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Enzyme Immunoassay for Tenuazonic Acid in Apple and Tomato Products

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ABSTRACT: The *Alternaria* mycotoxin tenuazonic acid was derivatized with succinic anhydride and conjugated to keyhole limpet hemocyanin (KLH) and to horseradish peroxidase (HRP), respectively. The KLH conjugate was used to produce polyclonal antibodies in rabbits. A competitive direct enzyme immunoassay (EIA) for tenuazonic acid was established, which was moderately sensitive for tenuazonic acid [50% inhibition concentration (IC_{50}): 320 ± 130 ng/mL] but strongly reacted with tenuazonic acid acetate (IC_{50}: 23.3 ± 7.5 ng/mL). Therefore, an optimized EIA protocol was established, which employed acetylation of standard and sample extract solutions. The mean standard curve detection limit (IC_{30}) for tenuazonic acid acetate was 5.4 ± 2.0 ng/mL, enabling detection limits for tenuazonic acid in apple and tomato products of 25−50 ng/g (150 ng/g in tomato paste). Recoveries in a concentration range of 50−2000 ng/g were 60−130% in apple juice and tomato juice and 40−150% in other tomato products. Tenuazonic acid was detected in apple juice and tomato products from German retail shops at levels of 50−200 ng/g. In conclusion, this novel EIA for tenuazonic acid could be useful within a screening program for *Alternaria* mycotoxins in food.

KEYWORDS: Mycotoxin, polyclonal antibodies, immunoassay, acetyl tenuazonic acid, apple, tomato, *Alternaria*

INTRODUCTION

L-Tenuazonic acid [(5*S*)-3-acetyl-1,5-dihydro-4-hydroxy-5-[(1*S*)-1-methylpropyl]-2*H*-pyrrol-2-one] (Figure 1) is a tetramic acid derivate commonly listed within the group of *Alternaria* mycotoxins. Tenuazonic acid was originally isolated by Rosett et al.[1] from *Alternaria* culture, and its chemical structure was established by Stickings et al.[2] Tenuazonic acid is considered to have the highest acute toxicity among the *Alternaria* mycotoxins.[3] The oral LD_{50} of tenuazonic acid was 225 ± 20 mg/kg bw in mice and 100−150 mg/kg bw in primates (*Macaca fascicularis*).[4] The primary mode of action of tenuazonic acid is the inhibition of the protein biosynthesis by suppression of the release of new proteins from the ribosome.[5]

Tenuazonic acid is a major compound of the *Alternaria* mycotoxin complex, which includes several structurally unrelated compounds such as the dibenzo-α-pyrones (e.g., alternariol), tentoxin, and the altertoxins.[6] Reports on the production of tenuazonic acid by other fungal species, namely, *Phoma sorghina*, *Pyricularia oryzae*, and, more anecdotally, *Aspergillus* and *Spaeropsidales* species, have been reviewed.[3,7] Tenuazonic acid-producing fungi are ubiquitous in many biological environments and are able to infest most plant species.[6] L-Tenuazonic acid is not very stable and undergoes isomerization in solution, and toxicity may be reduced after extended heating at 100−121 °C.[7,8] However, these processes may be rather slow in a food sample matrix; therefore, tenuazonic acid contamination was found in a large variety of cereals, vegetables, fruits, and seeds.[3,6,9] The frequent occurrence of tenuazonic acid, in some cases at levels exceeding 1 mg/kg, has been particularly reported for tomato and tomato products.[7,10−13]

Several chromatographic methods have been described for the determination of tenuazonic acid in foods and feeds, including thin-layer chromatography (TLC), gas chromatography, and liquid chromatography, the latter using UV detection.[9,14] More recently, liquid chromatography methods with mass spectrometric detection have been developed, some employing either precolumn derivatization[15] or stable isotope dilution assay[12] to overcome the difficulties in tenuazonic acid chromatography arising from its strong acidic and metal complexing properties.[16] A recent liquid chromatography−tandem mass spectrometry (LC-MS/MS) multimethod for *Alternaria* toxins includes tenuazonic acid at a detection limit of 2 μg/kg.[13]

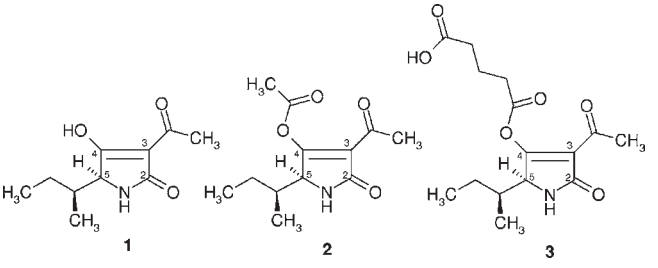

Figure 1. Structures of L-tenuazonic acid (**1**) and supposed structures of its derivatives tenuazonic acid acetate (**2**) and tenuazonic acid hemisuccinate (**3**).

