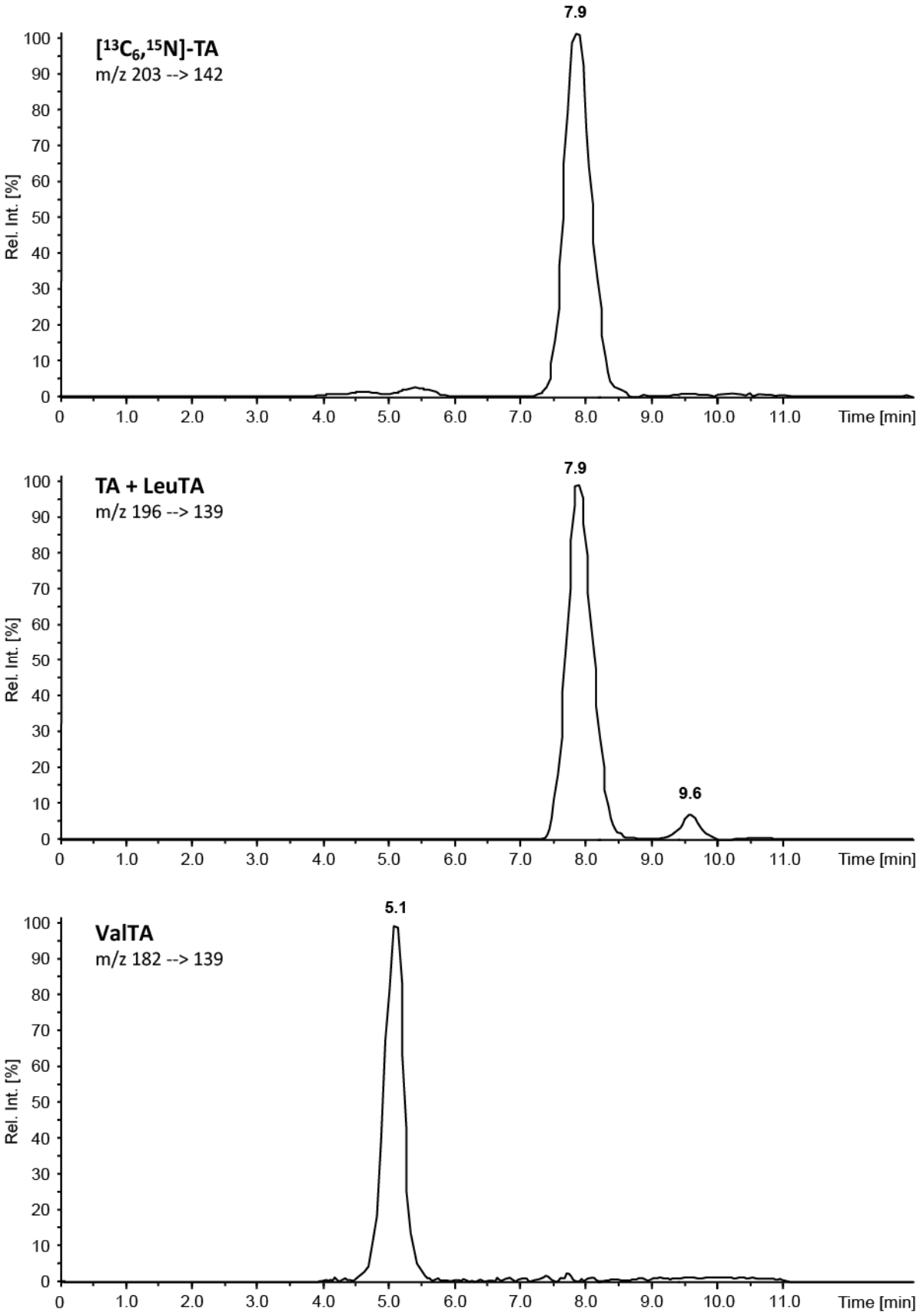
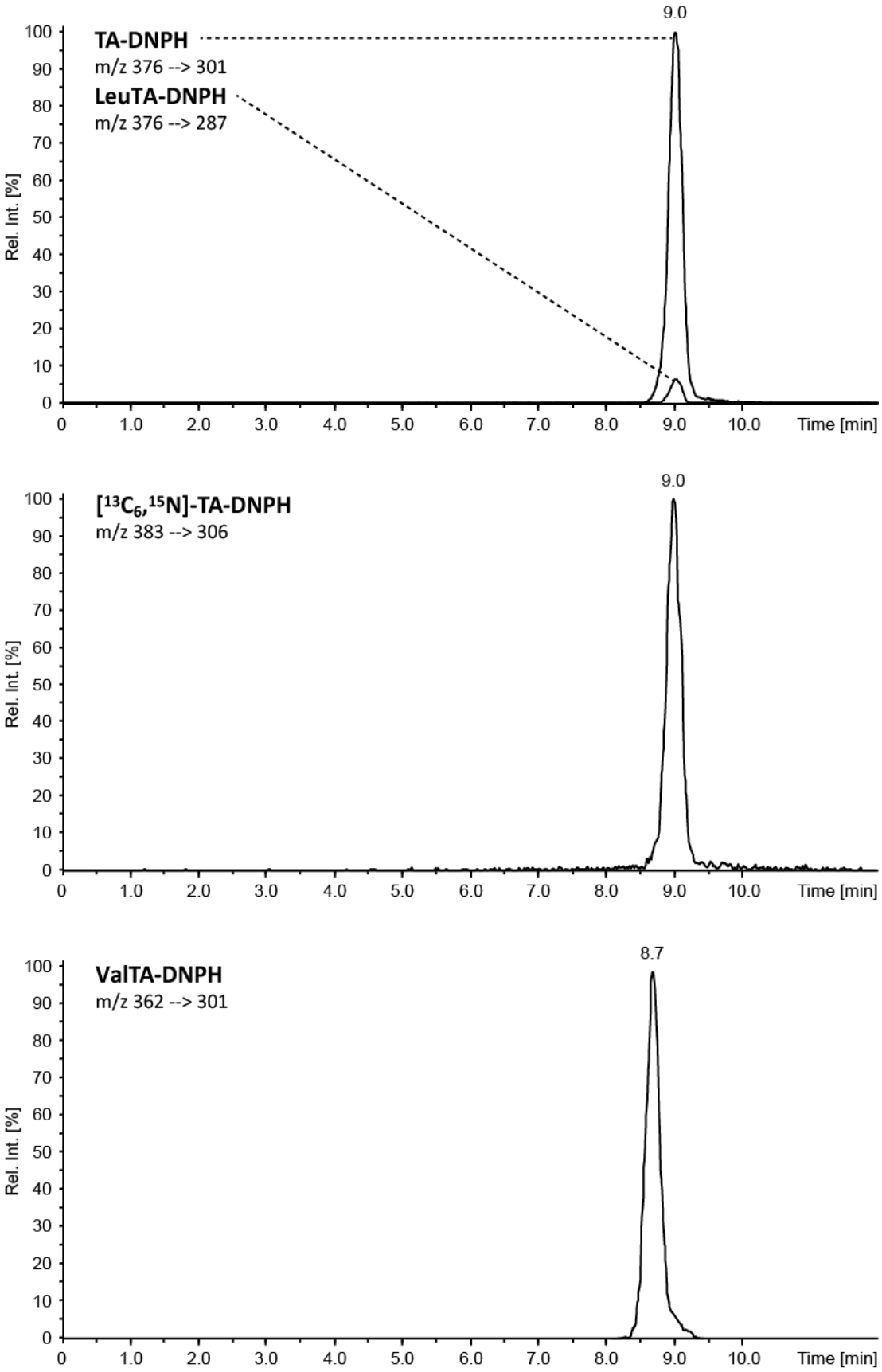

7.9
[13C6,15N]-TA
m/z 203 --> 142
Rel. Int. [%]
Time [min]
7.9
TA + LeuTA
m/z 196 --> 139
9.6
Rel. Int. [%]
Time [min]
5.1
ValTA
m/z 182 --> 139
Rel. Int. [%]
Time [min]

Figure 5



562

563 Figure 6