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Determination of tenuazonic acid in human urine by means of a stable isotope dilution assay

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Abstract

The content of tenuazonic acid in human urine was determined by a stable isotope dilution assay (SIDA) that was recently developed for the analysis of food commodities and extensively re-validated for urine matrix in this study. Linearity of the response curve was proven between molar ratios n(labeled standard)/n(analyte) of 0.02 – 100. The limit of detection (LOD) and the limit of determination (LOQ) were 0.2 µg/L and 0.6 µg/L, respectively. The mean recovery of the stable isotope dilution assay was 102 ± 3 % in the range between 1.0 – 100 µg/L. Interassay precision was 6.7 % (relative standard deviation of three triplicate analyses of a human urine sample during three weeks). The method was applied to two studies dealing with urinary excretion of tenuazonic acid: In the first study, tenuazonic acid was quantified in the 24-hour urine of six volunteers from Germany (3 female, 3 male) in a concentration range of 1.3–17.3 µg/L or 2.3–10.3 ng/mg^{-1} creatinine, respectively. In the second study, two volunteers (1 female, 1 male) ingested 30 µg tenuazonic acid by consumption of naturally contaminated whole meal sorghum infant cereals and tomato juice, respectively. The urinary excretion of the ingested tenuazonic acid was 54–81 % after six hours, depending on matrix and volunteer. After 24 hours 87–93 % of the ingested amount of tenuazonic acid was excreted, but the fate of the remaining about 10 % is open. Thus, it is not possible to exclude potential health hazards for the consumer, completely.

Keywords:

Tenuazonic acid, mycotoxin, stable isotope dilution assay, SIDA, human urine

Introduction

The tetramic acid derivative *L*-tenuazonic acid [(5*S*,8*S*)-3-acetyl-5-*sec.*-butyl-pyrrolidine-2,4-dione] (TA, Fig. 1) is a mycotoxin that has attracted increasing attention during the last years. Tenuazonic acid was originally isolated from a culture of the black mold *Alternaria* [1], a virulent plant pathogen invading a series of plants that is also involved in the postharvest decay of fruits, grains, and vegetables. However, other fungi like *Phoma sorghina* (isolated from sorghum) [2] and *Piricularia oryzae* (isolated from rice) [3, 4] also have been reported to produce tenuazonic acid.

Tenuazonic acid inhibits protein biosynthesis by suppression of the release of new proteins from the ribosome [5] and exhibits manifold biological activity. In detail, tenuazonic acid has been reported to exert antiviral [6], antitumor, antibacterial, cytotoxic [7] and phytotoxic properties [8] and also to be acutely toxic in mammals [9]. Oral LD_{50}-values of 182 or 225 mg kg^{-1} body weight for male mice [6, 9] and 81 mg kg^{-1} body weight for female mice [6] have been determined, but further toxicological data are missing. No data on chronic toxicity are available and, therefore, neither toxicological limits such as the tolerable daily intake (TDI) have been calculated so far, nor has the introduction of regulatory limits been considered, yet.

The analysis of tenuazonic acid in plant material and feed or food derived thereof generally is performed by high performance liquid chromatography. However, as tenuazonic acid is a strong acid and a metal chelating compound that shows irreproducible chromatographic behavior, the addition of modifiers like $Zn(II)SO_4$ to the mobile phase is necessary [10]. Although these methods are limited to UV-detection, the selectivity and sensitivity was sufficient to determine tenuazonic acid in tomatoes, especially [11–15].

However, tenuazonic acid analysis had been significantly improved in the last years due to the development of a derivatization method with 2,4-dinitrophenylhydrazine (DNPH) [16] and a stable isotope dilution assay using [$^{13}C_6$,^{15}N]-tenuazonic acid as internal standard [17] that both use mass spectrometric detection. The first approach converts tenuazonic acid to a stable derivative that can be separated chromatographically without special modifiers and shows high sensitivity in tandem mass spectrometry, whereas the SIDA approach counterbalances varying derivatization yields and ionization interferences in the ion source of the mass spectrometer and, therefore, provides most reliable results.

Consequently, tenuazonic acid was determined in a series of food commodities like flour and bakery products (e. g. crisp bread) [16], beer [18], tomato products (e. g. ketchup) [17], fruit juices and spices [19] and even infant food [20]. The degree of human exposure with tenuazonic acid can be assumed to be rather high, therefore, but the assessment of adverse health effects is essentially hampered, as no toxicokinetic data about absorption, metabolism and excretion of this mycotoxin are available. However, due to the polarity of tenuazonic acid, predominant urinary excretion can be assumed.

Recently, several methods have been published that focus on the determination of mycotoxins and their metabolites in human urine as biomarkers of exposure. The substances analyzed in these studies were deoxynivalenol (DON) and DON-glucuronide [21–23], ochratoxin A (OTA) and OTA-alpha [24, 25], fumonisins (B_1, B_2), either together with aflatoxins (B_1, B_2, G_1, G_2, M_1) and OTA [26], or separately [27], and zearalenone [28]. However, current methods based on liquid chromatography tandem mass spectrometry (LC-MS/MS) are able to determine most of these substances simultaneously [29–31]. Nevertheless, to the best of our knowledge, none of these methods included tenuazonic acid into their analytical scope.

