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Development of a Stable Isotope Dilution Assay for Tenuazonic Acid

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Supporting Information

ABSTRACT: A stable isotope dilution assay (SIDA) for the *Alternaria* mycotoxin tenuazonic acid was developed. Therefore, [$^{13}C_6$,^{15}N]-tenuazonic acid was synthesized from [$^{13}C_6$,^{15}N]-isoleucine by Dieckmann intramolecular cyclization after acetoacetylation with diketene. The synthesized [$^{13}C_6$,^{15}N]-tenuazonic acid was used as the internal standard for determination of tenuazonic acid in tomato products by liquid chromatography tandem mass spectrometry after derivatization with 2,4-dinitrophenylhydrazine. Method validation revealed a limit of detection of 0.1 μg/kg and a limit of quantitation of 0.3 μg/kg. Recovery was close to 100% in the range of 3−300 μg/kg. Determination of tenuazonic acid in two samples of different tomato ketchups (naturally contaminated) was achieved with a coefficient of variation of 2.3% and 4.7%. Different tomato products (n = 16) were analyzed for their content of tenuazonic acid using the developed SIDA. Values were between 15 and 195 μg/kg (tomato ketchup, n = 9), 363 and 909 μg/kg (tomato paste, n = 2), and 8 and 247 μg/kg (pureed tomatoes and comparable products, n = 5).

KEYWORDS: *Alternaria*, mycotoxins, tenuazonic acid, stable isotope dilution assay, SIDA, LC-MS/MS

INTRODUCTION

Molds of the genus *Alternaria* are ubiquitous plant pathogenic and saprophytic species that may invade a variety of substrates, including textiles, wallpapers, soil, and most commonly organic materials like leaves, fruits, vegetables, and crops. In addition to economical losses due to mold infestation, production of toxic metabolites, so-called mycotoxins such as alternariol, alternariol monomethyl ether, altenuene, and tenuazonic acid pose a potential health hazard for the consumer if accumulated in food and feed.[1] Concerning toxicity, *Alternaria* mycotoxins were suspected to be the cause of a hemorrhagic syndrome in poultry. Subsequent investigations showed that cultural extracts of *A. alternata* were toxic to chick embryos and day-old chicks, with tenuazonic acid being the major agent, whereas no effects were observed with alternariol, alternariol monomethyl ether, or altenuene.[2] Furthermore, it has been suggested that *Alternaria*-infested grains could be responsible for an increased incidence of human esophageal cancer in the Chinese province of Linxian.[3] Tenuazonic acid, a tetramic acid derivative (Figure 1), is acutely toxic against mice, day-old chicks, dogs, rats, and other animals. For male mice an oral LD_{50} of 182[4] and 225[5] mg/kg body weight (BW) has been determined, whereas the value was 81 mg/kg BW for females,[4] thus rendering tenuazonic acid the *Alternaria* toxin with the highest acute toxicity.[1,6]

Several methods for determination of tenuazonic acid in food have been reviewed recently[7,8] and are generally based on high-performance liquid chromatography (HPLC) with UV detection. Tenuazonic acid was quantified with this technique in tomato paste,[9] fresh tomatoes,[10,11] tomato puree,[12] and other tomato products.[13] However, as tenuazonic acid is a strong acid and a metal chelating compound it shows irreproducible chromatographic behavior that is remedied by adding additives, e.g., Zn(II)SO_4, to the mobile phase.[7] Unfortunately, this approach cannot be used when mass spectrometric detection is applied, which is the favored technique when analyzing complex food matrices that contain many interfering compounds. However, a recent paper[14] describes an excellent approach to overcome this difficulty by derivatization of tenuazonic acid with 2,4-dinitrophenylhydrazine. The generated tenuazonic acid hydrazone both showed reliable chromatographic and mass spectrometric behavior and allowed determination of tenuazonic acid in flour, bakery products,[14] and beer.[15]

Nevertheless, it is well known that derivatization reactions have to be thoroughly optimized in terms of concentration of the derivatization reagent and derivatization time for every different matrix to ensure complete derivatization of the analyte. It has been shown that the use of stable isotope-labeled standards compensates for derivatization uncertainties, for example, during derivatization with trimethylsilylchloride before gas chromatographic analysis of the mycotoxin patulin.[16] Consequently, it was the aim of this work to synthesize stable isotope-labeled tenuazonic acid and to develop a stable isotope dilution assay that compensates for any incomplete derivatization reaction. Besides simplification of the analytical workload, precise and reliable analytical results are available with this technique, especially when more complex matrices than grains are analyzed.

MATERIALS AND METHODS

Safety Note. Tenuazonic acid exhibits severe acute and possibly also chronic toxicity. It is recommended to handle this compound with

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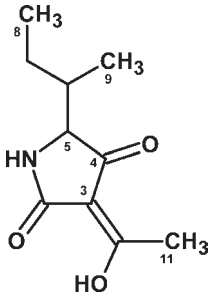

Figure 1. Structure of tenuazonic acid.

extreme care. Contaminated material should be treated with an aqueous solution of sodium hypochlorite (5%).

Chemicals and Reagents. Tenuazonic acid, copper(II) salt, [$^{13}C_6$,^{15}N]-L-isoleucine, 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one, sodium methylate, 2,4-dinitrophenylhydrazine, 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).

