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Determination of *Alternaria* Toxins in Food by SPE and LC-IDMS: Development and In-House Validation of a Candidate Method for Standardisation

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Abstract: *Alternaria* toxins (ATs) are frequently found contaminants in foodstuffs (e.g., alternariol), often reaching high concentrations (e.g., tenuazonic acid). They can spoil a wide variety of food categories (e.g., cereals, vegetables, seeds and drinks) and storage at fridge temperatures does not prevent the growth of *Alternaria* fungi. Therefore, reliable and validated analytical methods are needed to protect human health and to ensure a transparent and fair trade. This paper describes new technical features that improved a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the analysis of ATs in tomato, wheat and sunflower seeds. This analytical method should be simple to implement in different laboratories across the EU and thus be an attractive candidate for standardisation. The major element for improvement was the use of isotopically labelled internal standards, only recently commercially available, thereby reducing the sample handling and improving the accuracy of the results. In addition, the sample extraction and the solid-phase extraction (SPE) enrichment/clean-up were fine-tuned, whereas a more suitable analytical column (XSelect HSS T3) with improved selectivity was also employed. Overall, this method shows adequate precision (repeatability < 5.7% RSD; intermediate precision < 7.0% RSD) and trueness (recoveries ranging from 74% to 112%). The limits of quantification in wheat (the most analytically demanding matrix) vary between 0.19 and 1.40 µg/kg. These figures were deemed satisfactory by the European Committee for Standardization (CEN) and have formed the basis for a subsequent interlaboratory validation study. The corresponding standard was published by CEN in 2021.

Keywords: *Alternaria* toxins; tomato; wheat; sunflower; LC-MS/MS; validation; isotope dilution mass spectrometry

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1. Introduction

Alternaria toxins (ATs) have gained attention as a potential health risk due to their mutagenicity, cytotoxicity and genotoxicity and can be classified as emerging mycotoxins [1,2]. As a result, they are under consideration for regulation by the European Commission with the current candidates being tenuazonic acid (TeA), altenuene (ALT), alternariol (AOH), tentoxin (TEN) and alternariol monomethyl ether (AME) [3].

Alternaria species are widely distributed saprophytic, endophytic and pathogenic fungal species causing foodstuffs to decay during transport, processing and storage. They can proliferate under a wide range of environmental conditions, including refrigeration or freezing conditions [4,5].

ATs are commonly found in cereals [1,5–7], oilseeds [5], fruits and juices [4,5,8,9], vegetables [5,10,11], wine and beer [5,9]. Several reports and review papers [2,12–17] have been published on the occurrence of ATs in foodstuffs worldwide, covering the period 2010–2019. All of them indicate a high frequency of contamination as well as high

occurrence levels in food products (e.g., TeA was present in unprocessed wheat samples up to 4 mg/kg [13]). Often, the exposure values in the EU exceeded the threshold of toxicological concern established for AOH and AME (2.5 ng/kg bw) and for TeA and TEN (1500 ng/kg bw) [1,14,16,18].

The European Food Safety Authority (EFSA) published an opinion on ATs already in 2011. It indicated the need for harmonised and appropriate analytical methods in order to make sound exposure assessments [1].

The 'gold standard' for the analysis of ATs is liquid chromatography-tandem mass spectrometry (LC-MS/MS) [19,20]. Escrivá et al. [20] noted that LC-MS/MS was the predominant analytical technique used in the period 2005–2017 (80% of the cases), whereas Shi et al. [21] demonstrated that it has been employed to analyse the vast majority of food matrices. Several review papers addressing LC-MS/MS approaches for the determination of ATs in food were published [19,20,22,23]. The use of ultraviolet (UV) and diode array (DAD) detection coupled to HPLC did not account for more than 14%, jointly, of the applications for the analysis of cereals and 28% for the analysis of fruits and vegetables [20]. The use of liquid chromatography with fluorescence detection has been used in selected cases for the analysis of AOH, AME and ALT in cereals [7] and tomato products [24].

A restricted number of studies covered the determination of the five most relevant ATs (investigated in the present work) and most of them addressed vegetables, fruits and products thereof [3,9,25–27] (excluding cereals and oil seeds). Furthermore, methods based on a simple solid- or liquid-liquid extraction, following the 'dilute and shoot' or QuEChERS protocols, were generally insufficient to achieve sub-µg/kg LOQs in complex matrices [20,28], as required for exposure assessment studies [29,30].

Alternatively, a few bioanalytical methods, namely immunoassays, were developed for screening purposes [31,32]. They targeted mostly one single AT (e.g., tenuazonic acid). Whereas, the open sandwich enzyme-linked immunosorbent assay (OS-ELISA) developed by Liang et al. [31] demonstrated high sensitivity and specificity and is inherently a rapid and portable test, it lacks multi-toxin recognition and confirmation capabilities. To tackle these drawbacks, Tsagkaris et al. [33] proposed the use of the direct analysis in real time ion source coupled to orbitrap mass spectrometry (DART-Orbitrap MS), affording both screening and quantification/confirmatory abilities.