Therefore, it was the aim of our study to apply and re-validate the recently developed stable isotope dilution assay for the sensitive and precise measurement of tenuazonic acid in human urine.

Materials and Methods

Chemicals and reagents.

Tenuazonic acid, copper(II) salt, 2,4-dinitrophenylhydrazine (phlegmatized with 30 % water), undecylic aldehyde, Dowex 50 WX80 (100-200 mesh) cation-exchange resin, picric acid solution (0.9–1.1 %), creatinine hydrochloride, and β-glucuronidase from *Helix pomatia* (Type HP-2, aqueous solution, ≥ 100,000 units/mL, secondary activity: ≤ 7,500 units/mL sulfatase) 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 [17].

Preparation of standard solutions

Commercial tenuazonic acid copper(II) salt was converted into its free form as described in the literature [16, 17, 32]. Stock solutions of tenuazonic acid and [$^{13}C_6$,^{15}N]-tenuazonic acid (~10 µg/mL, respectively) were prepared in methanol and the concentration was determined by UV-spectroscopy using the molar extinction coefficient of 1.298×10^4 L mol^{-1} cm^{-1} according to the literature [11, 16, 17, 32]. Working solutions (0.1–1 µg/mL) were prepared by further dilution. All solutions were stored in the dark at −20°C to ensure stability [33].

Preparation of derivatization and quenching reagent.

Following a modified procedure from literature [16, 34], the derivatization reagent was prepared from 2,4-dinitrophenylhydrazine (150 mg, 0.5 mmol) in 2 M hydrochloric acid (65 mL) to give a stock solution (7.7×10^{-3} mol/L). It was used either directly or after dilution (1:10) with 2 M hydrochloric acid. Undecylic aldehyde (0.05 % in ethyl acetate, 2.4×10^{-3} mol/L) was used as quenching reagent in order to destroy excess derivatization reagent after the derivatization step [16, 17].

Sample preparation.

Food samples.

The homogenized sample (2 g) was weighted in a 50 mL-centrifugation tube (Sarstedt AG & Co., Nümbrecht, Germany) and spiked with labeled standard (1 µg/mL; 30 µl ≡ 15 µg/kg). Afterward, the derivatization reagent (0.77×10^{-3} mol/L; 15 mL) was added, followed by 10 min ultrasonication and 20 min shaking. After adding the quenching reagent (10 mL) shaking was continued for another 10 min. The centrifugation tube was centrifuged (5 min, 4000 g, 25 °C) by means of a Heraeus Multifuge 3 L-R (Thermo Fisher Scientific Inc., 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 phase was combined with the first portion in the 25 ml-pear shape flask and brought to dryness by means of a rotary evaporator.

The remainder was taken up in acetonitrile (1 mL) and transferred to a 10 ml-centrifugation tube (Sarstedt AG & Co., Nümbrecht, Germany). Water (3 mL) was added followed by centrifugation (5 min, 4000 g, 25 °C). The supernatant was used for C_{18} solid phase extraction as described in the following.

Urine samples.

An aliquot (10 mL) of the sample was precisely transferred into a 50 mL-centrifugation tube (Sarstedt AG & Co., Nümbrecht, Germany) and spiked with labeled standard (1 µg/mL; 30 µl ≡ 3 µg/kg). The pH of the sample was roughly adjusted to pH 2–3 with about 2–3 drops of hydrochloric acid (3 mol/L). Afterwards, the derivatization reagent (7.7×10^{-3} mol/L; 1.5 mL) was added and the sample was treated analogously as described above.

Solid phase extraction (SPE).

A 6-mL C_{18}-SPE column (500 mg, 50 µm, 70 Å, Discovery® DSC-18, SUPELCO Analytical, Bellefonte, PA, US) was attached to a vacuum manifold and preconditioned with acetone, methanol, and water (4 mL each) 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 water (5 mL) and acetonitrile/water (30/70; v/v; 3 mL) and rapidly dried by aspirating air after the last washing step. Elution of the target compounds was carried out with of acetonitrile (2 x 2.5 mL). The solvent was removed by means of a rotary evaporator and the residue taken up in acetonitrile/water (30/70; v/v; 500 µL). The extract was membrane filtered (0.22 µm; regenerated cellulose, Whatman plc, Maidstone, UK) before LC-MS/MS analysis.