Synthesis of [$^{13}C_6$,^{15}N]-Tenuazonic acid. *Preparation of [$^{13}C_6$,^{15}N]-L-Isoleucine Methyl Ester.* [$^{13}C_6$,^{15}N]-L-Isoleucine (13 mg, 0.1 mmol) was dissolved in dry methanol (1 mL). The solution was cooled in an ice bath before thionylchloride (50 μL, 0.69 mmol, 7 equiv) was added dropwise. Thereafter, the ice bath was removed and the solution was stirred for 4 h under reflux. After removal of the solvent the crude reaction mixture was dissolved in cold water (2 mL) and extracted with diethyl ether (2 × 2 mL). The aqueous phase was carefully alkalized (pH 10) with ammonium hydroxide (25%) and extracted with diethyl ether (2 × 2 mL). The organic phases were combined and dried over anhydrous sodium sulfate. Removal of the solvent gave [$^{13}C_6$,^{15}N]-L-isoleucine methyl ester as a colorless oil (9.3 mg; 64% yield), *m*/*z* 153 ($M + H^+$, 55%), 92 (100%), 74 (10%).

Preparation of Methyl N-Acetoacetyl-[$^{13}C_6$,^{15}N]-L-Isoleucinate. [$^{13}C_6$,^{15}N]-L-Isoleucine methyl ester (9.3 mg, 0.06 mmol) was suspended in toluene (0.2 mL). Afterward, 2,2,6-trimethyl-4*H*-1,3-dioxin-4-on (20 μL, 0.15 mmol, 1.5 equiv) was added and the solution was refluxed for 0.5 h. The solvent was removed under reduced pressure, and the reaction mixture was dissolved in ethyl acetate (2 mL). The solution was washed with hydrochloric acid (1 mol/L, 2 mL) and saturated aqueous sodium hydrogen carbonate. Evaporation of the solvent gave a dark, reddish oil. The crude product was chromatographed on silica gel (25 g, hexane-ethylacetate 50:50) to yield methyl *N*-acetoacetyl-[$^{13}C_6$,^{15}N]-L-isoleucinate as a yellow oil (11 mg; 76% yield), R_f 0.5 (hexane−ethylacetate 50:50), *m*/*z* 237 ($M + H^+$, 35%), 179 (100%), 176 (75%), 153 (15%).

Preparation of [$^{13}C_6$,^{15}N]-Tenuazonic Acid. Methyl *N*-acetoacetyl-[$^{13}C_6$,^{15}N]-L-isoleucinate (11 mg, 0.046 mmol) was dissolved in methanol (0.3 mL), and sodium methoxide (25 wt % in methanol, 12.5 μL, 0.046 mmol) was added. After refluxing for 2 h the reaction mixture was diluted with 2 mL of water and carefully acidified (pH 2) with hydrochloric acid (1 mol/L). The mixture was extracted with diethyl ether (2 × 2 mL). The extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness under reduced pressure to obtain [$^{13}C_6$,^{15}N]-tenuazonic acid as a colorless oil (5.2 mg; 55% yield), *m*/*z* 203 ($M - H^-$, 100%), 184 (15%), 142 (10%). UV_{max} = 277 nm. ^{13}C NMR, δ/ppm (CD_3OD, 125 MHz): 198.9 (C-4), 174.6 (C-4′), 67.1 (C-5), 57.9 (C-5′), 38.6 (C-6), 26.4 (C-7), 15.9 (C-9), 11.8 (C-8).

Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{13}C NMR experiments were performed on a 500 MHz Avance III spectrometer (Bruker, Rheinstetten, Germany). The sample was dissolved in CD_3OD (Sigma-Aldrich, Steinheim, Germany). Data processing was performed using Top Spin software version 2.0 (Bruker, Rheinstetten, Germany).

MS and MS/MS Measurements. 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 in the negative ESI mode, apart from the MS spectra of the first two synthetic steps that were recorded in the positive mode. The source parameters were set as follows: curtain gas, 10 psi; temperature, 550 °C; spray gas (GS1), 50 psi; dry gas (GS2), 70 psi; ion spray voltage, −4500 V (or +5500 V, respectively).

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 as described above. The following transitions were monitored (in parentheses, collision energy, CE; collision cell exit potential, CXP; declustering potential, DP): tenuazonic acid dinitrophenylhydrazone: *m*/*z* 376 → 182 (CE −34 V, CXP −9 V, DP −75 V), 376 → 122 (CE −64 V, CXP −7 V, DP −75 V), 376 → 301 (CE −30 V, CXP −7 V, DP −75 V). [$^{13}C_6$,^{15}N]-Tenuazonic acid dinitrophenylhydrazone: *m*/*z* 383 → 182 (CE −36 V, CXP −5 V, DP −70 V), 383 → 122 (CE −64 V, CXP −7 V, DP −70 V), 383 → 306 (CE −32 V, CXP −7 V, DP −70 V). Both quadrupoles were set at unit resolution.

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 × 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 and 0% A 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. Data acquisition was carried out using Analyst 1.4.2 software (Applied Biosystems INC, Foster City, CA).