Antibodies against tenuazonic acid have not been described so far, and thus, immunochemical methods, as a complementary analytical technique for rapid screening purposes of food, are not

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available. Recently, we have described highly sensitive immunoassays for alternariol and found that this *Alternaria* toxin is frequently detectable in apple and tomato products.[17] Here, we describe the development of polyclonal antibodies against tenuazonic acid and their use in a sensitive enzyme immunoassay (EIA) for tenuazonic acid in apple and tomato products, employing a simple acetylation procedure, which has been previously used for trichothecenes.[18,19]

MATERIALS AND METHODS

Chemicals, Buffers, and Equipment. Tenuazonic acid copper salt was from Sigma-Aldrich (Deisenhofen, Germany). For comparison purposes, pure tenuazonic acid was obtained from another source (Enzo Life Sciences, Lörrach, Germany). The UV absorbance maxima for tenuazonic acid copper salt were determined in acetonitrile and found at 291, 225, and 194 nm, while pure tenuazonic acid in acetonitrile had maxima at 277, 240, and 194 nm. The latter is in agreement with published UV spectra of tenuazonic acid.[7,8]

Keyhole limpet hemocyanin (KLH; molecular weight used for calculations, 3 000 000) and horseradish peroxidase (HRP; molecular weight, 40 000) were from Roche Diagnostics (Mannheim, Germany). Goat antirabbit IgG, 3,3′,5,5′-tetramethylbenzidine (TMB), dimethylformamide (DMF), dicyclohexyl carbodiimide (DCC), *N*-hydroxysuccinimide (NHS), dimethylaminopyridine, and all other reagents (reagent grade or better) were from Sigma-Aldrich. The acetylation reagent was a stock solution of acetonitrile containing 4 mg/mL 4-dimethylaminopyridine, to which 20 μL of acetic anhydride per mL was added shortly before use.

A tenuazonic acid acetate (copper salt) stock solution was prepared using a protocol described earlier for deoxynivalenol.[19] In brief, 100 μL of a tenuazonic acid standard solution in acetonitrile (2 mg/mL) was mixed with 900 μL of acetylation reagent and incubated for 1 h at ambient temperature (approximately 20 °C). Then, the mixture was diluted 1:19 with phosphate-buffered saline (PBS; 0.01 M phosphate buffer, pH 7.3, containing 0.1 M NaCl), corresponding to a nominal tenuazonic acid concentration of 10 μg/mL. EIA standard concentrations were prepared from this stock solution by dilution with 5% acetonitrile/PBS.

The dilution buffer for coating type MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) with antirabbit IgG was 0.05 M sodium-bicarbonate buffer, pH 9.6. The blocking solution for microtiter plates was PBS containing 20 g/L sodium caseinate. The wash solution was distilled water containing 8.5 g/L of NaCl and 0.25 mL/L of Tween 20. Hydrogen peroxide−citrate buffer solution, pH 3.95, for enyzme substrate/chromogen solution was 8.3 g of citric acid, 49 mL of 1 M KOH, 160 mL of distilled water, and 72 μL of 30% aqueous H_2O_2. The enzyme substrate/chromogen solution was prepared as used earlier.[17] Briefly, 50.4 mg of TMB was dissolved in 1 mL of acetone and 9 mL of methanol. Before use, 0.5 mL of TMB solution was mixed with 10 mL of H_2O_2−citrate buffer solution. EIA absorbance values were measured at 450 nm with a model Sunrise plate reader (Tecan, Männedorf, Switzerland) and evaluated by Magellan EIA calculation software (Tecan) with parameters as described earlier.[17] Briefly, the absorbance was measured at 450 nm, with a 620 nm reference filter. Seven standard concentrations (six serial dilutions of tenuazonic acid standard in buffer solution plus one buffer solution blank, B_0) were pipetted on each plate, and four duplicate wells were analyzed for all standard and sample solutions. After transformation of absorbance values (absorbance B_0 = 100%), the 50% inhibition concentration (IC_{50}) and the 30% inhibition concentration (IC_{30}) values of the standard curves were recorded, to check the stability and detection limit of the EIA over the period of analysis. Animal manipulations were performed in compliance with the respective of the German laws and guidelines and with the formal allowance by the regional Hessen authority (Regierungspräsidium Giessen).

Sample Materials. Apple juice (n = 7), tomato juice (n = 15), tomato ketchup (n = 18), and tomato paste (n = 10) were purchased as offered to the consumer in original packages or bottles from retail shops in the area of Giessen, Germany.