In 2013, the European Committee for Standardization (CEN) issued a call for tender to develop a candidate LC-MS/MS method for ATs in wheat, tomato and sunflower seed samples leading to international standardisation. It has been proposed that a fit-for-purpose method should provide limits of quantification (LOQs) equal to or below 10 µg/kg for TeA, 5 µg/kg for TEN, and 1 µg/kg for ALT, AOH and AME.

The simultaneous chromatographic separation of TeA and other ATs is challenging, as TeA is a vinylogous acid with metal-chelating properties [9], resulting in poor chromatographic peak shape in reversed-phase HPLC. The issue was first tackled by derivatising TeA with 2,4 dinitrophenylhydrazine. The method was in-house validated for tomato products and successfully applied in a proficiency test organised by the Federal Institute for Risk Assessment (BfR, Berlin, Germany) [3]. When this method was proposed for standardisation (CEN/TC 275/WG 5), the members of the working group argued whether the derivatisation was required to obtain suitable precision parameters. Despite the numerous challenges faced when determining ATs spanning a wide polarity range in a single solid-phase extraction (SPE) and chromatographic separation were stressed, the disapproval remained. Consequently, it was decided to skip the derivatisation step in the method proposed for standardisation.

After a feasibility trial, a modified method (without derivatisation of TeA) was collaboratively validated by 16 laboratories analysing 15 test materials. While most of the performance parameters were deemed acceptable, too low recoveries of AME were observed in the three tested matrices (below 70% at 8 µg/kg level). In addition, the reproducibility of AME and TeA resulted in HorRat (Horwitz ratio) values above 2.0 in one

of the spiked tomato juices and in three naturally contaminated sunflower seed samples, respectively [34].

Until 2018, the quantification of ATs was mostly based on matrix-matched calibration, as isotopically labelled analogues were not commercially available for all ATs. This strategy revealed limitations due to the frequent unavailability of fully matching blank matrices (e.g., unpeeled sunflower seeds). Additionally, several compromises were accepted in the reconstitution of extracts after SPE enrichment in view of the ATs' rather different polarities.

In order to improve the performance characteristics mentioned above, the analytical method was further optimised taking into account the constraints identified.

This manuscript presents the approaches used to improve the precision and trueness of the method, taking advantage of the latest technical developments and available consumables. It focuses on (i) the implementation of a superior HPLC separation of all ATs after eliminating the pre-column derivatisation of TeA; (ii) the optimisation of the sample extraction and SPE enrichment/clean-up applied to cereals, tomato products and sunflower seed samples (peeled and unpeeled); and (iii) the quantification of the ATs using an isotope dilution mass spectrometry (IDMS) approach employing all corresponding isotopically labelled internal standards.

2. Materials and Methods

2.1. Standards, Solvents and Instruments

The analytical standards of TeA, ALT, AOH, TEN and AME were purchased as dried-down films from Romer Labs (Tulln, Austria). The isotopically labelled internal standards TeA-(acetyl-$^{13}C_2$), ALT-(methoxy-d_3, methyl-d_3), AOH-(methyl-d_3), TEN-d_3 and AME-(1-methyl-d_3) were obtained from ASCA (Berlin-Adlershof, Germany). TeA-(acetyl-$^{13}C_2$) was supplied as a mixture of diastereomers in methanol with a concentration of 640 µg/mL, while the remaining standards were provided in crystalline form (approximately 1 mg).

Solvents (analytical grade methanol and ethyl acetate, and LC-MS grade methanol), reagents (acetic acid glacial Ph. Eur.), the mobile phase additives ((ammonium acetate LC-MS Ultra (Honeywell/Fluka) and ammonium hydroxide LC-MS LiChropur (Supelco)) were purchased from VWR (Oud-Heverlee, Belgium). Polysorbate 20 (Tween®20, $C_{58}H_{114}O_{26}$) was supplied by Sigma-Aldrich/Fluka (Merck-Sigma group, Schnelldorf, Germany). Deionised water and LC-MS grade ultrapure water were produced by Milli-Q® HX7040 and Milli-Q® Advantage A10 apparatus, respectively (Millipore/Merck, Darmstadt, Germany).

Strata-XL SPE cartridges (6 mL, 200 mg, 100 µm) were obtained from Phenomenex (Utrecht, The Netherlands). PTFE® syringe filters (13 mm, 0.22 µm pore size) and 1 mL syringes with needle (Terumo/Shibuya, Tokyo, Japan) were obtained from VWR.