LC-MS/MS

Analysis of the tenuazonic acid dinitrophenylhydrazine derivative (TA-DNPH) was performed on a hybrid triple quadrupole/linear ion trap mass spectrometer (API 4000 QTrap; Applied Biosystems, Foster City, CA, USA) as published in literature [17]. The toxin was measured in the negative electro spray ionization (ESI) mode. The source parameters were set as follows: curtain gas (CUR): 10 psi; temperature (TEM): 550 °C; spray gas (GS1): 50 psi; dry gas (GS2): 70 psi; ion spray voltage: -4500 V. For quantification, the mass spectrometer was operated in the MRM (multiple reaction monitoring) mode. Fragmentation was effected by collision-induced dissociation (CID) and respective MS/MS transitions were measured as follows (in parentheses, collision energy, CE; collision cell exit potential, CXP):

TA-DNPH: m/z 376 → 182 (CE -34 V, CXP -9 V), m/z 376 → 152 (CE -52 V, CXP -7 V), m/z 376 → 122 (CE -64 V, CXP -7 V), m/z 376 → 329 (CE -24 V, CXP -9 V), m/z 376 → 330 (CE -26 V, CXP -9 V), m/z 376 → 301 (CE -30 V, CXP -7 V).

[$^{13}C_6$,^{15}N]-TA-DNPH: m/z 383 → 182 (CE -34 V, CXP -9 V), m/z 383 → 152 (CE -52 V, CXP -7 V), m/z 383 → 122 (CE -64 V, CXP -7 V), m/z 383 → 336 (CE -24 V, CXP -9 V), m/z 383 → 337 (CE -26 V, CXP -9 V), m/z 383 → 306 (CE -30 V, CXP -7 V).

The declustering potential (DP) was -70 V for all compounds. Both quadrupoles were set at unit resolution. The selective transitions (labeling of the internal standard not being completely lost during fragmentation) m/z 376→ 301 and m/z 383 → 306 were chosen as quantifiers, with all other transitions serving as qualifiers.

HPLC separation was performed on a Shimadzu LC-20 A prominence HPLC system (Shimadzu, Kyoto, Japan) that was linked to the mass spectrometer. A Synergi Polar RP column (50 x 2 mm, 4 µm; 80 Å Phenomenex, Aschaffenburg, Germany) was used as the stationary phase, whereas the mobile phase consisted of variable mixtures of water (A) and methanol (B) that followed a linear binary gradient as follows: initial conditions were 50% A and 50% B. After 1 min isocratic delivery of the solvents, the content of B was linearly raised during the next 1.5 min to obtain 100% B and 0% A 2.5 min after injection. These conditions were continued until the end of the run after 6.5 min. Injection volume was 10 µL, flow rate 0.2 mL/min, and equilibration time between two runs was 10 min.

Calibration and quantification

Response solutions were prepared by mixing analyte (A) and labeled standard (S) in 13 molar ratios n(A)/n(S) from 0.01 to 100. In detail, mixtures of n(A)/n(S) of 1:100, 1:50, 1:20, 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1, 50:1 and 100:1 were prepared by adding constant amounts of labeled standard (30 µL, 1 µg/mL) to varying amounts of analyte (30–300 µL; 0.01–10 µg/mL). After addition of the derivatization reagent (0.77 x 10^{-3} mol/L; 50 µL) and ultrasonication (10 min), the quenching reagent was added (30 µL) 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). After measuring these solutions with LC-MS/MS, a response curve was constructed from the obtained signal area ratios A(A)/A(S) against the respective molar ratios n(A)/n(S) using linear regression. Mandel's fitting test was performed for checking linearity and the calibration point n(A)/n(S) = 0.01 had to be rejected from the curve as an outlier on a 95 % probability level. The content n(A) of TA in samples was calculated from the recorded signal area ratios A(A)/A(S), the equation of the response curve and the amount of labeled standard n(S) added to the respective sample. All data were based on duplicate analyses and duplicate injections.

Method validation

For determining the limits of detection (LODs) and limits of quantitiation (LOQs), the blank urine of one volunteer was spiked in triplicate with TA at four different concentration levels (0.3 µg/L; 1.0 µg/L; 2.0 µg/L; 3.0 µg/L). 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 [35].

The recovery of the SIDA method was determined by spiking the blank urine of one volunteer (in triplicate) with 9 different amounts of TA in the range of 1.0-100 µg/L.

After sample preparation, clean-up and LC-MS/MS measurement, the recovery was calculated as the mean of the spiking experiments.

Interassay precision was determined by analyzing a urine sample of one volunteer (4.2 µg/L) three times in triplicate during 3 weeks.

Generation of urine samples.

In a first study, human volunteers (n = 6; 3 female, 3 male) were asked to collect their total urine over 24 h while keeping their individual dietary habits.

In a second study, two further volunteers (female, 24 a, 67 kg; male, 48 a; 72 kg) ingested food of animal origin (milk and milk products, without spices and additives) and water, exclusively, for three days of wash-out. On the fourth day no food was consumed to obtain a blank urine free of tenuazonic acid. On the fifth day in the morning the volunteers ingested whole meal sorghum infant cereals (80 g dry product; 375 µg/kg tenuazonic acid ≡ ingested amount: 30 µg) prepared with milk (3.5 % fat, 200 mL) and water (200 mL). The volunteers were asked to provide urine samples every hour after food intake for the following six hours without ingesting any other food. During the whole study the volunteers were allowed to drink water ad libitum. After a recovery phase the study was repeated just as described above, but with tomato juice (400 mL; 75 µg/kg tenuazonic acid ≡ ingested amount: 30 µg) as test food. All urine samples were stored at -20 °C.

