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ORIGINAL ARTICLE

Survey of *Alternaria* toxin contamination in food from the German market, using a rapid HPLC-MS/MS approach

Sebastian Hickert[1,2] · **Marian Bergmann**[1] · **Seyma Ersen**[1] · **Benedikt Cramer**[1] · **Hans-Ulrich Humpf**[1,2]

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Abstract A HPLC-MS/MS-based method for the quantification of nine mycotoxins produced by fungi of the genus *Alternaria* in various food matrices was developed. The method relies on a single-step extraction, followed by dilution of the raw extract and direct analysis. In combination with an analysis time per sample of 12 min, the sample preparation is cost-effective and easy to handle. The method covers alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), altenuene (ALT), iso-altenuene (isoALT), tentoxin (TEN), altertoxin-I (ATX-I), and the AAL toxins TA_1 and TA_2. Some *Alternaria* toxins which are either not commercially available or very expensive, namely AOH, AME, ALT, isoALT, and ATX-I, were isolated as reference compounds from fungal cultures. The method was extensively validated for tomato products, bakery products, sunflower seeds, fruit juices, and vegetable oils. AOH, AME, TeA, and TEN were found in quantifiable amounts and 92.1 % of all analyzed samples (n=96) showed low level contamination with one or more *Alternaria* toxins. Based on the obtained results, the average daily exposure to *Alternaria* toxins in Germany was calculated.

Keywords Mycotoxin · *Alternaria* · Mass spectrometry · Isolation · Liquid chromatography · Multi method · Exposure

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✉ Hans-Ulrich Humpf
humpf@wwu.de

[1] Institute of Food Chemistry, Westfälische Wilhelms-Universität Münster, Corrensstraße 45, 48149 Münster, Germany

[2] NRW Graduate School of Chemistry, Wilhelm-Klemm-Str. 10, 48149 Münster, Germany

Introduction

Fungi of the genus *Alternaria* occur worldwide and infect various foodstuffs (e.g., tomato (Hasan 1995), potato (van der Waals et al. 2004), grain (Kosiak et al. 2004), citrus fruits (Akimitsu et al. 2003), apple (Rang et al. 2002), and sunflower seeds (Carson 1985). Besides, they are known to infect decaying material such as rotting wood (Yang 2005) or building materials (Gravesen et al. 1999). *Alternaria* fungi produce a variety of about 70 toxic secondary metabolites (Loggrieco et al. 2009; EFSA 2011a). The toxicological properties have been recently reviewed by Ostry and the European Food Safety Authority (EFSA) (Ostry 2008; EFSA 2011a). Tenuazonic acid (TeA) has acute toxic effects on various mammals. The LD_{50} values described are, e.g., 81 mg/kg b.w. (female mice), 186 mg/kg b.w. (male mice), or 168 mg/kg b.w. (female rats) (Pero et al. 1973). Alternariol (AOH) and alternariol monomethyl ether (AME) show no strong acute toxic effects but have been shown to be mutagenic in cell culture assays (Brugger et al. 2006; An et al. 1989) and lead to DNA double strand breaks (Pfeiffer et al. 2007). The altertoxins (ATX) are more potent mutagens than AOH and AME and cause DNA strand breaks. ATX-II is described as the most potent substance among the altertoxins (Fleck et al. 2012; Stack et al. 1986). AAL toxins exhibit mostly phytotoxic effects (Abbas et al. 1993) but have been shown to disrupt the sphingolipid metabolism in a similar way as fumonisins (Abbas et al. 1994) which have been correlated with esophageal cancer and animal diseases (D´Mello et al. 1999; Stockmann-Juvala and Savolainen 2008). Altenuene (ALT) shows the highest acute toxicity among the toxins covered by this study with a LD_{50} value of 50 mg/kg b.w. (mice) (Pero et al. 1973).

There are various methods for the quantification of single *Alternaria* toxins (or groups of related toxins) relying on solid phase extraction (SPE) or the QuEChERS ("Quick, Easy,

Cheap, Effective, Rugged and Safe") system (Delgado and Gómez-Cordovés 1998; Lau et al. 2003; Lohrey et al. 2013) and HPLC-separation with UV or MS detection. The applications of gas chromatography (Scott et al. 2006) for the determination of *Alternaria* toxins are limited due to the laborious derivatization reactions needed. There are up-to-date two methods described in literature for the quantification of AOH, AME, TeA, ALT, ATX-I, and TEN simultaneously (Noser et al. 2011; Walravens et al. 2014). However, most methods fail to cover all toxins of interest as they are limited to a narrow range of polarity. To the best of our knowledge, the method presented here is the first to cover AOH, AME, ALT, isoALT, ATX-I, AAL TA_1, and TA_2, TeA and tentoxin (TEN) (see Fig. 1 for structures). Recently, many multi-mycotoxin methods relying on a dilute-and-shoot approach without time-consuming sample preparation have been developed (Hickert et al. 2015; Malachová et al. 2014; Sulyok et al. 2007, 2010). Some of these methods cover up to seven out of nine toxins analyzed in this study (Malachová et al. 2014) but are mostly not validated for the matrices analyzed here. These methods accept a slight decrease in sensitivity (due to dilution of the raw extract and matrix effects) and cope with it by using high sensitive mass spectrometers. Such a dilute-and-shoot approach has been chosen for this study as well. AOH, AME, TeA, and TEN have been shown to occur in food samples frequently (Liu and Rychlik 2013; Ackermann et al. 2011; Lohrey et al. 2013), while the occurrence of ALT, isoALT, ATX-I, and AAL toxins is of much lower incidence. The presence of AAL toxins has even not been reported in food samples up to date. Due to their structural and toxicological similarity to the well-studied fumonisins, they are nevertheless included in this study. ALT has been found in linseed in low levels in the Czech Republic by Králová et al. (2006) and in apple juice, tomato products and beers by Prelle et al. (2013). The presence of isoALT has not been reported to the best of our knowledge. ATX-I was recently reported in low levels by Liu et al. in grain products (Liu and Rychlik 2015) and in feed by Streit et al. (2013). The EFSA reviewed the occurrence and toxicology of several *Alternaria* toxins and concluded that there is a need for additional occurrence data (EFSA 2011a). The method presented here provides an easy to handle tool to provide these data.

