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ORIGINAL PAPER

Determination of six *Alternaria* toxins with UPLC-MS/MS and their occurrence in tomatoes and tomato products from the Swiss market

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Abstract An ultra performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) method was developed for the determination of the *Alternaria* toxins tenuazonic acid, alternariol, alternariol monomethyl ether, altenuene, altertoxin I and tentoxin. Owing to its instability, altenusin could not be determined. The sample preparation includes an acidic acetonitrile/water/methanol extraction, followed by SPE clean-up step, before injection into the UPLC-MS/MS system. The separation was made on an Acquity UPLC column using a water/acetonitrile gradient with ammonium hydrogen carbonate as a modifier. Matrix compounds of real samples led to enhancement as well as suppression of the target compounds, depending on analyte and matrix. The recoveries were between 58 and 109% at a level of 10 μg/kg. Eighty-five tomato products, consisting of peeled and minced tomatoes, soup and sauces, tomato purées and concentrates, ketchup as well as dried and fresh tomatoes, were taken from the Swiss market in 2010. Tenuazonic acid was found most frequently (81 out of 85 samples) and in the highest levels of up to 790 μg/kg. Alternariol and alternariol monomethyl ether were found in lower concentrations, ranging from <1 to 33 μg/kg for alternariol and <5 to 9 μg/kg for alternariol monomethyl ether. Only a few samples were positive for altenuene and tentoxin. Altertoxin I was never detected.

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Introduction

Alternaria species are ubiquitous in the atmosphere, in soil and in vegetables. Cucumber, eggplants, potatoes and tomatoes are especially susceptible to mould infestation (Battilani et al. 2008). Due to their growth even at low temperatures, they are responsible for food spoilage during refrigerated transport and storage, which results in economic losses (Ostry 2008). Apart from the substrate, mould growth depends on temperature and water activity (a_w value). On a synthetic tomato medium *Alternaria alternata* isolated from tomato fruits grew best at 21°C and at an a_w of 0.982, the water activity being the most important parameter for the mould growth (Pose et al. 2009).

Several different toxins are produced by *A. alternata*, the most important are alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altenusin (ATS), altertoxins I-III (ATX-I-III), tentoxin (TEN) and tenuazonic acid (TeA) (Bottalico and Logrieco 1998).

Tomatoes and many other soft-skinned vegetables and fruits can easily be infected by fungi. *Alternaria* spp. have been reported to be the most common fungi infecting tomatoes. Under optimal growth conditions, moulds on tomatoes can produce *Alternaria* toxins. AOH, AME and TeA were found at different temperatures, water activities and pH conditions on agars and tomatoes (Pose et al. 2004, 2010; Graf and Geisen 2010).

In 11 of 19 naturally infected tomatoes, TeA was determined in concentrations of up to 13.9 mg/kg (Stinson et al. 1981). AOH, AME and ALT were found at much lower levels than TeA and ATX-I was absent. Another study

reports high TeA concentrations in fresh but visibly mouldy tomatoes used for ketchup production in California and in midwestern and eastern states, ranging from 0.4 to 69.7 mg/kg (Mislivec et al. 1987). The authors found that 73 out of 146 samples contained TeA; however, *Alternaria* spp. were not found in 35 of the 73 positive samples. Tomato and tomato products from the Brazilian market were checked for TeA, AOH, AME and cyclopiazonic acid. Mycotoxins were found in juices, but seven out of 22 pulp samples contained TeA up to 11 μg/kg. In four out of 22 purée samples TeA was found in concentrations of up to 76 μg/kg (da Motta and Valente Soares 2001). Thirty-nine out of 80 samples of Argentinian tomato purée contained one or two of the mycotoxins TeA, AOH or AME (Terminiello et al. 2006). Two official food control laboratories in Germany found TeA in concentrations of up to 520 μg/kg and AOH up to 13 μg/kg in tomatoes and tomato products (Chemisches und Veterinäruntersuchungsamt Sigmaringen and Umweltschutz 2005, 2006; Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit LAVES 2007). In commercial products from Germany, TeA was found using ELISA in four out of 15 tomato juices in the range of 20–200 ng/ml and two of 18 tomato ketchups contained TeA with 55 and 67 ng/ml respectively (Gross et al. 2010).