The studies were performed with permission of the ethics commission of the Technische Universität München.

Glucuronidase treatment

Aliquots (10 ml) of the urine sample of one volunteer (47.2 µg/L) were spiked with labeled standard (1 µg/mL; 30 µl ≡ 3 µg/kg), adjusted to pH 5 with hydrochloric acid (about 2-3 drops; 2 mol/L) and treated with different amounts of β-glucuronidase solution (50 µl, 100 µl, 150 µl) and incubated for different times (3 h, 4 h, 5 h), respectively. The reaction was stopped by adding the derivatization reagent (7.7×10^{-3} mol/L; 1.5 mL) and the samples were subdued to further sample preparation as described above.

Creatinine determination

The content of creatinine in the urine samples was determined according to a procedure reported in the literature [36]. For this purpose, urine samples were diluted (1:50; v/v) with distilled water and aliquots (0.5 mL) were reacted with picric acid solution (about 1 % in water; 2 mL). After 20 min standing in the dark at room temperature, the samples were measured spectrophotometrically (λ = 492 nm). Creatinine calibration standards (2.4-67 mg/L) were reacted and measured, analogously, and the creatinine content in the samples was calculated using the calibration function obtained after linear regression.

For determination of recovery, synthetic urine (solution of inorganic salts, urea and creatinine in typical composition and concentration) was prepared according to literature [37] and two different concentrations of creatinine (0.8 g/L and 1.8 g/L) were chosen for the determination of the recovery.

For determining the precision, a urine sample of one volunteer (creatinine concentration: 0.6 g/L) was analyzed ten-times.

Results and Discussion

Method improvement and validation

For the analysis of tenuazonic acid in food matrices a stable isotope dilution assay (SIDA) has been developed and successfully applied to several food commodities of plant origin, recently [17, 19, 20]. It was based on the use of [$^{13}C_6$,^{15}N]-tenuazonic acid as internal standard [17] and on the derivatization of tenuazonic acid with 2,4-dinitrophenylhydrazine [16].

To analyze urine samples, this method had to be modified to cope with the differing matrix. Thereto, the pH of the urine samples was adjusted to 2–3 to ensure the completeness of the derivatization reaction and the derivatization reagent was added as concentrated solution to avoid large dilution of the sample. To assure unambiguous identification of the analyte, two more diagnostic fragment ions of tenuazonic acid dinitrophenylhydrazone (TA-DNPH) were included in the LC-MS/MS method (m/z 329 and m/z 330). In the negative electrospray ionization mode, the mass spectrum of the TA-DNPH 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) are fragments of the DNPH moiety [17] that appear in the spectrum of the labeled standard as well, and are not characteristic for the analyte, therefore. The advanced LC-MS/MS method now includes the mass transitions to m/z 329 and m/z 330 (as well as m/z 337 and m/z 336 for the internal standard) as additional qualifiers to improve the selectivity of the method. All measurements were checked for constant ion intensity ratios between the diagnostic ions before accepting the quantitative value. By the use of a shorter (50 mm) LC-column the duration of the single measurement was shortened distinctively in order to provide a high sample throughput (Fig. 3).

The SIDA for tenuazonic acid in urine samples was extensively re-validated due to the alterations of the method compared to food matrices. The limit of detection (LOD) and limit of determination (LOQ) was determined following a method described in literature [35] that is comparable to DIN EN standard 32645. As blank matrix a human urine sample free of tenuazonic acid was used. The LOD calculated with this method was 0.2 µg/L and the LOQ was 0.6 µg/L for the SIDA of tenuazonic acid in urine. In comparison with the SIDA of tenuazonic acid in other matrices similar values were obtained for fruit juices [19], whereas the values were higher for solid matrices that are more difficult [16, 20].

The recovery of the SIDA method was determined by spiking human blank urine in 9 different concentration levels from 1.0-100 µg/L. The mean recovery over all concentration levels was 102 ± 3 %, which is a typical value for stable isotope dilution assays that compensate for any losses of analyte or ion suppression in the ion source of the mass spectrometer [17, 19, 20].

Interassay precision was determined by the triplicate analysis of a urine sample of a volunteer (0.6 µg/L) during three weeks, which revealed a relative standard deviation of 6.7 %. The concentration of the chosen urine sample was close to the LOQ, but as most of the measured urine samples contained tenuazonic acid in rather low concentrations, the reliability of the method had to be proven at this concentration level. However, better precision values have been reported for tomato products (2.3 %), cereals (3.5 %) and juices (4.2 %) with the identical method [17, 19], albeit at higher contamination levels. Without the use of a stable isotope labeled standard precision values of around 10 % have been reported for cereals at a contamination level of 50 – 5000 µg/kg [16].

Taken together the improved and newly validated SIVA is well suited for the determination of tenuazonic acid in human urine.