Materials and methods

Chemicals and reagents

All solvents used were of gradient grade and if not stated otherwise purchased from VWR (Darmstadt, Germany). Water was purified with a Milli-Q Gradient A10 system from Millipore (Schwalbach, Germany). TEN and AAL toxin (mixture of isomers TA_1 and TA_2) were purchased from Sigma Aldrich (Steinheim, Germany). AOH, AME, ATX-I, ALT, and isoALT were isolated from fungal cultures as described below. TeA was synthesized according to Lohrey et al. (see Supplementary Material for NMR data) (Lohrey et al. 2013).

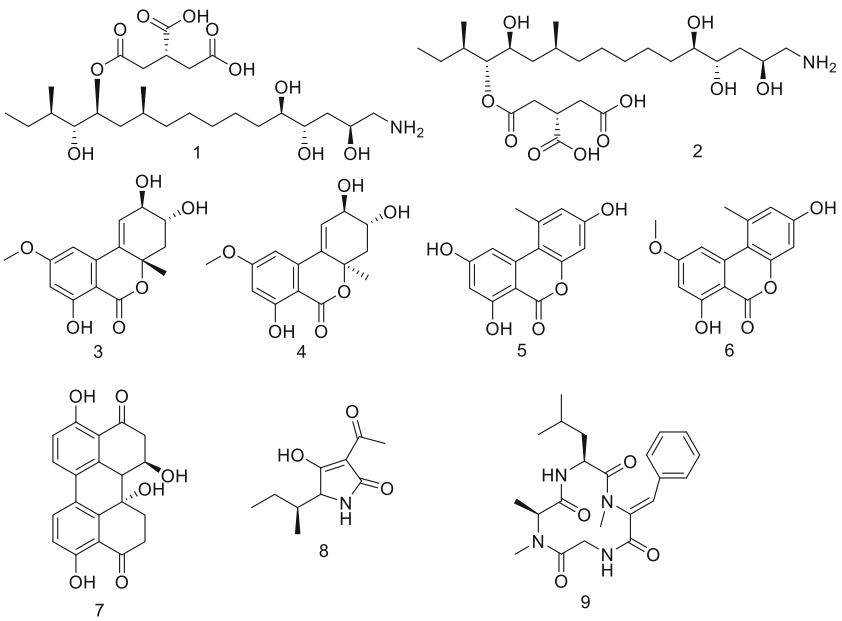

Fig. 1 Structures of the analyzed toxins. *1*: AAL toxin TA_1, *2*: AAL toxin TA_2, *3*: iso-altenuene (isoALT), *4*: altenuene (ALT), *5*: alternariol (AOH), *6*: alternariol monomethyl ether (AME), *7*: altertoxin I (ATX-I), *8*: tenuazonic acid (TeA), *9*: tentoxin (TEN)

Isolation of reference compounds from fungal cultures

AOH, AME, ALT, isoALT, and ATX-I were isolated as reference compounds from fungal cultures of *Alternaria alternata*. A detailed description of the isolation procedure as well as spectroscopic data can be found in the Supplemental Material.

Preparation of standard solutions

Commercially bought mycotoxin standards (purity >95 %) were dissolved in acetonitrile (ACN) to obtain stock solutions of 200 μg/mL. Standard solutions of ALT and isoALT were prepared by dissolving solid substance in ACN. The correct concentrations were determined by UV spectroscopy (DU 800 Beckman Coulter GmbH, Krefeld, Germany) using the molar absorptivity value of $\varepsilon = 1.00 \times 10^4$ L × $mol^{-1} \times cm^{-1}$ at 278 nm in ethanol (EtOH) described by Pero et al. (1971). Standard solutions of 394 μg/mL (ALT) and 158 μg/mL (isoALT) were prepared in ACN. Stock solutions of AOH (92.0 μg/mL) and AME (103 μg/mL) in ACN were prepared using the molar absorptivity values (AOH: 4.06×10^4 L × $mol^{-1} \times cm^{-1}$ at 256 nm in ACN, AME: 4.76×10^4 L × $mol^{-1} \times cm^{-1}$ at 256 nm in ACN) described by Asam et al. (2009). The stock solution of ATX-I (53.6 μg/mL) was obtained using the molar absorptivity value (3.46×10^4 L × $mol^{-1} \times cm^{-1}$ at 256 nm in ACN) given by Stack et al. (1986). A stock solution of 10.8 μg/mL TeA was prepared using the molar absorptivity value of 4.13×10^4 L × $mol^{-1} \times cm^{-1}$ at 277 nm in EtOH given by Stickings (Stickings 1959). By mixing, all toxin solutions were combined in one working solution in ACN. The resulting values are given in Table 1. The working solution was stored at −20 °C in the dark until further use. No decomposition of the working solution could be observed over a period of 1 month.

Calibration

Matrix matched calibration was used to quantify the compounds of interest. Calibration sets for tomato puree, whole grain bread, apple juice, ground sunflower seeds and sunflower seed oil were prepared with food samples containing none of the analyzed toxins above the limit of detection. As no tomato puree without contamination of TeA, AOH, and AME could be found, a puree containing only traces (below LOQ) of these substances was used for the calibration, and all calculations were performed regarding the natural contamination of the matrix. The matrix samples were extracted according to the extraction procedure for the samples mentioned below. The stock solution (Table 1) was evaporated under a stream of nitrogen and resuspended with this matrix solution to obtain eight calibration points within the working range. The exact concentrations can be found in the Supplementary Material (Tables S2 and S3). For tomato products, only seven out of eight calibration points for AAL TA_1, ALT, and isoALT were used because the lowest calibration point lead to S/N values <10 in the matrix matched calibration. The same applies for AAL TA_1 in sunflower seeds and vegetable oils as well as AAL TA_1 and TA_2 in bakery products.