Synthesis of Immunochemicals. Tenuazonic acid (copper salt) was derivatized with succinic anhydride and then conjugated to KLH and to HRP via an activated ester method[20] under conditions used earlier.[21] Tenuazonic acid copper salt (corresponding to 3 mg of toxin) and succinic anhydride (30 mg) were dissolved in pyridine (500 μL) and reacted at 97 °C for 3 h in a glass vial. The pyridine was evaporated with nitrogen. The brown solid residue was dissolved with 1 mL of ethyl acetate and 2 mL of HCl (0.01 mol/L) and mixed for 30 s on a wrist-action shaker. The organic phase was removed, and the aqueous phase was mixed again with 1 mL of ethyl acetate. Both ethyl acetate fractions were combined, the solvent was evaporated under nitrogen, and the tenuazonic acid hemisuccinate residue finally was redissolved with 1 mL of methanol.

The tenuazonic acid hemisuccinate was checked by TLC using Merck silica gel plates (with 254 nm fluorescence indicator) and either chloroform/methanol (90/10 v/v; solvent A) or chloroform/methanol/acetic acid (90/10/1 v/v/v; solvent B) as the mobile phase. The derivate was further checked by high-performance liquid chromatography (HPLC)-UV using a method similar as described by Scott and Kanhere[7] and equipment described previously.[17] The analytical column was a 125 mm × 4 mm i.d., 5 μm, Li-Chrospher 100 RP-18 (Merck, Darmstadt, Germany), the mobile phase was (A) methanol and (B) water containing 0.5 g of $ZnSO_4$ monohydrate per liter in a linear gradient from 70 to 50% B within 20 min, at a flow rate of 1 mL/min. Tenuazonic acid and its hemisuccinate were detected at 280 nm (200−400 nm diode array scan).

The tenuazonic acid hemisuccinate solution (≈2.7 mg of tenuazonic acid hemisuccinate) was then evaporated to dryness under nitrogen and redissolved with 0.4 mL of DMF. An activated ester intermediate of the tenuazonic acid hemisuccinate was formed by mixing the toxin derivative solution with 2.9 mg of NHS and 10.3 mg of DCC (in 0.4 mL of DMF) and stirring the mixture for 16 h at ambient temperature (approximately 20 °C). KLH (51.3 mg) and HRP (20.5 mg) were each dissolved with 1.7 mL of a 0.13 M aqueous $NaHCO_3$ solution (pH 8.3). Portions of each 0.3 mL of the activated tenuazonic acid hemisuccinate were added over a period of 1 min to the KLH and HRP solutions, and the conjugation mixtures were stirred at room temperature for 2 h. Then, the tenuazonic acid−KLH and the tenuazonic acid−HRP conjugates were separately dialyzed against each three changes (each 5 L) of PBS for 3 days. The concentration of the conjugates was estimated from their UV absorbance at 280 (KLH) and 403 nm (HRP) by comparison with KLH and HRP stock solutions. However, because both conjugates contained UV-absorbing impurities that interfered at these wavelengths, an exact determination was not possible. The conjugates were stored in small portions at −18 °C.

Immunization and Antibody Titer Determination. For use as the immunogen, 0.3 mL of the tenuazonic acid−KLH conjugate solution (corresponding to approximately 4.5 mg of tenuazonic acid −KLH) was mixed with 1.2 mL of PBS and emulsified with 4.5 mL of Freund's complete adjuvant. Three female chinchilla bastard rabbits (Charles River, Kisslegg, Germany) were each immunized with 2 mL portions of the immunogen by using multiple intradermal injections. Intramuscular booster immunizations with the same composition and volume of immunogen were made depending on the development of the individual antibody titers. Blood was collected every second week from the large ear artery. The relative antibody titer was determined in a double antibody solid phase EIA essentially as described previously,[21] using tenuazonic acid−HRP in a dilution of 1:1000. Titer was defined as the antiserum dilution that gave 0.3 absorbance units; the preimmune control sera gave maximum absorbance values of <0.1 units under these

conditions. The serum of one rabbit collected 22 weeks after primary immunization was used for further characterization.

Competitive Direct EIA. A microtiter plate was coated with anti rabbit IgG (10 μg/mL coating buffer, 100 μL per well) overnight at room temperature. Free protein binding sites were blocked for 30 min with sodium caseinate solution (200 μL per well), and then, the plate was washed three times and made semidry. To each well, 35 μL of tenuazonic acid standard or tenuazonic acid acetate solution (in 5% acetonitrile/PBS), 35 μL of anti tenuazonic acid antiserum dilution (1:3000 in PBS), and 35 μL of tenuazonic acid−HRP solution (1:3000 in 1% sodium caseinate/PBS) were added. The plate was incubated for 1 h at ambient temperature (approximately 20 °C). Then, the plate was washed again, and an enzyme substrate/chromogen solution was added (100 μL per well). After 15 min, the enzyme reaction was stopped with 1 M H_2SO_4 (100 μL per well), and the absorbance at 450 nm was measured. Evaluation of the absorbance readings was essentially performed as described previously.[17] A cutoff value of 70% relative binding of the standard curve (IC_{30}) was set as the standard curve detection limit.