UV Spectroscopy. The concentrations of stock solutions were determined using a UV spectrometer Specord 50 (Analytik Jena, Jena, Germany), annually calibrated by the service of the manufacturer. The purity of commercial tenuazonic acid as well as of synthesized labeled standard was checked by UV spectroscopy and comparison to literature data.[17] The concentrations of stock solutions were determined using UV spectroscopy as described below.

Preparation of Standard Solutions. Stock solutions of free tenuazonic acid were prepared according to the literature:[14,17] 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 cation-exchange resin was filled into a 2 mL plastic syringe without needle ("column"), which was attached to a vacuum manifold.[17] The resin was activated by subsequently passing sodium hydroxide (10 mL, 0.5 mol/L), deionized water (10 mL), and hydrochloric acid (10 mL, 0.5 mol/L) through the column. Afterward, 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 × 2 mL). All eluates were collected, and the solvent was evaporated under a gentle stream of nitrogen. The resulting colorless, 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 by UV spectroscopy using the molar extinction coefficient of 1.298×10^4 L mol^{-1} cm^{-1} according to the literature.[9,14,17] Working solutions were obtained by further dilution in the range of 1 (5 μmol/L), 0.1 (0.5 μmol/L), and 0.01 mg/L (0.05 μmol/L). Accordingly, the concentration of the synthesized [$^{13}C_6$,^{15}N]-tenuazonic acid stock solution was determined by UV spectroscopy, and working solutions were prepared as described above. All solutions were stored at −20 °C in the dark to ensure stability as recommended in the literature.[18]

Preparation of Derivatization and Quenching Reagent. The derivatization reagent 2,4-dinitrophenylhydrazine was prepared as described in the literature.[14,19] Undecylic aldehyde (5% in ethyl acetate) was used as quenching reagent in order to destroy excess derivatization reagent after the derivatization step.[14]

Calibration and Quantitation. Constant amounts of labeled standard (S) were mixed with varying amounts of analyte (A) in molar ratios n(S)/n(A) between 0.10 and 10 (0.1:1; 0.2:1; 0.5:1; 1:1; 2:1; 5:1; 10:1) before the derivatization reagent was added (1.3 mol/L, 50 μL, >100 equiv). After ultrasonication (10 min) the quenching reagent was added (2.4 mol/L, 30 μL, >100 equiv) followed by further ultrasonication (10 min). Thereafter, the solvent was removed by a stream of nitrogen, and the residue was taken up in methanol−water (100 μL, 50/50; v/v) to give a final concentration of 0.1 mg/L of the labeled standard. After LC-MS/MS measurement, the peak area ratios [A(S)/A(A)] were determined. Calibration functions were obtained using simple linear regression. Linearity was checked by analysis of the residuals (homogeneity and normal distribution) after linear regression. The response was linear for the chosen molar ratios (0.1−10), and the content of tenuazonic acid in samples was calculated using the respective calibration function.

Sample Preparation. The preparation of food samples for analysis by LC-MS/MS was based on a method described in the literature[14] but supplemented with further solid-phase extraction on C_{18} material similar to the SIDA of alternariol and alternariol methyl ether[20] to obtain cleaner extracts from tomato products.

In detail, 2 g of tomato product was weighed in a 50 mL centrifugation vial (Sarstedt AG & Co., Nümbrecht, Germany) and spiked with labeled standard (30 μL, 1 mg/L, 15 μg/kg). However, if the natural content of tenuazonic acid exceeded 150 μg/kg, sample preparation had to be repeated with an increased amount of labeled standard (300 μL, 1 mg/L, 150 μg/kg), as the linearity of the SIDA method was only validated for the n(S)/n(A) ratios between 0.1 and 10. Afterward, the derivatization reagent (1.3 mol/L, 15 mL) was added, followed by 15 min ultrasonication and 20 min shaking. After adding the quenching reagent (ethyl acetate/undecylic aldehyde, 95/5; v/v, 10 mL) shaking was continued for further 10 min. The centrifugation vial was centrifuged (5 min, 4600 rpm, 25 °C) by means of a Heraeus Multifuge 3 L-R (Thermo Fisher Scientific Inc., Waltham, MA), and the organic phase was transferred into a 50 mL pear shape 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 50 mL pear shape flask and rotary evaporated to dryness. The remainder was taken up in acetonitrile (2 mL) and transferred to a 10 mL centrifugation vial (Sarstedt AG & Co., Nümbrecht, Germany). Water (6 mL) was added followed by centrifugation (5 min, 4600 rpm, 25 °C). The supernatant was used for C_{18} solid-phase extraction as described previously.[20]

Limit of Detection (LOD) and Quantification (LOQ). LOD and LOQ for tenuazonic acid were determined according to the literature.[21] Self-made pureed tomatoes as blank matrix were prepared from whole, sound tomatoes by means of a GRINDOMIX GM 200 laboratory mixer (Retsch GmbH, Haan, Germany) and stored at −20 °C until use. Preliminary LC-MS/MS analysis confirmed that the pureed tomatoes prepared that way contained no tenuazonic acid. For determination of LOD and LOQ, the pureed tomatoes were spiked (each in triplicate) with tenuazonic acid at four different concentration levels (0.3−3 μg/kg). After addition of [$^{13}C_6$,^{15}N]-tenuazonic acid 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 a published method.[21]

Precision. Interassay precision was determined by analyzing two different naturally contaminated samples three times in triplicate during 3 weeks. For this purpose, two samples of tomato ketchup that naturally contained tenuazonic acid were chosen.