Method performance

The estimation of the limits of detection (LODs) and limits of quantification (LOQs) was made by dilution of the working solution in a blank matrix extract to the respective signal-to-noise ratio (S/N) of approximately 3 for the LOD and S/N >10 for the LOQ. Recoveries were determined by spiking 1 g of blank sample at three different levels in duplicate with the stock solution. It is common to use less sample for spiking experiments than for food samples to lower the amount of standard substances used (Malachová et al. 2014; Sulyok et al. 2007). Levels close to the 2nd, 4th, and 6th calibration points were chosen. This was achieved by spiking the material

Table 1 Concentrations of the analyzed toxins in the stock solution [μg/mL] and the working ranges

Toxin	Concentration in stock solution [μg/mL]	Working range[a] [μg/L]	Working range[b] [μg/kg]
AAL TA_1	5.00	2.5–250	12.5–1250
AAL TA_2	5.00	2.5–250	12.5–1250
isoALT	15.8	8.0–800	40–4000
ALT	15.8	8.0–800	39–3900
AOH	10.1	5.0–500	25–2500
AME	1.03	0.5–50	2.6–260
ATX-I	40.0	20–2000	100–10,000
TeA	53.9	27–2700	135–13,500
TEN	20.0	10–1000	50–5000

[a] The working range applies for juices

[b] The working ranges apply for tomato products, bakery products, vegetable oils, and sunflower seeds

with 10, 50, and 150 μL of the working solution (see Table 1). For juices (as there is no extraction step), the working solution was diluted fivefold with water before spiking. The exact spiking levels can be found in the Supplementary Material (Tables S2 and S3). The matrices were thoroughly homogenized with the spiking solution, and the solvent was evaporated at room temperature overnight. Juice samples were left open in the refrigerator at 7 °C overnight. As no toxin-negative tomato sample could be obtained, a tomato puree with all toxins below the LOQ was chosen. In this case, the peak area of a spiked sample was corrected by subtracting the peak area obtained for the same sample without spiking. The recovery samples were extracted analogue to the samples (with 4 mL instead of 20 mL extraction solvent). Intraday performance was assessed by extracting and measuring one spiked sample (close to the 6th calibration point) per matrix ten times on the same day. Interday repeatability was assessed by working up three spiked samples at three different concentrations (close to the 2nd, 4th, and 6th calibration point) per matrix separately on three different days in duplicate. The method performance characteristics for all substances, and all matrices in detail can be found in Table S1 in the Supplementary Material. A summary of the method performance characteristics is given in Table 2. The LOD and LOQ vary, depending on the food matrix investigated. The values for each matrix can be found in Table S1 in the Supplementary Material; in Table 2, the range covering the lowest and highest value is given. The recovery values represent averages over all three spiking levels; detailed information can be found in the Supplementary Material.

Samples

Tomato products, fruit and vegetable juices, bakery products, vegetable oils, and sunflower seeds were purchased from German retail stores in the area of the cities Münster and Arnsberg, respectively. The samples were stored at ambient temperature or refrigerated at 7 °C according to the food label instructions; extraction of *Alternaria* toxins was performed within 2 weeks. Fully worked up sample extracts were stored at −20 °C in the dark before analysis. Approximately 40 g of sample material for each sample were stored at −20 °C in polypropylene tubes as retain samples.

Sample preparation

Inhomogeneous tomato, sunflower seed, and bakery product samples were homogenized using a blender (BL 900, Kenwood Electronics, Bad Vilbel, Germany). Five grams (±0.05 g) of ground samples was weighted in a 40-mL polypropylene tube. Twenty milliliters of the extraction solvent (ACN/H_2O/formic acid (FA), 49+50+1, *v*/*v*/*v*) was added, and the samples were extracted for 1 h at 150 rpm on a laboratory shaker. Afterwards, they were centrifuged for 3 min at 3541×*g* (Napco 2019 R, Spectrum Chemical MFG Corp, New Jersey, USA), and 200 μL of the supernatant was added to 800 μL of water. As the diluted solutions sometimes clouded if stored overnight at 7 °C, they were filtered through a syringe filter (rectified cellulose, 0.45 μm, Phenomenex, Aschaffenburg, Germany) to avoid clogging of the HPLC-column. Juices (5.0 mL) were diluted 5 times with 20 mL H_2O/ACN/FA (84:15:1, *v*/*v*/*v*) stored at −20 °C overnight and centrifuged at 3541×*g* for 3 min. One milliliter of the supernatant was transferred to a autosampler vial and analyzed. Plant seed oil samples showed insufficient recovery rates (below 50 %) if extracted with the extraction solvent described above. These oil samples were extracted with ACN/H_2O/FA (75:24:1, *v*/*v*/*v*) for 1 h on a laboratory shaker at 150 rpm. 5.0 g (±0.05 g) of oil were extracted with 20 mL of this extraction solvent. Oils have a higher density than the extraction solvent, therefore, the supernatant was used. The samples were centrifuged for 20 min at 3541×*g* and 3 °C. Cooling of the samples leads to a better phase separation as the viscosity of the oil increases. Two hundred microliters of the obtained supernatant were mixed with 800 μL of water (1 % FA, *v*/*v*). The samples were frozen at −20 °C overnight. The remaining oil in the samples separated from the solution while freezing and could be removed after defrosting with another centrifugation at 3 °C and 3541×*g* for 20 min. Six

Table 2 Method performance characteristics. The lowest and highest values for each toxin (representing different food matrices) are shown

Compound	LOD [μg/kg]	LOQ [μg/kg]	Average recovery [%]	Interday repeatability [%]	Intraday repeatability [%]
AAL TA_1	2.8–5.4	9.3–18	63±8.0–100±19	6.8–20	4.1–9.1
AAL TA_2	1.2–17	3.8–55	57 ± 5.1–99±19	5.6–24	2.3–9.1
isoALT	1.3–19	4.4–62	64 ± 6.5–106 ± 3.1	4.3–20	4.2–9.1
ALT	0.8–24	2.5–81	78 ± 13–107 ± 4.5	5.3–17	4.0–7.7
AOH	0.2–2.8	0.6–9.3	64 ± 10–113 ± 6.4	10–17	7.5–13
AME	0.04–0.4	0.1–1.2	52 ± 5.0–98 ± 4.5	7.9–22	9–17
ATX-I	2.1–14	6.9–48	68 ± 9.1–106 ± 5.2	3.4–13	6.7–9.8
TeA	3.6–34	12–110	52 ± 6.9–102 ± 3.8	5.4–18	3.2–9.0
TEN	0.1–2.0	0.5–6.6	75 ± 8.8–103 ± 4.3	4.2–15	3.2–8.0

hundred microliters of the aqueous phase of the oil samples was transferred to an autosampler vial and analyzed.