Glucuronidase treatment

The content of tenuazonic acid in the urine samples that were treated with different amounts of β-glucuronidase for different time did not differ from the samples without enzyme treatment, if the analytical uncertainty of 6.7 % (RSD of the precision of the method) is taken into account. Thus, it can be concluded that tenuazonic acid is not metabolized to a glucuronide or sulfate (as the used glucuronidase had an sulfatase secondary activity) during its passage through the human body. This finding is reasonable to some degree as tenuazonic acid is highly water soluble already, which facilitates urinary excretion. This could not be expected from the very first, because the similar polar *Fusarium* mycotoxin deoxynivalenol is converted to a glucuronide by humans, anyway [21-23, 30].

Creatinine determination

In the literature, the content of mycotoxins in human urine is often given in ng/mg^{-1} creatinine, which is necessary, if no 24-hour urine is available and the samples have to be corrected for dilution effects. To facilitate comparison, creatinine was also determined in the 24-hour urine samples of all volunteers in our first study, but not taken into consideration during the second study, as fasting and keeping a special diet mixed up the creatinine excretion of the volunteers in this time period. Creatinine was determined photometrically after reaction with picric acid according to literature [36]. The linear working range for urine samples was 0.1-3.4 g/L creatinine. Recovery from spiked artificial urine [37] was 97.9 ± 3.6 % (at 1.8 g/L) and 99.8 ± 4.6 % (at 0.8 g/L). The precision (n = 10) of the creatinine determination in human urine (0.6 g/L) was possible with a relative standard deviation of 2.4 %.

Determination of TA in human urine samples

In a first study, the optimized and re-validated method was applied for the analysis of tenuazonic acid in the 24-hour urine of six volunteers (3 female, 3 male; 24 – 32 years of age) from Germany. Tenuazonic acid was quantified in the urine samples of all volunteers in the range of 1.3–17.3 µg/L or 2.3-10.3 ng/mg^{-1} creatinine, respectively (Table 1). Tenuazonic acid has not been determined in human urine before, which hampers the assessment of this finding.

However, in a recent study with 27 Austrian volunteers, the total deoxynivalenol concentration (free deoxynivalenol and deoxynivalenol glucuronides) in human first morning urine was 5-28 ng/mg^{-1} creatinine [38]. In another study with 9 Belgian volunteers, deoxynivalenol (3.7-67 ng/mg^{-1} creatinine), ochratoxin A (0.04-0.3 ng/mg^{-1} creatinine), ochratoxin α (2.5-6 ng/mg^{-1} creatinine), zearalenone (3.2-10.8 ng/mg^{-1} creatinine) and β-zearalenol (2.5-20 ng/mg^{-1} creatinine) were quantified in human first morning urine, also [29]. The values of tenuazonic acid found in our study indicate that tenuazonic acid can occur in similar or even higher concentrations in human urine in comparison to other mycotoxins. However, as tenuazonic acid was found to occur frequently in the human diet [16–20], elevated urinary values could be expected.

In a second study with two volunteers the urinary excretion rate of tenuazonic acid after consumption of naturally contaminated food was monitored. To generate urine samples free of tenuazonic acid (blank urine) it was necessary to avoid the ingestion of vegetable food like cereals, vegetables and fruits that are known to be frequently contaminated with tenuazonic acid [39]. The nutrition of the two volunteers consisted of cheese, milk and milk products, without the addition of spices and other additives, therefore. However, by monitoring the content of tenuazonic acid in the 24-hour urine of the volunteers, it become obvious that even with this diet it was not possible to generate urine samples with a content of tenuazonic acid below the limit of detection of the method. Although foods of animal origin contained tenuazonic acid only in traces that could be not quantified in the food itself, the ingestion of large amounts of these products lead to unambiguous detection of tenuazonic acid in the urine of the volunteers. It was necessary for the volunteers to abstain from food intake for one day completely to wash out traces of tenuazonic acid and to generate blank urine, therefore.

Following the washout phase the volunteers ingested 30 µg tenuazonic acid by the consumption of naturally contaminated foods that were commercially available. In the first part of the study whole meal sorghum infant cereals were consumed and in the second tomato juice. Both parts were separated by another washout phase. During the study the volunteers collected their urine in one-hour intervals for six hours and in a seventh interval after 18 further hours to give a complete urine sampling time of 24 hours. All urine samples were analyzed for their tenuazonic acid content. The results of this study are shown in Figure 4 and Table 2.

Of the ingested amount of tenuazonic acid 88 ± 4 % (volunteer A) and 54 ± 4 % (volunteer B) were excreted after consumption of sorghum infant cereals and 61 ± 1 % (volunteer A) and 81 ± 3 % (volunteer B) after the ingestion of tomato juice after six hours. It is unclear, why the excretion ratios are interchanged between ingested food and volunteer, but neither ingestion speed nor the amount of accompanying liquid intake was strictly controlled in this study. However, both volunteers showed similar excretion profiles. Due to the complexity of the food matrix, tenuazonic acid appeared to be more readily absorbed from tomato juice than from sorghum cereals. Thus, the excretion curve reached its maximum 2 hours after ingestion of tomato juice and 3 hours after ingestion of sorghum cereals for both volunteers.