Recovery. Samples of blank pureed tomatoes were spiked (each in triplicate) with three different amounts of tenuazonic acid (3, 30, and 300 μg/kg) and analyzed by LC-MS/MS. The recovery was calculated as the mean of the addition experiments.

Comparison with Alternative Methods. A naturally contaminated sample of tomato ketchup that contained 41 μg/kg tenuazonic acid determined by SIDA was quantified using the standard addition technique. Therefore, the sample was analyzed in duplicate (i) without addition of tenuazonic acid, (ii) with addition of 11 μg/kg tenuazonic acid, and (iii) with addition of 22 μg/kg tenuazonic acid. After derivatization and LC-MS/MS analysis a standard addition curve was constructed using linear regression, and the amount of tenuazonic acid in the sample was calculated from the x intercept of the curve. Furthermore, the same samples were also quantified using external calibration. Therefore, a calibration curve was constructed (linear regression) from the results of LC-MS/MS measurements of solutions of tenuazonic acid in acetonitrile and water (30:70; v/v) in concentrations between 0.2 and 1.2 mg/L and respective derivatization. Using this equation the content of tenuazonic acid in the samples without addition of analyte was determined. From the spiked samples the absolute recovery was calculated and used for correction of the content of tenuazonic acid in the native samples.

RESULTS AND DISCUSSION

Synthesis of Labeled Tenuazonic Acid. The syntheses of tenuazonic acid and related tetramic acids have been described several times.[22] Different approaches have been developed to obtain the tetramic acid nucleus, but the route involving Dieckmann intramolecular cyclization after *C*-acetylation of *N*-acyl amino esters was generally favored due to its rapidness and convenience. Using this approach for the synthesis of tenuazonic acid, isoleucine methyl ester was acetoacetylated with diketene and the resulting product was cyclized to the tetramic acid under basic conditions. However, preliminary microscale syntheses of tenuazonic acid via the Dieckmann cyclization route indicated a very low yield (<5%). We suspected that the high reactivity of diketene produced an excess of byproducts that were responsible for the low yield. To overcome this drawback we chose 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one as acetoacetylation reagent that has been reported to be a convenient alternative to diketene.[23,24] This compound is a diketene−acetone adduct that is stable at room temperature and releases diketene slowly during heating. Even with this reagent yields did not exceed 30% in our microscale approach (10 mg isoleucine), whereas yields of 78% were described when the concentration was 200-fold higher.[25] We assumed that the high temperature of refluxing toluene that is needed to release diketene from its acetone adduct leads to a degradation of the isoleucine methyl ester either by forming piperazine-2,5-diones or linear polypeptides. As the yield was

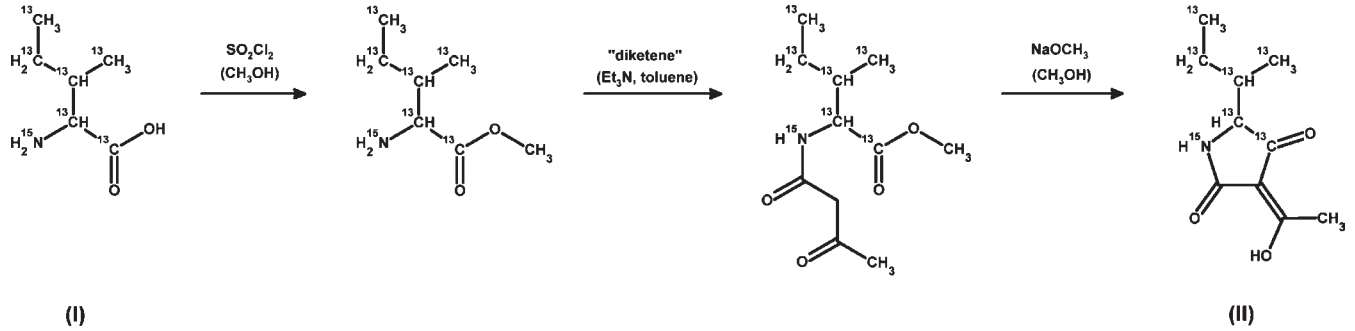

Figure 2. Synthetic pathway leading from [$^{13}C_6$,^{15}N]-isoleucine (I) to [$^{13}C_6$,^{15}N]-tenuazonic acid (II).

reproducible, we decided to choose this approach, anyhow. Hence, [$^{13}C_6$,^{15}N]-tenuazonic acid was prepared from [$^{13}C_6$,^{15}N]-isoleucine with a yield of about 30% (Figure 2).

The identity of the synthesized [$^{13}C_6$,^{15}N]-tenuazonic acid was checked against a commercial unlabeled tenuazonic acid standard material using UV spectroscopy, LC-MS, and LC-MS/MS. The UV spectrum of labeled [$^{13}C_6$,^{15}N]-tenuazonic acid was identical to the spectrum of the unlabeled isotopologue, showing a maximum of absorbance at 277 nm. Both spectra were in accordance with literature data.[17] In the LC-MS spectrum (negative ESI-mode) of [$^{13}C_6$,^{15}N]-tenuazonic acid (data not shown) the corresponding $[M - H]^-$ peak (m/z 203.2) was 7 amu heavier than the respective $[M - H]^-$ peak of tenuazonic acid (m/z 196.2), indicating incorporation of [$^{13}C_6$,^{15}N]-isoleucine into the molecule.