HPLC-MS/MS settings

Chromatographic separation was carried out using a Nexera™ system (Shimadzu, Duisburg, Germany) with a LC-20ADXR solvent delivery module, a SIL-20AXR autosampler, a DGU-20A5R degasser, a CBM-20A communications bus module and a CT0-10ASVP column oven. The column used was a Halo™ RP-Amide column (2.1 mm × 100 mm, 2.7 μm, Advanced Materials Technology, Wilmington, USA). A binary gradient consisting of MeOH (A) and H_2O (B) (both with 1 % FA) with a flow rate of 400 μL/min was applied. Fifty microliters of sample solution was injected. Starting conditions were 20 % A held for 1 min, followed by a linear gradient to 55 % A at 7.5 min. The methanol content was increased to 100 % until 11.0 min. The mobile phase composition was returned to starting conditions at 11.01 min and held constant for 1 min (12.00 min). The temperature was held at 50 °C. To prevent a high entry of amino acids, salts and sugars into the mass spectrometer, a diverter valve was applied. This device directs the flow from the HPLC system directly into the solvent waste. The first 3 min of each chromatographic run was discarded. The mass spectrometer used was an AB Sciex (Darmstadt, Germany) QTRAP®5500 with a Turbo V™ ESI (Electrospray ionization) source. The curtain gas was set to 35 psi, the collision activated dissociation gas to "medium", GS1 to 35 psi and GS2 to 45 psi. The source temperature was 450 °C. The ion spray voltage was 5500 V in positive mode and −4500 V in negative mode. Entrance potentials of 10 and −10 V were used in positive and negative mode, respectively, and a dwell time of 10 ms per selected reaction monitoring (SRM) transition was applied. Unit resolution was applied. Data analysis was done with *Analyst*® software (AB Sciex, Darmstadt, Germany, version 1.5.2). Parent and fragment ions (quantifier and qualifier) for each analyte were chosen regarding to the best signal-to-noise ratios in a spiked sample. The potentials given in Table 3 were optimized after direct infusion with a syringe pump. Note that the SRM parameters for AAL toxin TA_1 and TA_2 as well as ALT and isoALT are identical due to the similar structure of the two pairs of isomers. The ratios of quantifier to qualifier SRM were determined based on one calibration curve for each food matrix, and the standard deviation (SD) is indicated. Representative chromatograms can be found in Fig. 2 and Fig. 3.

Statistical treatment of obtained data

The concentrations of the toxins were calculated with the average of two calibration curves. One was measured each day before the samples and one after the samples. The content of toxin in the injected sample extract was calculated using the linear regression function calculated from the calibration curves. This concentration was converted to the toxin content considering the recovery rate for the specific matrix and the exact sample weight. Each sample was worked up in duplicate and the toxin content calculated separately for both duplicates. The values given in Table 4 represent the average of both analysis and standard deviation. The recovery values (Table 2) were calculated analogously to the samples. The recovery rates are presented as an average over low, medium, and high spiking level, including the standard deviations. Intraday repeatability and interday repeatability (Table 2) are expressed as relative standard deviations (RSD) over the peak areas for the respective toxin and set of samples. If five or more samples showed quantitative co-occurrence of two or more toxins, the toxin concentrations were plotted against each other to investigate if a correlation between the amounts of both substances exists. Furthermore, the data obtained were analyzed for qualitative relationships of pairs of analytes. Therefore, the coefficient of

Table 3 SRM parameters

Compound	Parent ion [*m/z*]	t_R[min]	Quantifier/qualifier [Da]	Quantifier/qualifier [cps/cps]	DP [V]	CE [V]	CXP [V]
AAL TA_1	$[M+H]^+$ 522	5.74	310/328	1.3 ± 0.1	150	35/31	15/15
AAL TA_2	$[M+H]^+$ 522	5.47	310/328	1.3 ± 0.0	150	35/31	15/15
isoALT	$[M+H]^+$ 293	4.97	257/197	11 ± 0.3	150	19/35	10/10
ALT	$[M+H]^+$ 293	5.27	257/197	6.1 ± 0.2	150	19/35	10/10
AOH	$[M-H]^-$ 257	8.71	251/147	2.2 ± 0.1	−230	−34/−42	−10/−10
AME	$[M-H]^-$ 329	9.51	256/255	2.6 ± 0.2	−215	−30/−42	−12/−12
ATX-I	$[M-H]^-$ 351	6.42	263/297	2.1 ± 0.1	−180	−43/−35	−9/−9
TeA	$[M+H]^+$ 198	4.98	125/153	1.3 ± 0.0	160	21/18	9/9
TEN	$[M+H]^+$ 415	6.50	199/312	3.6 ± 0.2	207	17/32	12/12

t_R retention time, *DP* declustering potential, *CE* collision energy, *CXP* collision cell exit potential