Alternaria toxins were reported in different foods: mainly TeA in tomatoes and tomato products (Bottalico and Logrieco 1998). An overview article summarized qualitatively TeA, AOH, AME and ALT findings in tomatoes; TeA was reported in every cited work (Scott 2001). Scientific information provided to the European Food Safety Authority (EFSA) gives an overview on the published papers of *Alternaria* toxins in several commodities, including tomatoes and tomato products. TeA and AOH were most frequently analysed and were most often found; AME was not so frequently detected and occurred in lower concentrations (Battilani et al. 2008).

With the exception of TeA, the *Alternaria* toxins are very weak acute toxins. However, AOH and AME are teratogenic and fetotoxic and ATX-I is cytotoxic and mutagenic (Weidenbörner 2001). TeA and AME cause precancerous changes in the esophageal mucosa when fed to mice (Yekeler et al. 2001). AOH and AME also seem to be mutagenic and act as antagonists to topoisomerase; furthermore, AME provokes DNA strand breaks (Fehr et al. 2008, 2009; Boettler et al. 2009; Bächler et al. 2010). AOH can be very rapidly absorbed by the human intestinal lumen (Burkhardt et al. 2009).

There are only a few papers dealing with the simultaneous determination of six *Alternaria* toxins (TeA, AOH, AME, ALT, ATX-I, TEN) with liquid chromatography (LC)-tandem mass spectrometry (MS/MS) in food items. A multi-method for *Alternaria* toxins in edible oil and other food including tomato products has been presented (Kocher 2007). Other multi-methods are able to determine *Alternaria* toxins, but determination limits are rather high (Vishwanath et al. 2009). A method including five *Alternaria* toxins consists of a clean-up step with Bond Elut Plexa SPE-cartridges and LC-MS/MS detection (Rheinhold and Bartels 2007).

Here we report our newly developed rapid ultra performance (UP) LC-MS/MS method, which allows the simultaneous determination of six *Alternaria* toxins, and its application for a market survey of tomato produce in northwestern Switzerland.

Samples from the market

Eighty-five tomato samples were taken from the Swiss market in the autumn and winter of 2010. Samples consisted of 13 peeled and minced tomatoes, 24 soups and sauces, 17 purées and concentrates, 19 ketchups, 8 dried tomatoes, and 4 fresh tomatoes. All samples were stored as prescribed on the package or, in case of missing indications, room temperature. Fresh tomatoes were analysed within 2 days after picking.

The sample size was one food pack or at least 200 g tomatoes or tomato product.

Material and methods

Chemicals and materials

For all experiments, nanopure water (H_2O) provided by a Nanopure-Easypure-LF-system (Skan, Basel Switzerland) was used. Acetonitrile (MeCN, gradient grade), methanol (MeOH, gradient grade), formic acid (LC-MS quality), *o*-phosphoric acid (85%), ammonium hydrogen carbonate (LC-MS quality), sodium dihydrogenphosphate xH_2O (p.a.) were purchased from Sigma-Aldrich. The SPE-cartridges Bond Elut Plexa 500 mg were obtained from Varian. AOH, AME, TEN, ALT and TeA-copper salt were obtained from Sigma-Aldrich. A solution of 100 μg/ml ATX-I was kindly provided by Dr. M. Sulyok (IFA Tulln). ATS was purchased from Alexis Biochemie (Enzo Life Sciences, Lausen, Switzerland). All reference substances were on analytical quality and had a certificate.

Toxin stock solutions were prepared for each toxin by dissolving in MeCN, resulting in a concentration of 100 μg/ml and injected into the UPLC-MS/MS and checked for purity. Only AOH showed a small peak of about 5% of the area of the main peak. All other standards showed no other peaks. A standard mixture containing 1.0 μg/ml of all toxins was made with aliquots of the stock solutions and by

diluting with MeCN. The calibration solutions were prepared by diluting the 1.0 μg/ml mixture with 30% MeCN in water containing 0.1% formic acid.