The overall robustness of the EIA system was checked by recording the IC_{50} and IC_{30} values of standard curves performed over a period of 6 months. The stability of the tenuazonic acid acetate standard solution stored at 6−8 °C was evaluated in two experiments by comparing the IC_{50} values of standard curves performed with two batches of a tenuazonic acid acetate stock solutions (10 μg/mL in 5% acetonitrile/PBS) stored over a period of up to 161 days.

Cross-reactivity of the antitenuazonic acid antibodies with other *Alternaria* toxins was tested by competition experiments with standard solutions of alternariol, alternariol monomethyl ether, and altenuene at concentrations of up to 10 μg/mL under the conditions of the competitive direct EIA.

Sample Preparation for EIA Analysis. Apple or tomato juice (4 mL) was extracted with 8 mL of ethyl acetate for 3 × 4−5 s on a wrist-action shaker at full speed, followed by centrifugation (1500*g*, 15 min, 4 °C). Tomato ketchup or tomato paste (5 g) was diluted with distilled water (8 mL), and 4 mL of the diluted sample was extracted for 30 s on a wrist-action shaker with 8 mL of ethyl acetate. The organic phase of each extract was transferred into an 25 mL evaporation flask, and the solvent was evaporated in a rotary evaporator at 40 °C under reduced pressure. The residue was dissolved with 1 mL of acetylation reagent. After 1 h, the acetylation reaction was stopped by the addition of 19 mL of PBS. In the case of apple juice and tomato ketchup, this solution was directly used for EIA analysis, while for tomato juice and tomato paste, the solution was further diluted with 5% acetonitrile/PBS at least 1:2 and 1:8, respectively, to eliminate sample matrix effects. At least two dilutions of all sample extracts were analyzed by EIA, and four replicate wells were analyzed for each dilution.

For recovery studies, tenuazonic acid standard solution was added to apple and tomato products in a final concentration range of 50−2000 ng/g before extraction and acetylation. To check whether a single ethyl acetate extraction is sufficient to recover tenuazonic acid, a preliminary experiment was performed in which samples of apple juice ($n = 3$) were spiked with tenuazonic acid at a concentration of 2 μg/mL, and 4 mL portions of each sample were extracted two times with 8 mL each of ethyl acetate.

RESULTS AND DISCUSSION

Antibodies against Tenuazonic Acid. Tenuazonic acid is a relatively small hapten (mol wt, 197), which makes synthesis of suitable immunogens difficult. An initial attempt, employing direct condensation of tenuazonic acid to proteins with formaldehyde, which was successfully used for other mycotoxins,[22,23] failed to yield a specific immune response in rabbits (unpublished data). Tenuazonic acid was therefore derivatized with succinic anhydride to introduce a reactive carboxyl group for conjugation to proteins. By TLC, tenuazonic acid and its hemisuccinate had R_f values of 0.38 and 0.48 in solvent A and 0.44 and 0.5 in solvent B. No underivatized tenuazonic acid could be detected in the hemisuccinyl product. In HPLC, tenuazonic acid had a retention time of 4.1 min (λ_{max}, 281 nm), while the tenuazonic acid hemisuccinate eluted slightly earlier (3.8 min; λ_{max}, 278 nm). On the basis of peak area comparison between toxin standard and derivative, the estimated yield from 3 mg of tenuazonic acid was 2.7 mg of the hemisuccinyl derivative, assuming identical UV absorbance for both compounds.

The tenuazonic acid−hemisuccinate was converted to its NHS ester and conjugated to KLH as the immunogen and to HRP as the labeled antigen, using a standard activated ester reaction.[20] The tenuazonic acid−KLH conjugate demonstrated a moderate immunogenic activity in rabbits. Four weeks after the primary immunization, antibody titers binding to tenuazonic acid−HRP could be detected in all three animals. However, serum of one rabbit failed to give competitive binding inhibition by free tenuazonic acid at concentrations up to 10 μg/mL. While the serum of a second rabbit yielded weak competitive binding inhibition by tenuazonic acid in the μg/mL range, the serum of the third rabbit gave the most sensitive standard curve, with a IC_{50} concentration of 320 ± 130 ng/mL and a IC_{30} concentration (detection limit) of 90 ± 29 ng/mL ($n = 10$). We chose the IC_{30} as a cutoff value, which is a very conservative approach, to include a safety margin covering the overall variability of the extraction and acetylation procedure, thus avoiding the risk of reporting of false-positive results. If certified tenuazonic acid reference materials (positive and negative) for the full range of matrices would be available, a more precise and probably much lower detection limit could be established. For analysis of tenuazonic acid buffer solutions only, an IC_{20} could probably be used as the detection limit.

To our best knowledge, this is the first study describing a successful procedure to generate antitenuazonic acid antibodies. Therefore, it is not possible to directly compare this antiserum with other reports. However, as compared with, for example, antibodies against alternariol, another important *Alternaria* mycotoxin,[17] which enabled a detection limit in the low pg/mL range, the antitenuazonic antibodies are only moderately sensitive for tenuazonic acid.