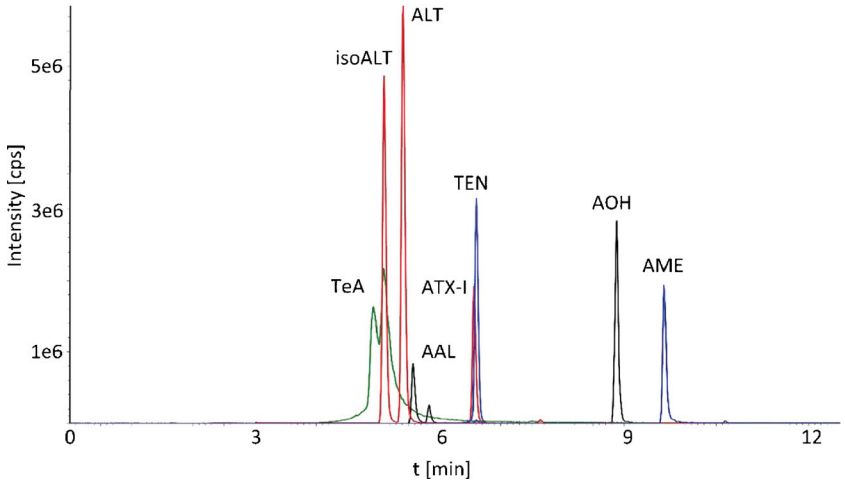

Fig. 2 HPLC-MS/MS chromatogram of spiked sunflower seeds. Spiking level represents the 2nd highest calibration point

contingency Φ was calculated for all analyte pairs using the following equation (Köhler et al. 2007)

$$\Phi = \sqrt{\frac{\sum \frac{(f_b - f_e)^2}{f_e}}{N}}$$

where f_b represents the empiric incidence and f_e represents the theoretical incidence if no correlation between both parameters existed. Φ describes the degree to which the presence of one toxin influences the presence of a second toxin. Φ ranges between 0 (no correlation) and 1 (perfect correlation).

This calculation leaves quantitative data aside and only regards qualitative presence of the analytes.

To calculate the exposure of the German population, food consumption data for adult Germans were extracted from the Comprehensive European Food Consumption Database (EFSA 2008). The database provides consumption data both chronic and acute on the basis of 10,419 adults (chronic consumption) or 20,838 days (acute consumption). For chronic consumption data given in the database, intake statistics have been calculated based on individual average consumption over the total survey period, whereas for acute consumption, calculation is based on every single reporting day (EFSA 2011b). The consumption data are categorized in four categories of different complexity. We chose the Foodex L3 level as the used categories and subcategories fits best with the purpose of this study. To calculate the chronic exposure from one class of analyzed food samples, different food categories were summed up. "Tomato products" represent the sum of tomato puree and tomato ketchup; "bakery products" consist of wheat bread and rolls, multigrain bread and rolls, mixed wheat and rye bread and rolls, rye bread and rolls, other bread and bread products; and "juices" contain the sum of apple juice, multifruit juice, tomato juice, mixed fruit juice and vegetable juice. "Sunflower seeds" is only represented by sunflower seeds. The chronic food intake data extracted from the EFSA database are summarized in Table 5. The exposure was calculated

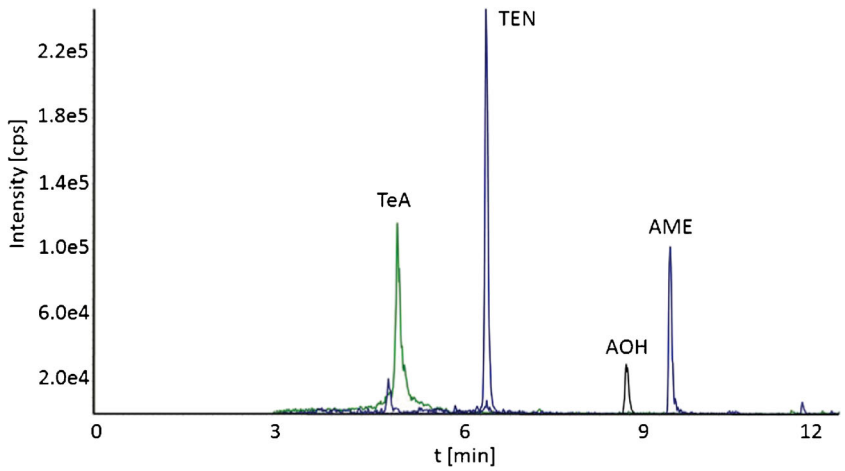

Fig. 3 HPLC-MS/MS chromatogram of a naturally contaminated sunflower seed sample. TeA content: 490 μg/kg, TEN content 800 μg/kg, AOH: 39 μg/kg, and AME: 21 μg/kg