Furthermore, ^{13}C nuclear magnetic resonance (NMR) spectroscopy was applied to verify the identity of the synthesized [$^{13}C_6$,^{15}N]-tenuazonic acid. Usually it is not possible to record ^{13}C NMR spectra with such low quantities of substance that we obtained from the synthesis, but as the molecule was labeled with ^{13}C, clear and distinctive signals were observed for all positions bearing labeled carbons, whereas the unlabeled positions were invisible. The signals occurred as multiplets due to coupling between the neighboring ^{13}C atoms. According to literature data,[26,27] the signals of the carbon atoms 4, 5, 6, 7, 8, and 9 of tenuazonic acid (Figure 1) could be assigned due to their chemical shift. These are the positions that are derived from [$^{13}C_6$,^{15}N]-isoleucine during synthesis. Furthermore, signal doubling at C-4 and C-5 due to formation of external tautomers was observed as described for tenuazonic acid in the literature.[26] Thus, the correct incorporation of [$^{13}C_6$,^{15}N]-isoleucine into [$^{13}C_6$,^{15}N]-tenuazonic acid was proven by ^{13}C NMR spectroscopy.

Unfortunately, no statements can be made on the extent of epimerization during the synthesis of [$^{13}C_6$,^{15}N]-tenuazonic acid. Since the absolute amount of [$^{13}C_6$,^{15}N]-tenuazonic acid was too low to achieve reasonable ^1H NMR spectra the amount of the unnatural (5*R*,6*S*)-diastereomer could not be measured. However, common analytical methods do not separate between both epimers. Thus, epimerization of the synthesized labeled standard does not pose a threat to the reliability of the method, at least as far as no immunologic steps are involved that discriminate against one diastereomer.

LC-MS/MS Experiments. After derivatization with 2,4-dinitrophenylhydrazine, LC-MS/MS spectra were recorded in the negative ESI mode for the respective hydrazones of tenuazonic acid and its labeled isotopologue (Figure 3). In these spectra the predominant fragment ion was m/z 182.1 for both molecules. This similarity of the native and labeled tenuazonic acid dinitrophenylhydrazone implies that the tenuazonic acid nucleus was no longer present in the fragment m/z 182 because the labels were lost. We assume that cleavage of the N−N bond of the hydrazone releases tenuazonic acid imine, while the charge passes over to a resonance-stabilized dinitrophenylimine moiety with m/z 182. Further fragmentation of this ion involves the 2-fold elimination of nitric oxide to generate m/z 152 and 122.

However, the LC-MS/MS spectra also showed fragmentation of the tenuazonic nucleus itself that allows the differentiation between native and labeled tenuazonic acid dinitrophenylhydrazone (Figure 3). From the respective molecular ion $[M - H]^-$ (m/z 376 and 383) both elimination of nitrogen dioxide (leading to the fragment ions m/z 330 and 337) and elimination of nitrous acid (leading to the fragment ions m/z 329 and 336) were observed. Whereas elimination of nitrogen dioxide seemed to be an end point of fragmentation, the break up of the tenuazonic acid nucleus was observed starting from the fragment ions m/z 329 and 336. By elimination of ethylene (28 amu, C_2H_4), the fragment ion m/z 301, and by elimination of methylene (14 amu, CH_2) the fragment ion m/z 315 was formed. The labeled isotopologue showed mass transitions of 30 ($^{13}C_2H_4$) and 15 amu ($^{13}CH_2$), respectively, resulting in fragment ions m/z 306 and 321. We assume that the fragment ions m/z 329 and 336 form biradicals during CID that break down either by cleavage (fragmentation I, releasing methylene) or by rearrangement (fragmentation II, releasing ethylene). Both reactions are similar to photochemistry as Norrish type I and II reaction, respectively. Fragmentation II is also known from GC-MS-EI and LC-MS/MS spectra as McLafferty rearrangement of radicals and ions,[28] respectively.

This specific fragmentation pattern of tenuazonic acid dinitrophenylhydrazone in the negative ESI mode differed significantly from the fragmentation observed in the positive mode.[14] Negative ion electrospray ionization was preferred anyway, because the background noise was significantly lower than in the positive mode, which enabled lower limits of detection. The sensitive transitions m/z 376 → 182 and 376 → 122 were used as qualifiers, whereas the selective transition m/z 376 → 301 was used as quantifier. As the latter transition involves fragmentation of the tenuazonic acid nucleus and allows distinctive separation of analyte and labeled standard, selectivity was assured. The respective transitions of the labeled standard were m/z 383 → 182 (qualifier 1), 383 → 122 (qualifier 2), and 383 → 306 (quantifier).