We therefore sought an approach to further improve the detection limit of the tenuazonic acid EIA. The low molecular weight of tenuazonic acid, and earlier immunochemical experience with succinyl/acetyl ester derivatives of the mycotoxin deoxynivalenol[19,24] suggested that the ester bond in the tenuazonic acid hemisuccinate may be an important feature for antibody recognition and that formation of the acetyl ester of tenuazonic acid could be suitable to enhance immunoassay antibody recognition. In fact, competition tests with tenuazonic acid acetate and tenuazonic acid hemisuccinate revealed a more than 10-fold higher sensitivity for the acetate, while the sensitivity for the hemisuccinate was even higher than that for tenuazonic acid acetate (Figure 2).

Because the acetylation procedure is relatively straightforward and easy to perform, the formation of tenuazonic acid acetate as an integral part of the sample preparation was used for further EIA development. Long-term evaluation of the tenuazonic acid acetate standard curve parameters (Table 1) showed that the mean detection limit was at about 5 ng/mL. On the basis of the comparison of IC_{50} values of standard curves for freshly acetylated

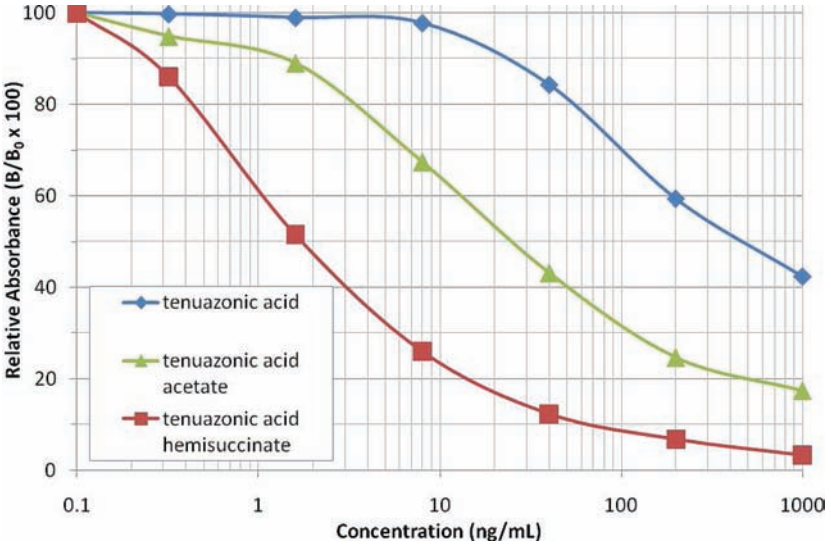

Figure 2. Typical standard curves of the competitive direct EIA for tenuazonic acid acetate and tenuazonic acid. Four replicate wells of all standard concentrations were analyzed. Mean IC_{50} concentrations were 320 ± 130 (tenuazonic acid), 23 ± 7.5 (tenuazonic acid acetate), and 4.9 ± 4.5 ng/mL (tenuazonic acid hemisuccinate).

Table 1. Long-Term Standard Curve Robustness of the Competitive Direct EIA for Tenuazonic Acid after Acetylation[a]

parameter	IC_{50}	IC_{30} (detection limit)
mean ± standard deviation (ng/mL)	23.3 ± 7.5	5.4 ± 2.0
relative standard deviation (%)	32.2	36.4
minimum (ng/mL)	12	3
maximum (ng/mL)	37	8

[a] Evaluation of 25 tests performed over a period of 6 months.

Table 2. Stability of the Tenuazonic Acid Acetate Standard Solution (in 5% Acetonitrile/PBS), as Determined from the IC_{50} of the EIA Standard Curve

	standard curve IC_{50} (ng/mL)	
days of storage	experiment 1	experiment 2
0	22.1	21.6
1	21.5	
2	18.3	
7		36.0
16		38.1
43		25.8
49		20.2
59		37.4
70		24.4
114		15.7
133		14.5
161		18.6
mean ± SD	20.6 ± 2	25.2 ± 8.9

tenuazonic acid and stored (6−8 °C) tenuazonic acid acetate solutions, the 10 μg/mL tenuazonic acid acetate stock solution was found to be stable for nearly 6 months (Table 2). Cross-reactivity with other *Alternaria* toxins (alternariol, alternariol monomethyl ether, and altenuene) was not detectable (<1%). This was not surprising, since the chemical structure of tenuazonic acid has no similarities with the other *Alternaria* toxins. Vice versa, antibodies against alternariol had no reactivity with tenuazonic acid.[17]

The advantages of the acetylation procedure are that it can easily be integrated into the sample preparation scheme, that it does not require the absolute absence of water, and that it can be performed at ambient temperature around 20 °C. Furthermore, the acetylation step enhances specificity of the analytical process. The disadvantage is that acetylation adds another working step and that it requires a relatively long reaction time. In this study, we used a reaction time of 1 h since this parameter has been found to be suitable earlier.[19,25] A possible reduction of the acetylation reaction time could be studied in future experiments.