Table 4 Mycotoxin contamination of analyzed food samples

Tomato products (N=34)						
	N/N^{pos}	N^{qual}	N^{quant}	Avg^{quan} [μg/kg]	Min^{quan} [μg/kg]	Max^{quan} [μg/kg]
AAL TA_1	34/0	0	0	–	–	–
AAL TA_2	34/0	0	0	–	–	–
isoALT	34/0	0	0	–	–	–
ALT	34/0	0	0	–	–	–
AOH	34/24	14	10	13	6.1 ± 0.9	25 ± 1.2
AME	34/27	19	8	2.5	1.2 ± 0.04	7.4 ± 0.5
ATX-I	34/0	0	0	–	–	–
TeA	34/31	16	15	200	52 ± 2.1	460 ± 5.6
TEN	34/9	9	0	–	–	–
Bakery products (N = 9)						
	N/N^{pos}	N^{qual}	N^{quant}	Avg^{quan} [μg/kg]	Min^{quan} [μg/kg]	Max^{quan} [μg/kg]
AAL TA_1	9/0	0	0	–	–	–
AAL TA_2	9/0	0	0	–	–	–
isoALT	9/0	0	0	–	–	–
ALT	9/0	0	0	–	–	–
AOH	9/8	8	0	–	–	–
AME	9/8	7	1	3.2	3.2 ± 0.1	3.2 ± 0.1
ATX-I	9/0	0	0	–	–	–
TeA	9/9	7	2	140	75 ± 1.3	210 ± 7.6
TEN	9/9	7	2	11	9.2 ± 1.0	12 ± 1.0
Juices (N = 23)						
	N/N^{pos}	N^{qual}	N^{quant}	Avg^{quan} [μg/kg]	Min^{quan} [μg/kg]	Max^{quan} [μg/kg]
AAL TA_1	23/0	0	0	–	–	–
AAL TA_2	23/0	0	0	–	–	–
isoALT	23/0	0	0	–	–	–
ALT	23/1	1	0	–	–	–
AOH	23/13	6	7	3.1	0.65 ± 0.02	16 ± 0.7
AME	23/10	7	3	1.8	0.14 ± 0.1	4.9 ± 0.1
ATX-I	23/0	0	0	–	–	–
TeA	23/12	1	11	73	21 ± 0.8	250 ± 8.5
TEN	23/11	10	1	1.0	1.0 ± 0.10	1.0 ± 0.10
Vegetable oils (N = 19)						
	N/N^{pos}	N^{qual}	N^{quant}	Avg^{quan} [μg/kg]	Min^{quan} [μg/kg]	Max^{quan} [μg/kg]
AAL TA_1	19/0	0	0	–	–	–
AAL TA_2	19/0	0	0	–	–	–
isoALT	19/0	0	0	–	–	–
ALT	19/0	0	0	–	–	–
AOH	19/9	8	1	6.0	6.0 ± 0.4	6.0 ± 0.4
AME	19/16	12	4	9.9	2.8 ± 0.1	14 ± 1.7
ATX-I	19/0	0	0	–	–	–
TeA	19/4	3	1	15	15 ± 1.3	15 ± 1.3
TEN	19/9	8	1	11	11 ± 0.4	11 ± 0.4
Sunflower seeds (N = 11)						
	N/N^{pos}	N^{qual}	N^{quant}	Avg^{quan} [μg/kg]	Min^{quan} [μg/kg]	Max^{quan} [μg/kg]
AAL TA_1	11/0	0	0	–	–	–
AAL TA_2	11/0	0	0	–	–	–
isoALT	11/1	1	0	–	–	–
ALT	11/1	1	0	–	–	–
AOH	11/6	3	3	27	16 ± 2.3	39 ± 3.0
AME	11/7	2	4	11	0.64 ± 0.1	21 ± 8.2
ATX-I	11/1	1	0	–	–	–
TeA	11/11	9	2	420	350 ± 19	490 ± 24
TEN	11/10	1	10	110	6.7 ± 0.7	800 ± 140

N/N^{pos} : Number of samples analyzed/number of positive samples (S/N > 3), N^{qual} : Number of samples above LOD but below LOQ, N^{quant} : Number of quantified samples, Avg^{quan} : Mean of quantified samples, Min^{quan} : Minimum of quantified samples, Max^{quan} : Maximum of quantified samples

Table 5 Chronic and acute food intake of the analyzed food groups based on the comprehensive food consumption database (EFSA 2008)

Food group	Mean chronic intake [g/day]	Median chronic intake [g/day]	Low chronic intake (5th percentile) [g/day]	High chronic intake (95th percentile) [g/day]	High acute intake[a] (95th percentile) [g/day]
Tomato products	7.7	0.0	0.0	65.2	251.2
Bakery products	136.7	0.0	49.5	542.0	262.0
Juices	154.1	5.0	5.0	937.5	1662.5
Vegetable oils	1.8	0.0	0.0	9.1	27.6
Sunflower seeds	0.1	0.0	0.0	0.0	0.0

For most food groups, the median equals 0.0 g/day due to the high number of non-consumers

[a] The acute intake is based on the most consumed food of each food group (tomato puree, rye bread and rolls, apple juice, rapeseed oil, and sunflower seeds)

for mean, median, low (5th percentile) and high (95th percentile) consumption of each class of food samples analyzed. According to the EFSA guidelines (EFSA 2011b), it is possible to assume that an individual is a chronic high level consumer of up to eight food categories.

To evaluate the effect of acute high consumption of one class of food sample, the acute exposition regarding the 95th percentile of the most consumed single product from one group of food samples was calculated. We chose to calculate this exposition based on one single product as it is very unlikely that one individual is a high consumer of two products of the same product class on the same day (e.g., wheat and rye bread). The products with the highest acute consumption are tomato puree (for tomato products), rye bread and rolls (for bakery products), rapeseed oil (for vegetable oils), apple juice (for juices), and sunflower seeds. The acute food intake data extracted from the EFSA database are summarized in Table 5. The average contamination of one class of food products was calculated by addition of all quantified results. Results between LOD and LOQ were treated as LOQ/2, and results below the LOD were taken into account as LOD/2. The average contamination levels can be found in Table 6. The exposition of the various consumers was calculated by multiplying the respective daily intake with the average contamination of the food group. The total exposition from all food groups was received by addition of the data for each group of consumers. The EFSA (EFSA, 2011a) defined thresholds of toxicological concern (TTC) values for AOH, AME, (both 2.5 ng/kg/b.w. per day) TeA, and TEN (both 1500 ng/kg/b.w. per day). Table 7 gives the percentage to which the total TTC is exhausted (TTC$^{\%}$) by the average exposure for a person of 60 kg body weight. This calculation has been done in accordance with that used in the recent EFSA report (EFSA 2011a).

Results and discussion

Method development

Figure 2 shows a typical HPLC-MS/MS chromatogram for an extract of spiked sunflower seeds. The peak for TeA is quite broad and also shows some tailing. There are some examples in literature where a better peak shape for TeA is achieved with eluents of (slightly) basic pH values (Lohrey et al. 2013; Noser et al. 2011). We nevertheless decided to use an acidic pH for the separation of the toxins as basic pH values (or less acidic pH values) fail to separate the pairs of isomers AAL TA_1/AAL TA_2 and ALT/isoALT. Furthermore, the TeA reference standard shows two partially separated peaks, while naturally contaminated sunflower seeds showed only one peak for TeA (Fig. 3). The second peak for TeA in the standard chromatograms is caused by the TeA isomer *allo*TeA, which is present in the synthetic standard we used in this study (Lohrey et al. 2013). The impaired peak shape did not influence the linearity for the calibration curves of TeA (R^2>0.99, see Table S1) and therefore had no obvious negative impact on quantitative analysis of this toxin. As there are no legal limits for *Alternaria* toxins in the European Union, no performance criteria for the analytical