Development of a Stable Isotope Dilution Assay. Determination of tenuazonic acid in food samples was performed according to the previously reported method including derivatization of the

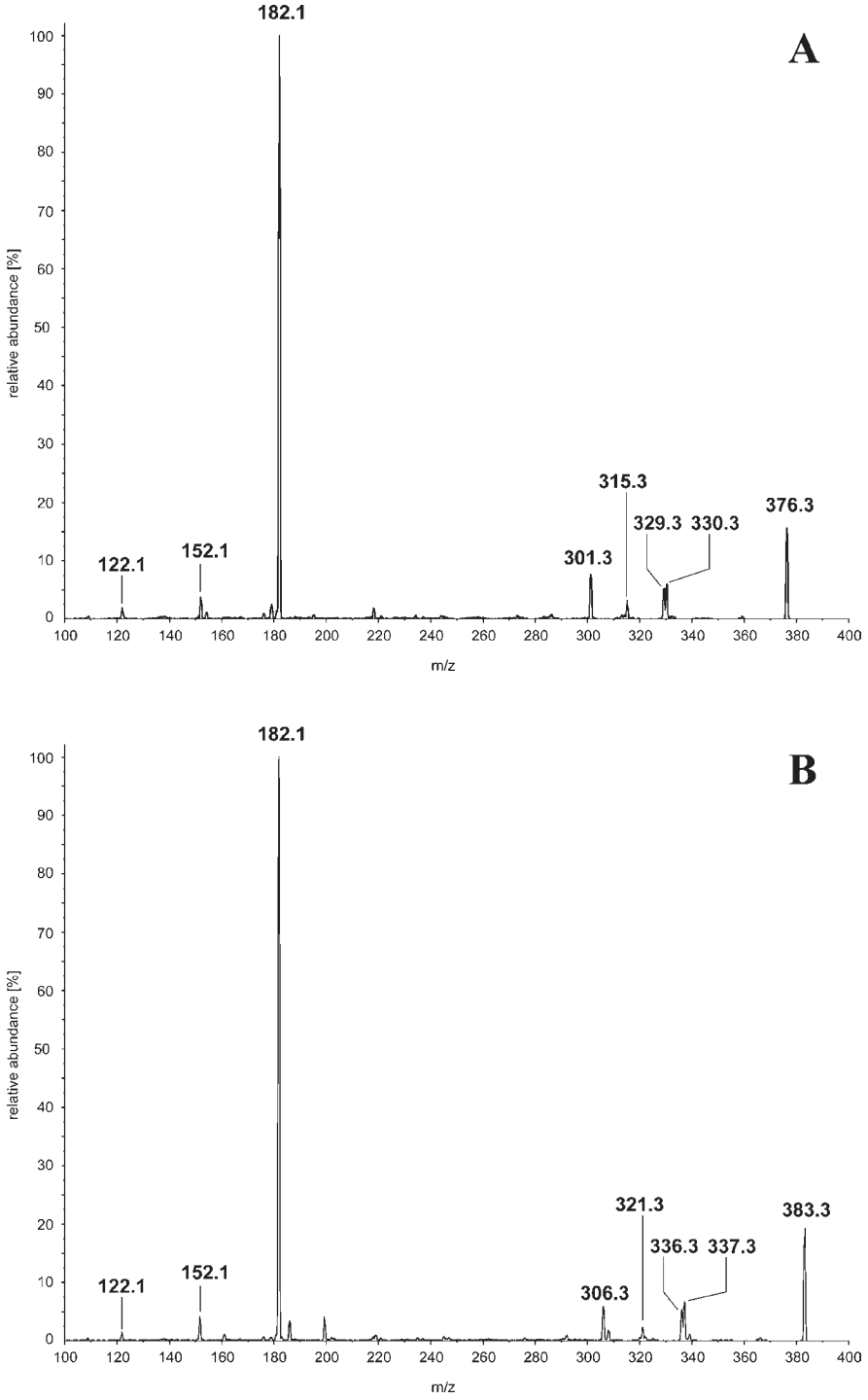

Figure 3. LC-MS/MS spectra (ESI negative; collision energy −30 V) of the dinitrophenylhydrazones of tenuazonic acid (A) and [$^{13}C_6$,^{15}N]-tenuazonic acid (B).

analyte with 2,4-dinitrophenylhydrazine.[14] However, reliable quantitative results were obtained using the SIDA technique, which requires addition of [$^{13}C_6$,^{15}N]-tenuazonic acid to the sample prior to extraction and derivatization. As we focused on rather complex matrices like tomato sauces or pastes, an additional cleanup step was introduced using RP-18 SPE cartridges that takes place after derivatization. This cleanup method was already applied successfully in the analysis of alternariol and alternariol monomethyl ether in beverages.[20] Samples that underwent derivatization and cleanup as described above resulted in LC-MS/MS chromatograms devoid of serious interferences (Figure 4). Consequently, we observed no decline neither in the sensitivity of the mass spectrometer nor in the separation capability of the LC column during the analysis of tomato samples.

Limit of Detection (LOD) and Limit of Quantitation (LOQ). Both values were determined following a recently published method,[21] which is comparable to DIN EN standard 32645.