After 24 hours the mean urinary excretion was about 89 ± 4 % of the ingested tenuazonic acid for both volunteers. Due to its design it cannot be deduced from our study, whether the approximately missing 10 % of ingested tenuazonic acid are excreted later than 24 hours after uptake, or are eliminated differently than by urinary excretion, or remain in the body.

Conclusion

The determination of tenuazonic acid in human urine has been described for the first time. Tenuazonic acid was detected in the urine of all six volunteers in concentrations similar or higher than the urinary values of most other mycotoxins reported in literature [29, 38]. This finding gives evidence that human exposure to tenuazonic acid from the diet is a matter of fact in Germany and the concerns about the health of the consumers are certainly justified in view of the unclear toxicology of this mycotoxin. However, our study with two volunteers revealed that ingested tenuazonic acid was rapidly absorbed from the food and nearly completely excreted via the urine. Nevertheless, it cannot be concluded from our results that tenuazonic acid does not pose a major threat to human health. Firstly, because of the limited number of participants in our studies and, secondly, because of the unclear disposition of the about 10 % of the ingested amount of tenuazonic acid that was not recovered from the urine after 24 hours. Altogether, this points out to the need of an extended study with another study design and more participants in order to reveal the fate of tenuazonic acid after incorporation and to exclude potential health hazards. Anyway, our study gives more evidence about the importance of the analysis of biomarkers to assess mycotoxin exposure of humans as has been reviewed recently [40, 41].

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Tables

Table 1 Content of tenuazonic acid (TA) and creatinine (CREA) in the 24-hour urine of human volunteers

Volunteer		TA [µg]	TA [µg/L]	CREA [g]	TA [ng/mg CREA]
♀	1	4.1	1.3	1.8	2.3
	2	5.6	4.7	1.2	4.6
	3	7.8	1.7	1.1	6.9
♂	4	8.7	1.7	2.4	3.6
	5	17.9	17.3	2.5	7.3
	6	20.8	14.1	2.0	10.3

536 Table 2 Urinary excretion of tenuazonic acid (TA) after ingestion of [1] sorghum infant cereals
537 and [2] tomato juice (equivalent to an uptake of 30 µg TA) by two volunteers (A & B)

Urine sample (time after ingestion)	Urinary excretion of TA [µg] [1] Sorghum infant cereals		Urinary excretion of TA [µg] [2] Tomato juice	
	Volunteer A	Volunteer B	Volunteer A	Volunteer B
1	3.9 ± 0.2	0.8 ± 0.1	6.0 ± 0.1	10.1 ± 0.5
2	4.3 ± 0.2	1.0 ± 0.01	7.7 ± 0.1	12.3 ± 0.2
3	8.8 ± 0.5	10.7 ± 0.9	1.4 ± 0.0	1.6 ± 0.1
4	5.9 ± 0.2	3.1 ± 0.1	1.9 ± 0.1	0.1 ± 0.01
5	1.2 ± 0.1	0.4 ± 0.01	0.9 ± 0.1	0.1 ± 0.01
6	0.2 ± 0.01	0.1 ± 0.01	0.3 ± 0.01	0.1 ± 0.01
Σ 6	**24.2 ± 1.1**	**16.1 ± 1.1**	**18.2 ± 0.4**	**24.2 ± 0.9**
Rel. excretion	81 ± 4 %	54 ± 4 %	61 ± 1 %	81 ± 3 %
Σ 24	**27.8 ± 1.3**	**26.3 ± 1.1**	**26.1 ± 0.8**	**26.5 ± 1.0**
Rel. excretion	93 ± 4 %	88 ± 4 %	87 ± 3 %	88 ± 3 %

538

539

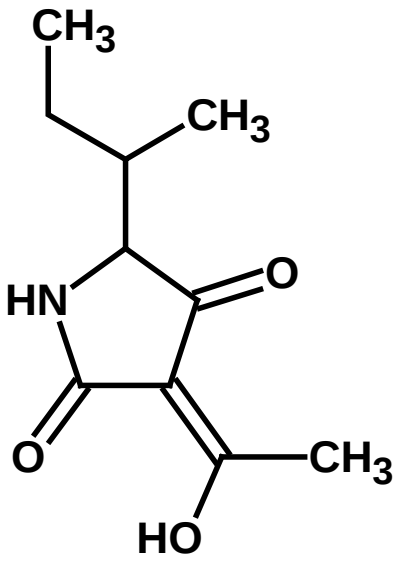
Legend to the figures

Figure 1 Structure of tenuazonic acid in its preferred tautomeric form according to the literature [42]

Figure 2 MS/MS spectra of tenuazonic acid and [$^{13}C_6$,^{15}N]-tenuazonic acid with proposed structures of fragment ions of both compounds. Abbreviations: "■" = ^{13}C, "♦" = ^{15}N

Figure 3 LC-MS/MS run of a urine sample containing 17 µg/L tenuazonic acid (only quantifier traces shown)