Table 6 Average contamination of the analyzed food groups with AME, AOH, TeA and TEN (see 'Materials and Methods' for calculation)

Toxin	Content in tomato products [μg/kg]	Content in bakery products [μg/kg]	Content in juices [μg/kg]	Content in vegetable oils [μg/kg]	Content in sunflower seeds [μg/kg]
AOH	8.3	13	1.5	3.2	8.7
AME	1.6	2.5	0.4	5.7	4.0
TeA	193.7	133.8	46.3	25.6	375.3
TEN	1.7	8.9	0.1	3.1	83.6

Table 7 Average daily exposition with *Alternaria* toxins calculated based on the results obtained in this study. The TTC values were defined by the EFSA (EFSA 2011a). $TTC^{\%}$ is the percentage of the TTC reached by the average daily exposition. Consumption data are taken from the EFSA food consumption database (see Table 5) (EFSA 2008). For average contamination levels of the analyzed food groups, see Table 6

Chronic exposition calculated based on mean consumption data

Substance	Total Exposition [μg/d]	TTC [ng/kg b.w. day]	$TTC^{\%}$ [%]	Exposition from bakery products [%]	Exposition from juices [%]	Exposition from tomato products [%]	Exposition from vegetable oil [%]	Exposition from sunflower seeds [%]
AOH	2.1	2.5	1400	86	11	3.1	0.3	0.04
AME	0.4	2.5	280	82	13	3.0	2.4	0.09
TeA	27	1500	30	68	27	5.5	0.2	0.14
TEN	1.3	1500	1.40	97	1.0	1.0	0.5	0.66

Chronic exposition calculated based on median consumption data

Substance	Total Exposition [μg/d]	TTC [ng/kg b.w. day]	$TTC^{\%}$ [%]	Exposition from bakery products [%]	Exposition from juices [%]	Exposition from tomato products [%]	Exposition from vegetable oil [%]	Exposition from sunflower seeds [%]
AOH	0.7	2.5	430	99	1.1	0	0	0
AME	0.1	2.5	85.0	99	1.4	0	0	0
TeA	6.9	1500	7.6	97	3.4	0	0	0
TEN	0.4	1500	0.49	100	0.1	0	0	0

Chronic exposition calculated based on low (5^{th} percentile) consumption data

Substance	Total Exposition [μg/d]	TTC [ng/kg b.w. day]	$TTC^{\%}$ [%]	Exposition from bakery products [%]	Exposition from juices [%]	Exposition from tomato products [%]	Exposition from vegetable oil [%]	Exposition from sunflower seeds [%]
AOH	0.007	2.5	4.9	0	100	0	0	0
AME	0.002	2.5	1.2	0	100	0	0	0
TeA	0.232	1500	0.3	0	100	0	0	0
TEN	0.000	1500	0.0	0	100	0	0	0

Chronic exposition calculated based on high (95^{th} percentile) consumption data

Substance	Total Exposition [μg/d]	TTC [ng/kg b.w. day]	$TTC^{\%}$ [%]	Exposition from bakery products [%]	Exposition from juices [%]	Exposition from tomato products [%]	Exposition from vegetable oil [%]	Exposition from sunflower seeds [%]
AOH	9.0	2.5	6000	78	15	6.0	0.3	0
AME	1.9	2.5	1200	74	18	5.7	2.8	0
TeA	130	1500	140	56	34	9.8	0.2	0
TEN	5.1	1500	5.6	96	1.5	2.1	0.6	0

methods have been developed. The criteria from CEN/TR 16059 were used to assess the quality of the method presented here (CEN 2011). This guideline allows recovery rates between 50 and 120 % for non-regulated mycotoxins in single laboratory validated methods. The method used in this study complies with these criteria for most toxins, only for AME slightly insufficient recoveries in bakery products at low and medium spiking levels were observed (49 % in both cases, see Table S1 in the Supplementary Material). This is regarded as acceptable because the recovery rates were highly reproducible. The results for TeA also showed a low recovery of 44 % (Table S1) at medium spiking level in sunflower seeds. For AAL toxins TA_1 and TA_2 and AOH, recoveries higher than 120 % were obtained at low spiking level in vegetable oils (126 % for both AAL toxins and 121 % for AOH, see Table S1). The major benefits of the presented method are the simple and rapid sample preparation, as no isotopically labeled standards have to be applied and no cleanup step or other laborious sample handling has to be performed. This leads to slightly higher LOD and LOQ values than described in literature (Zhao et al. 2015; Noser et al. 2011; Walravens et al. 2014) for some toxins and some matrices, but this minor decrease in sensitivity was regarded as acceptable as it allows the simultaneous detection of all toxins of interest. Inter- and intraday repeatability (Table 2, Table S1) are usually below 20 %, indicating satisfactory reproducibility.

Alternaria toxin content of analyzed samples

The results for the content of *Alternaria* toxins in the analyzed food samples are summarized in Table 4. Of all samples, 8.8 % were free of *Alternaria* toxins above the LODs, 15.4 %

Table 8 Coefficient of contingency. If one of the compared toxins occurs in all samples analyzed, no contingency can be calculated. This is indicated with n.d.* in the table