EIA Analysis of Tenuazonic Acid in Tomato and Apple Products. Sample extraction by liquid−liquid partitioning with ethyl acetate and acetylation of tenuazonic acid yielded detection limits in apple juice and tomato juice of 25 and 50 ng/mL, respectively. Tomato ketchup and tomato paste had to be diluted with water before liquid−liquid extraction, resulting in a detection limit for tomato ketchup of approximately 40 ng/g. Slightly stronger sample matrix effects were observed for extracts of tomato paste, requiring analysis of more diluted extracts, which resulted in a detection limit for tenuazonic acid in tomato paste of approximately 150 ng/g. The recoveries were as follows: apple juice, 78−130% at 50−500 ng/mL; tomato juice, 61−113% at 50−2000 ng/mL; tomato ketchup, 40−150% at 50−2000 ng/g; and tomato paste, 44−57% at 500−2000 ng/g (Table 3). It has to be pointed out that different brands of products, with considerable variation in recipe and composition, were used for recovery study within each type of food, which may account for some of the observed variability. However, relatively low and variable recoveries indicate the need to improve the sample preparation. Further work would aim at experiments improving the recovery of tenuazonic acid, for example, by repeating the liquid−liquid extraction with ethyl acetate. Although a preliminary experiment using repeated extraction with three different samples

Table 3. Recovery of Tenuazonic Acid from Artificially Contaminated Apple Juice and Tomato Products[a]

		tenuazonic acid found			
sample type	tenuazonic acid added (ng/mL or ng/g)	mean ± SD (ng/mL or ng/g)	RSD[b] (%)	recovery (%)	*n*
apple juice	50	65.4 ± 16.1	24.6	130	5
	100	104 ± 37.2	35.8	104	7
	500	388 ± 101	26	77.5	6
tomato juice	50	56.4 ± 27.1	48.1	113	3
	100	97.3 ± 54.5	56	97.3	3
	500	430 ± 236	54.8	86	6
	1000	595 ± 169.6	28.5	59.5	5
tomato ketchup	100	136 ± 32.6	23.9	136	3
	500	227 ± 95.4	42	45.2	4
	1000	436 ± 128	29.3	43.6	4
	2000	683 ± 215	31.5	34.2	3
tomato paste	500	287 ± 92.6	32.3	57.3	4
	1000	508 ± 192	20	50.8	4
	2000	871 ± 146	16.7	43.6	3

[a] All nonspiked samples were always analyzed in parallel for tenuazonic acid by EIA and consistently gave negative results. [b] RSD, relative standard deviation.

Table 4. Tenuazonic Acid in Apple Juice and Tomato Products from the German Market

sample type (no. of samples)	EIA detection limit (ng/mL or ng/g)	positive samples	tenuazonic acid levels (ng/mL or ng/g)
apple juice (7)	25	1	58
tomato juice (15)	50	3	61, 104, 227
tomato ketchup (18)	40	2	55, 67
tomato paste (10)	150	0	

of apple juice spiked with tenuazonic acid at a level of 2 μg/mL showed that only 12, 18, and 20% of the toxin were present in the second extract, the situation may still be different with other apple juices and with other food materials. Furthermore, matrix influence on the efficiency of the acetylation process, in particular in highly contaminated sample material, should also be studied.

A preliminary application study showed that tenuazonic acid at levels of 50−200 ng/g could be detected by EIA in apple juice, tomato juice, and tomato ketchup purchased from German retail shops. The negative results for tomato paste may be due to the relatively poor detection limit in these products (Table 4).

The largest amount of published data on the occurrence of tenuazonic acid in foods is available for tomato products. Scott and Kanhere[7] found small amounts of tenuazonic acid in tomato paste (range of 10−100 ng/g). At a detection limit of 11 ng/g, tenuazonic acid was found in tomato pulp/tomato purée from South America in concentration ranges of 29−111[10] and 39−4021 ng/g.[11] Considering analytical detection limits and tenuazonic acid frequency and levels, these results are mostly in the same range as those obtained in our study.

With the availability of highly sensitive LC-MS/MS methods, detection limits of 0.1[12] and 2 ng/g[13] were reported for tenuazonic acid in tomato products. In contrast to our findings, these studies found a contamination frequency of 100% in all processed tomato products from the German and Swiss markets, at levels of 15−909[12] and 2−790 ng/g,[13] respectively. For most products, the median tenuazonic acid values in these two studies were in a range of 30−70 ng/g. This would suggest that a high number of samples, in particular tomato paste, contain tenuazonic acid at levels that are below, or close to, the detection limit of the EIA. Further work should include a comparison study between immunoassay and LC-MS/MS, to more exactly establish the range of applicability of the EIA in various tomato products. In the light of the obviously widespread contamination of tomato products with tenuazonic acid, sometimes at relatively high levels, a toxicological evaluation of this mycotoxin would be desirable.