Figure 4 Urinary excretion of tenuazonic acid (TA) [µg] by two volunteers (A & B) after ingestion of sorghum infant cereals [1] and tomato juice [2]



550

551 Figure 1

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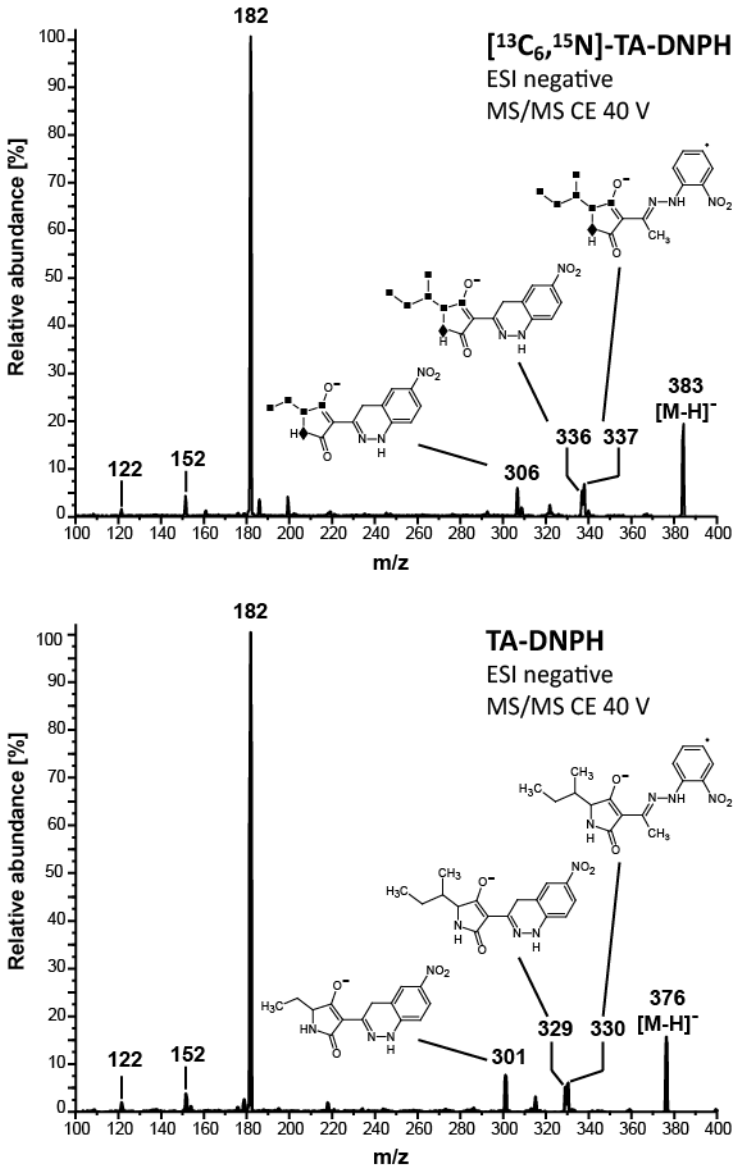