Tomato products	AOH	AME	TeA	TEN
AOH	–	0.68	0.13	0.47
AME	0.38	–	0.36	0.40
TeA	0.13	0.36	–	0.33
TEN	0.47	0.40	0.33	–
Sunflower seeds	AOH	AME	TeA	TEN
AOH	–	0.83	n.d.*	n.d.*
AME	n.d.*	–	n.d.*	n.d.*
TeA	n.d.*	n.d.*	–	n.d.*
TEN	n.d.*	n.d.*	0.83	–
Juices	AOH	AME	TeA	TEN
AOH	–	0.42	0.39	0.12
AME	0.42	–	0.49	0.24
TeA	0.39	0.49	–	0.65
TEN	0.12	0.24	0.65	–
Bakery products	AOH	AME	TeA	TEN
AOH	–	0.65	0.14	n.d.*
AME	0.65	–	0.14	n.d.*
TeA	0.14	0.14	–	n.d.*
TEN	n.d.*	n.d.*	n.d.*	–
Vegetable oils	AOH	AME	TeA	TEN
AOH	–	0.44	0.34	0.26
AME	0.44	–	0.27	0.57
TeA	0.34	0.27	–	0.61
TEN	0.26	0.57	0.61	–

contained one analyte, 23 % were positive for two toxins, 24 % showed contamination with three toxins and 26 % of the samples contained four toxins. Only 1.1 % showed presence of five or seven analytes; no sample with six toxins was found. In total, 91.2 % of all samples were positive for one or more toxins. The most prevalent toxins were AME (68 % positive, ranging from 0.14 to 21 μg/kg), TeA (67 % positive, ranging from 21 to 490 μg/kg), AOH (60 % positive, ranging from 0.65 to 39 μg/kg) and TEN (48 % positive, ranging from 1.0 to 800 μg/kg). ALT was found in two samples below the LOQ, isoALT and ATX-I each in one sample. No AAL toxins could be detected in any of the samples.

Correlation and contingency analysis

A correlation analysis regarding the respective concentrations could be performed for the analyte pairs TeA/AOH in juice samples and for TeA/AOH, TeA/AME, and AOH/AME in tomato products. No correlation between the concentrations of these analyte pairs could be established; R^2 values ranged from 0.05 (TeA/AOH in juice, N=7) to 0.33 (TeA/AME in tomato products, N=8). The results of the statistical contingency analysis are summarized in Table 8. The results showed a moderate to strong relationship between the occurrence of AOH and AME for all food matrices, with Φ ranging from 0.42 in juices to 0.83 in sunflower seeds. A strong correlation was observed for TeA and TEN in vegetable oils, sunflower seeds and juices. The correlation was much weaker in tomato products (Φ=0.33). A value for Φ was not calculable for bakery products because TeA and TEN occurred in all bakery products. These differences in co-occurrence could be due to the contamination of the raw material with different *Alternaria* strains with variations in their biosynthetic profile or the selective degradation of certain compounds during food processing.

Exposure assessment

Combining the food consumption data published by EFSA (2008) (Table 5) and the contamination data obtained in this study, (Table 6), the average daily intake (exposition) of *Alternaria* toxins was calculated, the results of which are summarized in Table 7. These calculations represent only a rough estimation, as there might have been other sources of exposition than the food samples analyzed, and only a limited number of samples per food group have been analyzed. The results for low (5th percentile) and median consumption are of little significance as the consumption scenario for these groups of

Table 9 Acute exposition to *Alternaria* toxins through high level (95th percentile) consumption of the given product class. The portion size is indicated (see Table 5 for high acute food intake and Table 6 for average contamination levels of the analyzed food groups)

Acute exposition calculated based on high (95th percentile) consumption data (consumption per day)					
Substance	Exposition from 260 g rye bread and rolls [μg]	Exposition from 1600 mL apple juice [μg]	Exposition from 250 g tomato puree [μg]	Exposition from 28 g rapeseed oil [μg]	Exposition from 77 g sunflower seeds [μg]
AOH	3.4	2.46	2.1	0.09	0.7
AME	0.7	0.59	0.4	0.16	0.3
TeA	35	77	49	0.71	29
TEN	2.2	0.1	0.4	0.09	6.4

foods by this type of consumer equaled zero consumption. The results for mean consumption (2.1 μg/day for AOH, 0.4 μg/day for AME, 1.3 μg/day for TEN) were in good accordance with those summarized by the EFSA in 2011 for AOH, AME, and TEN (EFSA 2011a). In this opinion, exposures of 0.1–2.3 μg/day for AOH, 0.05–0.3 μg/day for AME, and 2.2–8.5 μg/day for TEN are reported. The exposition calculated by the EFSA for TeA is ≤0.8 μg/day which is much lower than that calculated in this study (27 μg/day for mean consumption). The exposition of people consuming high amounts of the analyzed food groups is significantly higher (9.0 μg/day AOH, 1.9 μg/day AME, 5.1 μg/day TEN, and 130 μg/day TeA). For both the mean and high consumption scenario, the main source of exposition was bakery products, followed by juices, tomato products, and vegetable oil. The exposition from sunflower seeds was negligible. The $TTC^{\%}$ values for mean consumption for AOH and AME exceed the TTC values defined by the EFSA by 1400 and 280 %, respectively. As already stated by the EFSA, this also reflects the need for further toxicological studies for these compounds. The $TTC^{\%}$ for TeA reached 30 % of the TTC, while the $TTC^{\%}$ for TEN (1.4 %) was negligible. Expositions below the TTC are very unlikely to pose a risk to human health (Kroes et al. 2004). Individuals belonging to the low consumption group are faced with very low $TTC^{\%}$ values (maximum: 4.9 % for AOH). People belonging to the high consumption groups exceed the TTC for AOH by 6000 %, for AME by 1200 %, and for TeA by 140 %. This is in contrast to the 2011 EFSA report (EFSA 2011b) which concluded that the TTC values were only exceeded for AOH and AME because our results indicate that TeA might pose a risk for high consumers of contaminated food groups, as the $TTC^{\%}$ for TeA exceeds 100 %. This underlines the need to obtain further occurrence data for the *Alternaria* toxins to allow a more precise exposure assessment of the consumers. Furthermore, occurrence data from regions with a generally higher exposure to mycotoxins (e.g., Africa or South East Asia) would be valuable. The results of the acute exposition from Table 9 based on the high acute intake of analyzed food show the same trend as the consumption data for the chronic high consumption. However, it seems very unlikely that an individual person is a high acute consumer of two or more products (e.g., eating 260 g rye bread and 77 g sunflower seeds, and drinking 1600 mL apple juice). Therefore, the data should not be considered to be additive. Furthermore, foods which are consumed by a minority of persons, but then in large quantities (high consumers), such as sunflower seeds, can lead to an acute exposition to toxins to which the consumer is otherwise only rarely exposed (6.4 μg TEN from 77 g sunflower seeds).

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