UPLC-MS/MS

UPLC analysis was performed using a Waters Acquity-System consisting of a binary pump, autosampler, column oven and a photodiode array-detector. The separation of the toxins was performed using a 100 mm×2.1 mm i.d., 1.8 μm, Acquity UPLC HSS T3 column (Waters, Baden-Dättwil, Switzerland). The column temperature was set at 30°C. Solvent A was made by dissolving 40 mg ammonium hydrogen carbonate in 237.5 ml water and then adding 12.5 ml MeCN, resulting in 250 ml solvent A with a modifier concentration of 2 mM. Solvent B was made by dissolving 40 mg ammonium hydrogen carbonate in 12.5 ml water and then adding 237.5 ml MeCN, resulting in 250 ml solvent B with 2 mM NH_4HCO_3. A binary gradient at a flow rate of 0.5 ml/min was programmed as follows: 0–3 min isocratic 0% B, followed by a linear gradient to 100% B, ending at 10.5 min and from 10.5 to 11.0 min isocratic 100% B.

The retention times of the toxins were TeA 1.3 min, AOH 4.0 min, ALT 4.4 min, ATX-I 5.1 min, TEN 5.3 min and AME 6.5 min (Fig. 1). ATS has a retention time of 3.5 min and it is not shown in Fig. 1.

MS/MS analysis was performed on a triple quadrupole mass spectrometer "Xevo" (Waters, Baden-Dättwil, Switzerland) equipped with an electrospray ionization (ESI) source (Waters, Baden-Dättwil, Switzerland) heated at 150°C. The desolvation temperature was set at 500°C and the desolvation gas flow was 700 l/h nitrogen. Argon was used as a collision gas at a flow rate of 0.10 ml/min. Quantification was performed using multiple reaction monitoring (MRM) using the TargetLynx software (Waters, Baden-Dättwil, Switzerland). The following transition reactions of AOH, AME, ATS, ATX-I, ALT, TEN and TeA with the respective dwell times (DW), cone voltages (CV), collision voltages (CE) were recorded using the first mass transition for quantification: AOH: ESI negative m/z 256.96→147.03 (DW 0.055 s, CV 42 V, CE 34 V), ESI positive m/z 159.00→185.00 (DW 0.055 s, CV 40 V, CE 30 V). AME: ESI negative m/z 271.04→255.94 (DW 0.060 s, CV 36 V, CE 22 V), ESI positive m/z 273.04→128.06 (DW 0.060 s, CV 44 V, CE 42 V). ATS: ESI negative m/z 286.95→227.93 (DW 0.055 s, CV 38 V, CE 20 V), m/z 287.07→243.00 (DW 0.055 s, CV 38 V, CE 14 V). ATX-I: ESI negative m/z 351.06→314.94 (DW 0.055 s, CV 20 V, CE 20 V). ALT: ESI negative m/z 291.00→229.00 (DW 0.086 s, CV 26 V, CE 12 V), ESI positive m/z 293.10→257.00 (DW 0.086 s, CV 18 V, CE 16 V). TEN: ESI positive m/z 415.19→171.09 (DW 0.055 s, CV 18 V, CE 20 V), m/z 415.19→199.11 (DW 0.055, CV 30 V, CE 14 V). TeA: ESI positive m/z 198.10→125.05 (DW 0.055 s, CV 29 V, CE 18 V), m/z 198.10→153.10 (DW 0.055 CV 29 V, CE 14 V).

Sample preparation

Based on the methods described by Kocher (2007) and Rheinhold and Bartels (2007), the following preparation was used.

All samples were first homogenised with a Retsch Grindomix GM 200 (Schieritz & Hauenstein, Arlesheim, Switzerland).