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554 Figure 2

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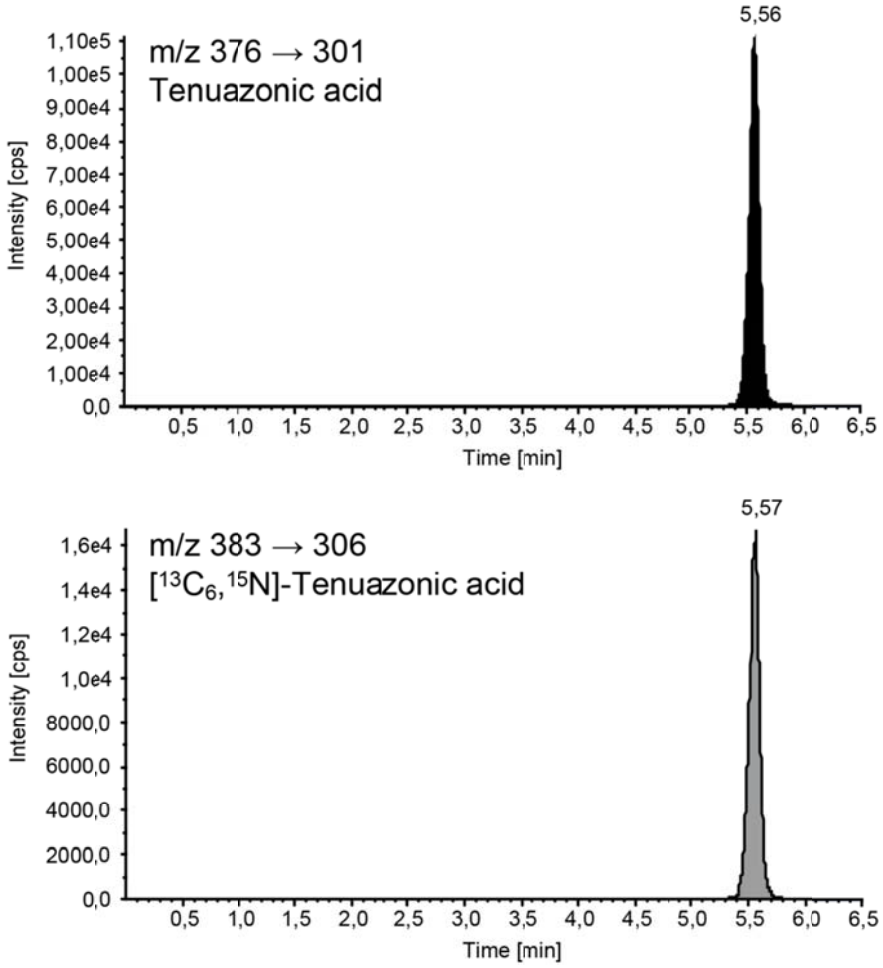
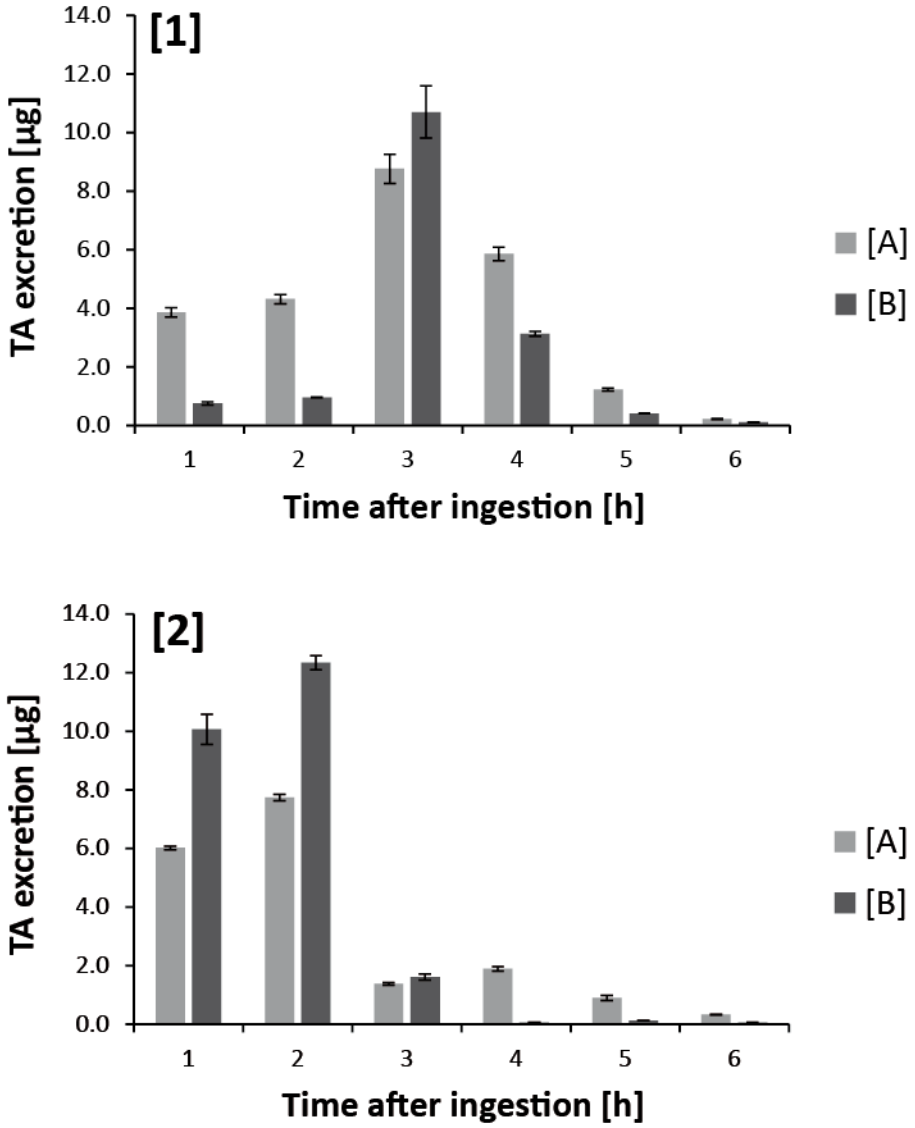

5,56
m/z 376 → 301
Tenuazonic acid
Intensity [cps]
1,10e5
1,00e5
9,00e4
8,00e4
7,00e4
6,00e4
5,00e4
4,00e4
3,00e4
2,00e4
1,00e4
0,0
0,5 1,0 1,5 2,0 2,5 3,0 3,5 4,0 4,5 5,0 5,5 6,0 6,5
Time [min]
5,57
m/z 383 → 306
[13C6,15N]-Tenuazonic acid
Intensity [cps]
1,6e4
1,4e4
1,2e4
1,0e4
8000,0
6000,0
4000,0
2000,0
0,0
0,5 1,0 1,5 2,0 2,5 3,0 3,5 4,0 4,5 5,0 5,5 6,0 6,5
Time [min]

Figure 3



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559 Figure 4