It is known that *Alternaria* grows well on apples, and tenuazonic acid has been reported to occur on decayed apples,[9] but its occurrence in apple juice or other apple products for direct consumption has not been reported so far. The mean daily consumption of apple juice by adults in Germany in different age groups is quite high (63−138 mL) and may be even higher in children.[26] The fact that one out of seven samples of apple juice was positive for tenuazonic acid in our study indicates the necessity of a larger survey, to clarify the relevance of apple juice for the overall exposure to this mycotoxin. Furthermore, such a survey ideally should include other fruit and vegetable juices and wines, because other *Alternaria* toxins have been found in these products.[27,28]

Because an estimate concerning the tolerable daily tenuazonic acid intake is not available, neither maximum acceptable levels in food nor minimum sensitivity requirements for analytical methods can be established. However, considering the ubiquitous growth of *Alternaria*, it seems to be advisible to identify potential sources of tenuazonic acid intake within the food catalogue and to closely monitor the contamination levels of relevant foods. Furthermore, because the presence of tenuazonic acid in foods is largely a consequence of postharvest fungal decay, it could also be an interesting approach to study its usefulness as a quality parameter in food control. The tenuazonic acid EIA is sufficiently sensitive to cover this toxin in a range similar as set, for example, as maximum levels for the mycotoxin patulin in apple products (10−50 ng/mL[29]).

In conclusion, the novel EIA for tenuazonic acid could be a useful tool to identify products containing critical levels of tenuazonic acid. A very recent study[30] showed that tenuazonic acid

contamination is very widespread over a large variety of foods from the German market, including juices other than apple juice, cereals, and spices. Extremely high values (>37 mg/kg) were found in spices. This further emphasizes the need for enhanced monitoring measures. With regard to the EIA, some further improvement of the sample preparation procedure seems to be necessary, and a further evaluation, for example, by comparison with LC-MS/MS analysis, would be advisible. However, the EIA has the potential to be implemented in a monitoring program for *Alternaria* toxins in processed foods.

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REFERENCES

(1) Rosett, T.; Sankhala, R. H.; Stickings, C. E.; Taylor, M. E. U.; Thomas, R. Studies in biochemistry of microorganism; 103: Metabolites of *Alternaria tenuis auct.*: culture filtrate products. *Biochem. J.* **1957**, *67*, 390–400.

(2) Stickings, C. E. Studies in the biochemistry of micro-organisms. 106. Metabolites of *Alternaria tenuis auct.*: The structure of tenuazonic acid. *Biochem. J.* **1959**, *72*, 332–340.

(3) Ostry, V. *Alternaria* mycotoxins: An overview of chemical characterization, producers, toxiticity, analysis and occurrence in foodstuffs. *World Mycotoxin J.* **2008**, *1*, 175–188.

(4) Miller, F. A.; Rightsel, W. A.; Sloan, B. J.; Ehrlich, J.; French, J. C.; Bartz, Q. R.; Dixon, G. J. Antiviral activity of tenuazonic acid. *Nature* **1963**, *200*, 1338–1339.

(5) Shigeura, H. T.; Gordon, C. N. The biological activity of tenuazonic acid. *Biochemistry* **1963**, *2*, 1132–1137.

(6) Bottalico, A.; Logrieco, A. Toxigenic *Alternaria* species of economic importance. In Sinha, K. K., Bhatnagar, D., Eds.; *Mycotoxins in Agriculture and Food Safety*; Marcel Dekker: New York, NY, 1998; pp 65−108.

(7) Scott, P. M.; Kanhere, S. R. Liquid chromatographic detection of tenuazonic acid in tomato paste. *J. Assoc. Off. Anal. Chem.* **1980**, *63*, 612–621.

(8) Combina, M.; Dalcero, A. M.; Torres, A. Spectrometric studies on stability of tenuazonic acid (TeA) solution in organic solvents. *Mycotoxin Res.* **1998**, *14*, 54–59.

(9) Scott, P. M. Analysis of agricultural commodities and foods for *Alternaria* mycotoxins. *J. AOAC Int.* **2001**, *84*, 1809–1817.

(10) Da Motta, S.; Soares, L. M. V. Survey of Brazilian tomato products for alternariol, alternariol monomethyl ether, tenuazonic acid, and cyclopiazonic acid. *Food Addit. Contam.* **2001**, *18*, 630–634.

(11) Terminiello, L.; Patriarca, A.; Pose, G.; Pinto, V. F. Occurrence of alternariol, alternariol monomethyl ether and tenuazonic acid in Argentinean tomato puree. *Mycotoxin Res.* **2006**, *22*, 236–240.

(12) Asam, S.; Liu, Y.; Konitzer, K.; Rychlik, M. Development of a stable isotope dilution assay for tenuazonic acid. *J. Agric. Food Chem.* **2011**, *59*, 2980–2987.