An aliquot of 2.5 g sample was weighed in a 50-ml centrifuge tube and 30 ml extraction mixture (MeCN/H_2O/MeOH, 45/45/10, v/v/v adjusted to pH 3 with *o*-phosphoric acid) was added. The mixture was blended by a Polytron at 10,000 rpm for 4 min. The pH was then adjusted to 3 with concentrated *o*-phosphoric acid. After centrifugation for 5 min at 4,000 rpm, 15 ml of the supernatant was diluted with phosphate buffer (0.05 M sodium dihydrogen phosphate adjusted to pH=3) to 50 ml and shaken for 1 min. Twenty millilitres of the diluted sample extract was passed through a conditioned Bond Elut Plexa SPE cartridge (conditioning of the SPE cartridges was made first with 5 ml MeOH, followed by 5 ml water). The cartridge was washed with 5 ml water, followed by air drying on the manifold. Elution of the *Alternaria* toxins was carried out with 5 ml MeOH and 5 ml MeCN sequentially. After evaporating to dryness, the cleaned-up extract was dissolved in 500 μl water/MeCN (3/7, v/v) containing 0.1% formic acid and then injected into the UPLC-MS/MS system.

Recovery rates were determined by adding 125 μl of the standard mixture to every sample before the extraction amounting to 50 μg/kg of each toxin. Every result was corrected by its recovery and analysis of samples containing over 100 μg/kg TeA was repeated at least twice and diluted tenfold.

Results and discussion

Altenusin (ATS)

Altenusin was not stable in the standard mixture containing 1.0 μg/ml of each toxin. Comparison of calibration solutions of varying concentrations showed that after 3 days storage at 4°C, ATS contents decreased to 50% of the original concentration. Furthermore, ATS was never found in spiked samples. According to M. Sulyok, IFA Tulln (personal communication), we concluded that ATS is not stable in solution and when using the suggested clean-up step.