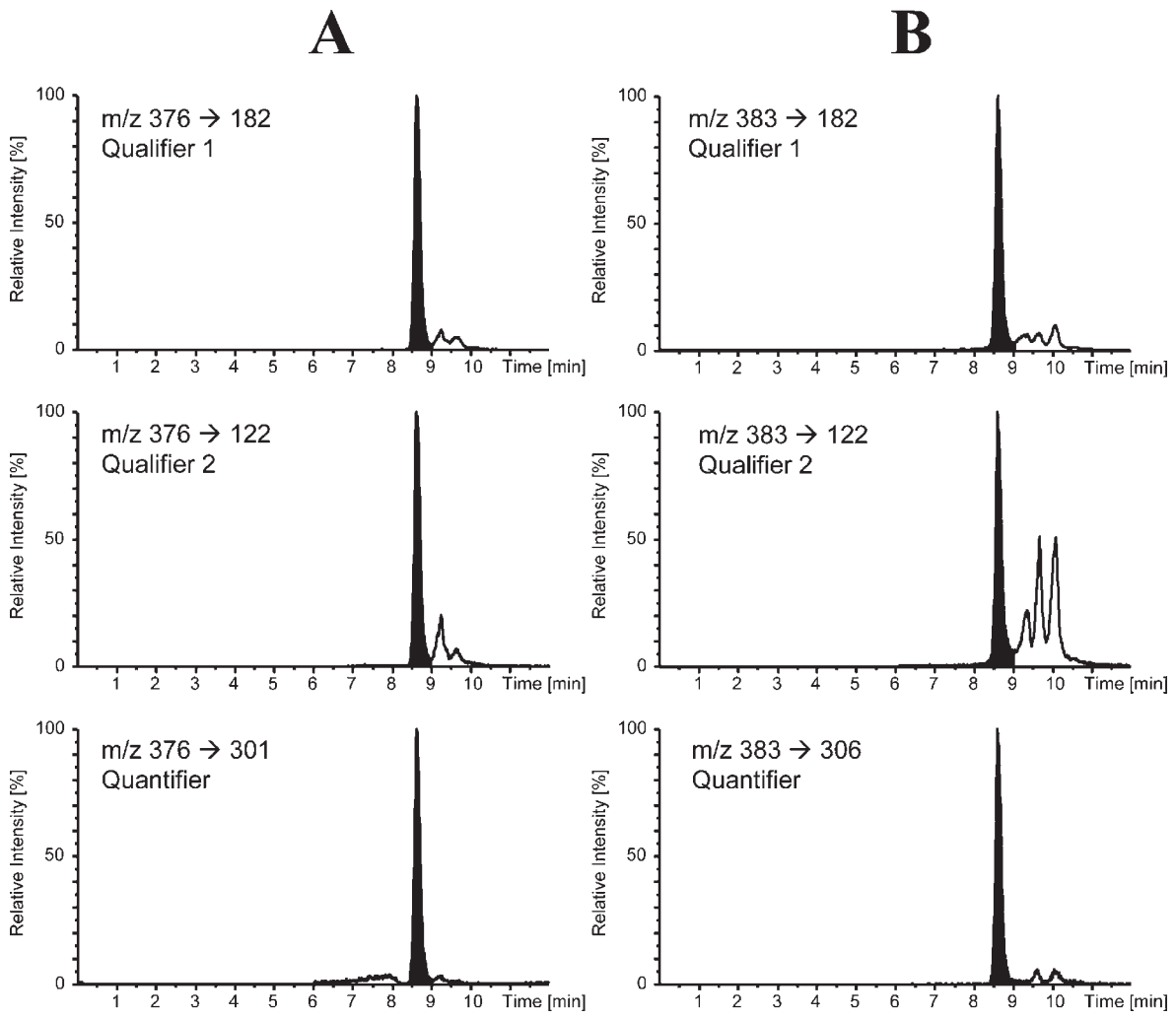

Figure 4. Negative ESI-LC-MS/MS chromatogram of an extract from pureed tomatoes containing 247 μg/kg tenuazonic acid: (A) analyte; (B) isotope labeled standard.

Hence, a LOD of 0.1 μg/kg and a LOQ of 0.3 μg/kg was obtained. The developed method, therefore, is 100 times more sensitive than the method reported for analysis of grains using no cleanup step.[14] Compared to the latter method, the described RP-18 SPE cleanup step allowed a concentration of the sample extract by a factor of 100 without facing matrix interferences in the ion source of the LC-MS/MS.

Recovery. Recovery was determined in the range of 3−300 μg/kg and was 108 ± 1.6% (3 μg/kg), 105 ± 1.7% (30 μg/kg), and 104 ± 2.5% (300 μg/kg). Recoveries around 100% are typical for SIDA as every loss of analyte is counterbalanced by the stable isotope-labeled standard and the resulting value is corrected, therefore. Although a comparison is difficult since different matrices were studied, the recovery of the method without correction with a stable isotope-labeled standard was reported to be 79 ± 11% for different cereal matrices[14] and 90 ± 22% for beer.[15]

Precision. For determination of interassay precision two naturally contaminated samples were analyzed three times in triplicate during 3 weeks. For tomato ketchup (content of tenuazonic acid 41 μg/kg) a coefficient of variation of 2.3% was obtained. In spicy tomato ketchup (content of tenuazonic acid 65 μg/kg) determination of tenuazonic acid was possible with a coefficient of variation of 4.7%. Compared to the respective method using no stable isotope-labeled standard,[14] a 2−3-fold increase of precision was obtained.