(13) Noser, J.; Schneider, P.; Rother, M.; Schmutz, H. Determination of six *Alternaria* toxins with UPLC-MS/MS and their occurrence in tomatoes and tomato products from the Swiss market. *Mycotoxin Res.* **2011**, *27*, 265–271.

(14) Abramson, D.; Delaquis, P.; Smith, D. Assessment of ochratoxin A and tenuazonic acid in Canadian ice-wines. *Mycotoxin Res.* **2007**, *23*, 147–151.

(15) Siegel, D.; Rasenko, T.; Koch, M.; Nehls, I. Determination of the *Alternaria* mycotoxin tenuazonic acid in cereals by high-performance liquid chromatography/electrospray ionization-multistage mass spectrometry after derivatization with 2,4-dinitrophenylhydrazine. *J. Chromatogr., A* **2009**, *1216*, 4582–4588.

(16) Lebrun, M. H.; Duvert, P.; Gaudener, A.; Deballon, A. Complexation of the fungal metabolite tenuazonic acid with copper (II), iron (III), nickel (II) and magnesium (II) ions. *J. Inorg. Biochem.* **1985**, *24*, 167–181.

(17) Ackermann, Y.; Curtui, V.; Dietrich, R.; Gross, M.; Latif, H.; Märtlbauer, E.; Usleber, E. Widespread occurrence of low levels of alternariol in apple and tomato products, as determined by comparative immunochemical assessment using monoclonal and polyclonal antibodies. *J. Agric. Food Chem.* **2011**, *59*, 6360–6368.

(18) Xu, Y.-C.; Zhang, G. S.; Chu, F. S. Enzyme-linked immunosorbent assay for deoxynivalenol in corn and wheat. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 945–949.

(19) Usleber, E; Märtlbauer, E. A limited survey of cereal foods from the German market for *Fusarium* toxins (deoxynivalenol, zearalenone, fumonisins). *Arch. Lebensmittelhyg.* **1998**, *49*, 42–45.

(20) Kitagawa, T.; Shimozono, T.; Aikawa, T.; Yoshida, T.; Nishimura, H. Preparation and characterization of hetero-bifunctional crosslinking reagents for protein modifications. *Chem. Pharm. Bull.* **1981**, *29*, 1130–1135.

(21) Usleber, E.; Dade, M.; Schneider, E.; Dietrich, R.; Bauer, J.; Märtlbauer, E. Enzyme immunoassay for mycophenolic acid in milk and cheese. *J. Agric. Food. Chem.* **2008**, *56*, 6857–6862.

(22) Abramson, D.; Usleber, E.; Märtlbauer, E. An indirect enzyme immunoassay for the mycotoxin citrinin. *Appl. Environ. Microbiol.* **1995**, *61*, 2007–2009.

(23) Latif, H.; Curtui, V.; Ackermann, Y.; Gross, M.; Usleber, E. Production and characterization of antibodies against fumigaclavine A. *Mycotoxin Res.* **2009**, *25*, 159–164.

(24) Usleber, E.; Abramson, D.; Gessler, R.; Smith, D. M.; Clear, R. M.; Märtlbauer, E. Natural contamination of Manitoba barley by 3,15-diacetyldeoxynivalenol and its detection by immunochromatography. *Appl. Environ. Microbiol.* **1996**, *62*, 3858–3860.

(25) Abramson, D.; Clear, R. M.; Usleber, E.; Gessler, R.; Nowicki, T. W.; Märtlbauer, E. Fusarium species and 8-keto-trichothecene mycotoxins in Manitoba barley. *Cereal Chem.* **1998**, *75*, 137–141.

(26) German Federal Institute for Risk Assessment. Aluminium in Apfelsaft: Lagerung von Fruchtsäften nicht in Aluminiumtanks. Gesundheitliche Bewertung Nr. 034/2008 des BfR vom 18. Juni 2008. Document available at http://www.bfr.bund.de/cm/343/aluminium_in_apfelsaft_lagerung_von_fruchtsaeften_nicht_in_aluminiumtanks.pdf (last accessed 2011−08−31).

(27) Scott, P. M.; Lawrence, G. A.; Lau, B. P.-Y. Analysis of wines, grape juices and cranberry juices for *Alternaria* toxins. *Mycotoxin Res.* **2006**, *22*, 142–147.

(28) Asam, S.; Konitzer, K.; Rychlik, M. Precise determination of the *Alternaria* mycotoxins alternariol and alternariol monomethyl ether in cereal, fruit and vegetable products using stable isotope dilution assays. *Mycotoxin Res.* **2011**, *27*, 23–28.

(29) European Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Commun.* **2006**, *L364*, 5−24.

(30) Asam, S., Lichtenegger, M.; Liu, Y.; Rychlik, M. Content of the *Alternaria* mycotoxin tenuazonic acid in food commodities determined by a stable isotope dilution assay. *Mycotoxin Res.* (DOI: 10.1007/s12550-011-0111-x).