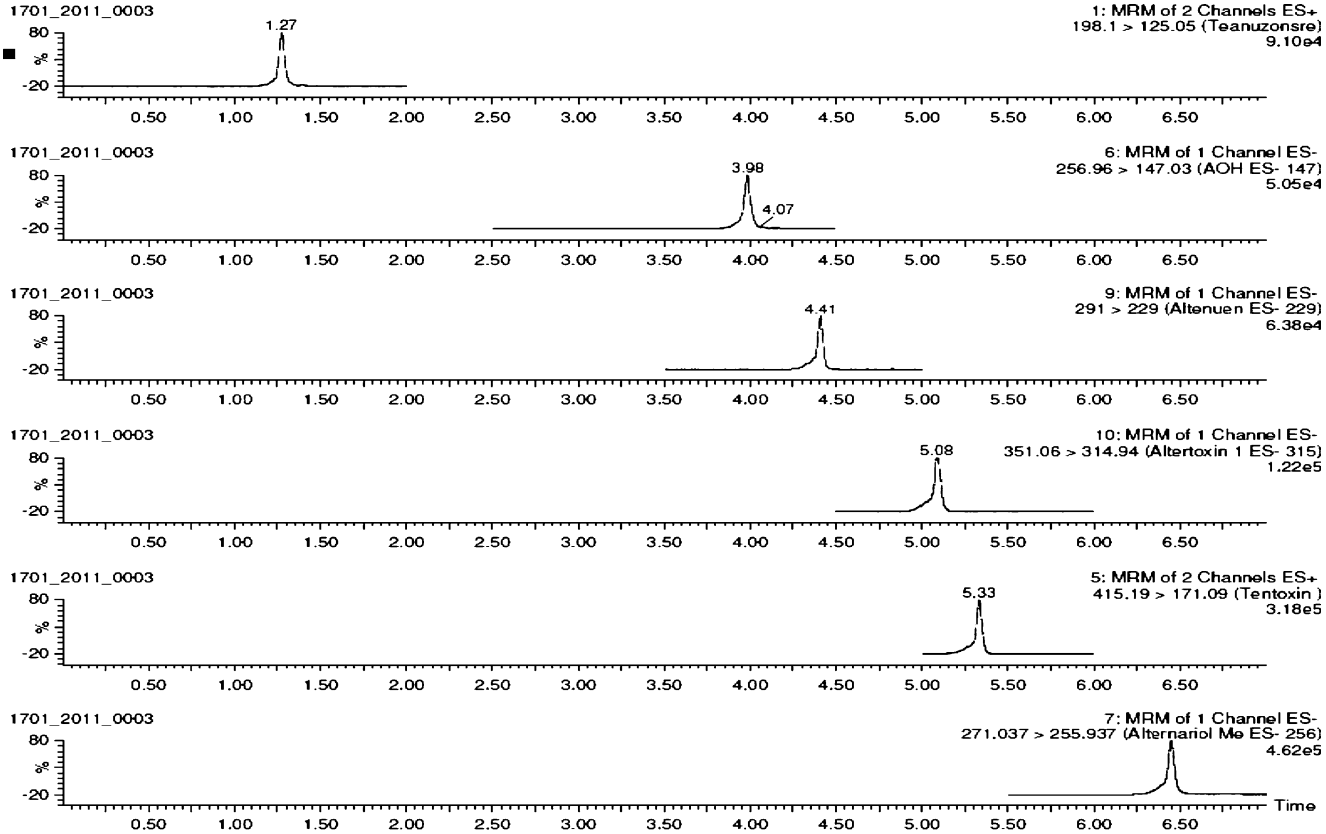

Fig. 1 Chromatograms of a calibration solution containing 50 ng/ml of each toxin with its quantitation transition. The chromatograms are tenuazonic acid (*top*), alternariol (*second*), altenuene (*third*), altertoxin I (*fourth*), tentoxin (*fifth*) and alternariol monomethyl ether. Not shown is altenusin. On the *right side* are the transitions and maximum total ion current are labelled

UPLC, linearity and recoveries

The pH of the eluents and using methanol instead of acetonitril can cause in two peaks for TeA and peak splitting for AOH and ALT. Ammounim hydrogen carbonate as modifier has given the best peak shapes and no splitting.

Calibration solutions between 2.5 ng/ml and 100 ng/ml were with coefficients of correlations better than 0.99 linear.

The overall recovery rates were depending on the sample matrices. For AOH the recovery rates were below 50%, for ATX-I and AME the recovery rates were partly below 50% and the other toxins gave rates above 50%. In Table 1 are the averages of the overall recoveries and their standard deviations of all samples sorted in matrix classes shown. More concentrated tomatoes like dried tomatoes or tomato concentrates tends to result in lower recovery rates than fresh or minced vegetables.

Matrix effects, recoveries of the clean-up step and detection limits

For the observation of matrix effects on the MS signal, the dried sample residue was dissolved in 500 µl standard mixture containing 50 µg/ml of each toxin. Table 2 shows

Table 1 Average of the overall recovery rates and their standard deviations of different tomato matrices. Every matrix group contains all associated samples and their repetitions. Addition was 50 µg/kg and data were not corrected by any matrix effects

Matrix	*n*	AOH (%)	AME (%)	ALT (%)	ATX-I (%)	TEN (%)	TeA (%)
Ketchup	22	36±11	77±17	62±21	46±7	84±18	94±28
Dried tomatoes	8	21±3	45±12	56±10	32±6	62±11	58±16
Fresh and whole tomatoes	3	29±2	87±6	60±4	61±10	87±6	98±11
Tomato puree and concentrates	20	28±8	62±12	52±12	56±10	58±11	61±25
Tomatoes peeled, minced	15	37±8	73±15	63±7	54±6	91±13	100±17
Tomato sauces, tomato soup	26	35±7	74±23	66±7	50±7	79±18	84±26

Table 2 Enhancement or suppression of different matrices on the MS signal of the *Alternaria* toxins (n=2). An enhancement gives a factor >1.0 and suppression gives a factor <1.0. There was always one sample for one matrix used

Matrix	AOH	AME	ALT	ATX-I	TEN	TeA
Ketchup	0.6	1.0	0.8	0.7	1.4	1.0
Tomatoes	0.3	1.0	0.8	0.7	0.7	1.6
Tomato puree	0.5	1.3	0.9	0.5	0.9	1.4
Apple juice	0.7	1.0	0.8	0.7	1.4	0.7

the factors for signal enhancement or suppression. The experiment was done with ketchup, tomatoes, purée and apple juice. AOH signals were severely suppressed.