Comparison of SIDA with Alternative Methods. In order to elucidate the accuracy of stable isotope dilution assays, other quantitation techniques were tested in comparison. Therefore, a naturally contaminated tomato ketchup (content of tenuazonic acid determined by SIDA 41 μg/kg) was also quantified using (i) the standard addition technique, (ii) external calibration, and (iii) external calibration with correction for recovery. Whereas the content of tenuazonic acid determined by standard addition (38 μg/kg) was quantified only with a low bias (−7.3%) in comparison to the SIDA value, external calibration failed without recovery correction (17 μg/kg; $\text{bias}_{\text{SIDA}}$ −59%) and was only partly satisfying with correction for recovery (recovery 56 ± 7%; 30 μg/kg; $\text{bias}_{\text{SIDA}}$ −27%). This points out that only the time- and labor-consuming standard addition technique results in values that are comparable with the values obtained by SIDA. Hence, the use of stable isotope-labeled standards is a great improvement in the analysis of tenuazonic acid.

Analysis of Tomato Products. Different tomato products were purchased from local supermarkets and analyzed for their content of tenuazonic acid. All samples contained tenuazonic

acid. In tomato ketchup ($n = 9$) values ranged from 15 to 195 μg/kg. The median level was 54 μg/kg. Tomato paste was significantly higher contaminated. In two samples a content of tenuazonic acid of 363 and 909 μg/kg was quantified. However, as both samples were triple concentrated, in the raw material contents below 300 μg/kg can be calculated. A series of further samples ($n = 5$) that included pureed and sieved tomatoes ("passata di pomodoro"), basic tomato sauce ("sugo"), ready-to-use pasta sauce, and basic pizza spread were also analyzed. Contamination with tenuazonic acid was rather heterogeneous in these samples ranging from 8 (pasta sauce) to 247 μg/kg (pureed tomatoes). A median value of 64 μg/kg was determined for these samples.

It is well known that tomatoes are readily colonized by *Alternaria* and tenuazonic acid as the predominant *Alternaria* mycotoxin was reported to occur in moldy tomato fruits in rather high values of 0.4−70[10,11] and 10.5−139 mg/kg.[29] Concerning processed tomato products, values were much lower anyway. Tenuazonic acid was determined in 23 out of 80 samples of Argentinean "tomato puree" (reprocessed from tomato paste) with values between 39 and 4021 μg/kg[12] and in tomato paste with values between 10 and 100 μg/kg.[9] In a survey of Brazilian tomato products, tenuazonic acid was quantified in 7 out of 20 samples of tomato pulp with values between 39 and 111 μg/kg and in 4 out of 22 samples of "tomato puree" with values between 29 and 76 μg/kg.[13] Compared to these studies we did not find tenuazonic acid by incidence but in every analyzed sample. As the determined values were higher than the quantification limits of the above-mentioned studies[12,13] this finding is not a consequence of the increased sensitivity of our LC-MS/MS method but of enhanced mycotoxin contamination. Additionally, we found higher maximum values of tenuazonic acid in tomato paste (909 μg/kg) and pureed tomatoes (247 μg/kg) than reported in the literature.[9,13] However, if the median values are considered, the mean content of tenuazonic acid in pureed tomatoes (64 μg/kg) in our study was similar to literature data (54 μg/kg calculated from the literature[13]). Tomato ketchup was not evaluated before, but our study showed that there is no great divergence of the content of tenuazonic acid (median values) between tomato ketchup (54 μg/kg) and other tomato products (64 μg/kg). Taken together, our study showed enhanced contamination of processed tomato products with tenuazonic acid whereby not the median value was increased but the incidence.

Risk Evaluation. Contamination of tomato products with tenuazonic acid was clearly detectable, but in general the median content was below 100 μg/kg. Therefore, the maximum intake of tenuazonic acid by consumers can be estimated to be below 1 μg/kg BW, which is 5 orders of magnitude below the data for acute toxicity in mice. Although a transfer of these toxicity data from animals to humans should be approached with caution, tomato products do not appear to pose an acute risk toward the health of the consumer. However, other studies showed contamination of cereals with tenuazonic acid in values very similar to those obtained for tomato products in our study.[14] Furthermore, tenuazonic acid was found in every analyzed sample in our study, which equals a contamination rate of 100%. Adverse effects of tenuazonic acid ingestion have to be considered on the basis of cumulative exposure, therefore. More data on the tenuazonic acid content in further food commodities and on its possible chronic toxicity and carcinogenicity are necessary in order to calculate this potential risk for human health.

■ ASSOCIATED CONTENT

Supporting Information. LC-MS/MS spectra of tenuazonic acid and [$^{13}C_6$,^{15}N]-tenuazonic acid (Figure S1), proposed MS/MS fragmentation schemes of tenuazonic acid and [$^{13}C_6$,^{15}N]-tenuazonic acid (Figure S2) and their respective dinitrophenylhydrazones (Figure S3) with special focus on the formation of the fragment ions m/z 301.3 and 315.3 (Figure S4), response curve of the developed SIDA (Figure S5), and table about the content of tenuazonic acid in tomato products (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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