The recovery experiments (Table 3) were done by spiking a sample aliquot before extraction, giving a matrix spiked addition with 10 μg/kg ketchup, purée and apple juice and for tomatoes. Aliquots from the same specimens were used to determine matrix effects. For establishing recovery rates, the results were corrected by the enhancement or suppression factor. This gives the recovery of the clean-up step. With the exception of AME in ketchup and tomatoes and TEN in tomatoes, the recovery rates were above 70% (Table 3).

The matrix effect can compensate for a recovery of less than 100% in the case of TEN in ketchup and in apple juice. Therefore it is necessary to know both individual matrix effects and the recovery rate of every compound. For AOH, recovery rates of about 80% were found, for AME 56–77%, for ALT 85–95%, for ATX I 80–93% and for TEN 56–89%. All analyses of one matrix class were done in 1 day, resulting in the intraday precision with standard deviations of <10% for all toxins. Recovery experiments of the clean-up step with standard solutions gave recovery rates of between 90 and 100%.

The detection limits (LOD) were calculated from the addition of 10 μg/kg to the different matrices. On this experiments the following LODs were estimated based on a signal/noise ratio of 4–5 and an injection volume of 10 μl: 2 μg/kg for TeA, 4 μg/kg for AOH, 1 μg/kg for AME, 2 μg/kg for ALT, 2 μg/kg for ATX-I, and 2 μg/kg for TEN.

Samples containing TeA were repeated on different days. The interday precision for a tomato mark sample containing 790 μg/kg TeA had a standard deviation of 75 μg/kg, for 30 μg/kg AOH a standard deviation of 3 μg/kg (n=3) and for AME 8 μg/kg±1 μg/kg, TEN was at detection limit of 2 μg/kg. A tomato sauce with 143 μg/kg TeA showed a standard deviation of 5 μg/kg, AOH and AME were at detection limits (n=3). And a sample of peeled, canned tomatoes with 200 μg/kg TeA had a standard deviation of 14 μg/kg, AOH and AME were at the detection limits (n=3).

Tomatoes and tomato products

TeA was detected in 81 out of 85 samples. The highest level was determined in a tomato purée sample containing 790 μg/kg TeA besides 30 μg/kg AOH, 2 μg/kg TEN and 8 μg/kg AME. Two other concentrates also contained high levels of TeA (610 μg/kg and 590 μg/kg). Both samples contained also AOH, AME and TEN. One third of the purées and concentrates contained over 100 μg/kg of TeA (Table 4). Due to the big difference between the mean and median values, tomato purées and tomato concentrates did not show a Gauss distribution of the samples.

In half of the peeled and minced tomatoes TeA levels were above 100 μg/kg. One sample of canned peeled tomatoes contained 200 μg/kg of TeA. Three sauces and one ketchup showed TeA levels over 100 μg/kg. Fresh tomatoes were the only samples where no TeA was detected. A sample of canned cherry tomatoes in tomato sauce had 37 μg/kg TeA.

Considering that tomatoes were concentrated two to three times for making tomato purée or concentrates, the relatively high levels of the purées and concentrates can be explained when compared with the peeled and minced tomatoes.

TeA was the major *Alternaria* toxin detected in this study. Much lower levels of AOH, AME, ALT and TEN were detected in the following order AOH > AME > ALT = TEN. The highest concentration of AOH was 33 μg/kg in a tomato concentrate and in the same sample a maximum level of AME was 9 μg/kg. AOH was detected in 27

Table 3 Recoveries and their standard deviation of the *Alternaria* toxins in different matrices. The enhancement or suppression was accounted for the recovery calculation. The same samples as for the matrix effects were used resulting in one sample per matrix group. Analysis of one matrix group was done within 1 day, resulting in the intraday precision

Matrix	n	Added level (μg/kg)	AOH (%)	AME (%)	ALT (%)	ATX-I (%)	TEN (%)	TeA (%)
Ketchup	6	10	85±3	58±5	89±4	86±3	89±6	106±5
Tomatoes	6	20	75±2	56±3	79±4	81±3	56±1	85±9
Tomato puree	6	10	82±5	73±5	85±3	80±3	87±4	94±8
Apple juice	2	10	85±1	73±1	95±1	93±1	82±1	109±2

Table 4 Overview of TeA in tomatoes and tomato products

Sample group	*n*	Positive	TeA range (μg/kg)	TeA mean (μg/kg)	TeA median (μg/kg)
Ketchup	19	19	3–141	37	31
Dried tomatoes	8	7	n.d.–166	52	45
Fresh and whole tomatoes	4	1	n.d.–37	9	0
Tomato puree, tomato concentrates	17	17	2–790	165	46
Tomatoes peeled, minced	13	13	25–200	81	74
Tomato sauces, tomato soup	24	24	4–144	40	35

n.d. not detectable, <2 μg/kg

samples, AME in 26, TEN in nine samples and ALT in two samples (Table 5). No ATX-I could be detected.

With the exception of three samples, AME and AOH were detected together. In these three exceptions, two samples contained ALT at 2 μg/kg and in one sample TEN was present at 2 μg/kg. Neither correlation was found between concentrations of TeA and AOH nor between concentrations of AOH and AME. However, the following observations were made: a level of AOH over 10 μg/kg was always in the presence of AME and levels of TeA higher than 150 μg/kg was always correlated with the presence of AOH in the sample.

In a laboratory test, five whole tomatoes were allowed to spoil during 3 weeks. The specimens were put in a plastic beaker closed with a lid, sprayed twice a week with water and stored at room temperature. After this time, two of the mouldiest tomatoes were pooled for toxin determination. They contained 53,000 μg/kg TeA, 175 μg/kg AOH, 40 μg/kg AME, 290 μg/kg TEN and 15 μg/kg ATX-I but no ALT. These findings correspond with the results of the market samples TeA >> AOH > AME, with the exception of TEN. This experiment confirms that TeA is the main toxin produced by fungi on tomatoes, followed by AOH and AME at much lower levels.

The findings of this study are not consistent with the data published from a survey of the Argentinian market, where 39 tomato pulp samples were analysed for TeA, AOH and AME (Terminiello et al. 2006). In contrast to our study, only about 60% of the tomato pulp samples contained TeA; however, concentrations were up to 4,000 μg/kg. AME was detected as frequently as TeA but at slightly lower concentrations of up to 1,700 μg/kg. AOH was less frequently found than TeA and AME.

In 60% of tomato pulp samples from the Brazilian market, TeA was detected up to a concentration of 111 μg/kg. The authors did not analyse the samples for other *Alternaria* toxins (da Motta and Valente Soares 2001).

EFSA gave an overview, citing authors who have analysed AOH and AME. They found these toxins at the same levels as we have (Battilani et al. 2008). Our findings are consistent with the results from the German food authorities (Chemisches und Veterinäruntersuchungsamt Sigmaringen and Umweltschutz 2005, 2006); nearly all tomato concentrates contained *Alternaria* toxins. TeA was also found at up to 520 μg/kg, whereas AOH and AME were at lower levels. In a recent study in Germany, AOH, AME and TeA were analysed in 15 tomato samples from the local market and TeA was also the main toxin in these samples. This paper confirms our findings (Asam et al. 2011).

The suggested sample preparation and the UPLC-MS/MS analysis were successfully applied in a market survey. Determination limits could be further decreased if necessary, e.g. for analysing apple juice by decreasing the volume of the cleaned-up extract solution. Our study shows that TeA is prevalent in tomato products and can occur in high amounts. More studies are therefore needed on the

Table 5 AOH, AME, ALT and TEN in tomato samples

Sample group	*n*	AOH		AME		ALT		TEN	
		Positive	Range (μg/kg)	Positive	Range (μg/kg)	Positive	Range (μg/kg)	Positive	Range (μg/kg)
Ketchup	19	3	4–5	3	1	0		0	
Dried tomatoes	8	1	4	3	2–7	2	2	2	2
Fresh and whole tomatoes	4	0		0		0		0	
Tomato puree, concentrates	17	11	4–33	12	1–9	0		6	1–3
Tomatoes peeled, minced	13	3	4–7	1	1	0		1	2
Tomato sauces, tomato soup	24	8	4–10	7	1–4	0		0	

occurrence of the *Alternaria* toxins in food items and its toxicological implications.

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