The samples were extracted in a wrist shaker CAT S50 (Ingenieurbüro CAT, M. Zipperer GmbH, Ballrechten-Dottingen, Germany) and centrifuged in an Eppendorf 5810 R centrifuge (Hamburg, Germany). When needed, a vigorous dispersion of the sample was applied with an UltraTurrax apparatus (IKA-Werke, Staufen, Germany). SPE was performed employing an Alltech manifold (Thermo Fisher Scientific, Waltham, MA, USA) and the sample extract was evaporated in a Techne Sample concentrator DB-3D (Cole Palmer, Vernon Hills, IL, USA). The analytical and precision balances were from Sartorius (Gottingen, Germany), while the pH meter WTW inoLab pH Level 2 was from Xylem Analytics (Weilheim, Germany).

The LC-MS/MS equipment was composed of a Nexera X2 chromatographic system from Shimadzu Corporation (Kyoto, Japan) coupled to a hybrid quadrupole linear ion trap mass spectrometer QTrap 6500 from ABSciex (Framingham, MA, USA) mounted with a Turbo Ionspray interface. The chromatographic system included two LC-30AD pumps, a DGU-20A5R degasser, a SIL-30AC autosampler, a CTO-20AC column oven and a CBM-20A controller. The data acquisition and evaluation were performed using the Analyst software 1.6.3 installed with the MultiQuant 3.0.2 and PeakView 2.1 packages.

2.2. Method Development

2.2.1. Chromatographic Conditions

The early elution of TeA observed in the predecessor method [34] was considered a limitation. Five alternative analytical columns were tested in this work: XSelect® HSS T3 (100 mm × 2.1 mm ID, 2.5 µm particle size (PS)) equipped with a XSelect® HSS T3 VanGuard pre-column (5 mm × 2.1 mm ID, 2.5 µm PS); Acquity® HSS T3 (100 mm × 2.1 mm ID, 1.8 µm PS) mounted with an HSS T3 VanGuard pre-column (5 mm × 2.1 mm ID, 1.8 µm PS), Atlantis T3 (100 mm × 2.1 mm ID, 3 µm PS) and Cortecs T3 (100 mm × 2.1 mm ID, 2.7 µm PS) all from Waters (Milford, MA, USA); and Gemini® NX-C18 (100 mm × 2.1 mm ID, 3 µm PS) attached to a SecurityGuard C18 pre-column (5 mm × 2.1 mm ID) both from Phenomenex (Utrecht, The Netherlands).

The mobile phase composition and the gradient programme were similar to the one used in the former method [34]: mobile phase A—5 mM ammonium acetate buffer at pH 8.0 (instead of 8.7); mobile phase B—methanol. The above columns were evaluated regarding their capacity factor and resolution for TeA (primarily), also taking into account their upper operational pH limit.

2.2.2. Extraction Conditions

A central composite design (CCD) with k = 3 independent variables generated by the R software, ver. 3.0.2 for Windows (http://www.r-project.org, accessed 8 May 2014) was deployed for optimising the extraction conditions. A response surface methodology was used to derive the optimum extraction conditions and ANOVA allowed for the estimation of the main effects and the interactions between the independent variables. A homogeneous and naturally contaminated wheat sample containing TeA (600 µg/kg), AOH (20.5 µg/kg) and AME (1.20 µg/kg) was used as a model matrix for implementing the experimental design.

According to the literature, the percentages of organic solvent and acid in the aqueous extraction medium are the critical variables that typically influence the extractability of polar to non-polar mycotoxins from food items [6,35]. These parameters were included as key factors in the CCD next to the extraction time (independent variables). The dependent variable was the chromatographic response (peak area) for TeA, AOH and AME, as indicative of the extraction efficiency of these ATs. The experimental domain of the CCD was defined taking into account the conditions found in the literature for different matrices [6]. The following ranges of the variables were used: 48.2 to 100% of methanol, 0.11 to 1.89% of acetic acid, and 18.2 to 71.8 min of extraction time. An orthogonal design with $\alpha = 1.7638$, two blocks and one replicate of the centre point was adopted (18 runs). The experimental conditions generated by the CCD are displayed in Table 1. The runs were randomised to minimise the effects of hidden variables and systematic errors.

Table 1. Central composite design experiments used for the optimisation of the extraction conditions.

Run. No.	Block	Methanol (%)	Acid (%)	Time (min)
C1.9	1	75	1	45
C1.1	1	60	0.5	30
C1.4	1	90	1.5	30
C1.8	1	90	1.5	60
C1.2	1	60	1.5	30
C1.10	1	75	1	45
C1.7	1	90	0.5	60
C1.3	1	90	0.5	30
C1.6	1	60	1.50	60
C1.5	1	60	0.5	60
S2.4	2	100	1	45
S2.3	2	48.2	1	45
S2.8	2	75	1	45
S2.6	2	75	1	71.8

Table 1. *Cont.*

Run. No.	Block	Methanol (%)	Acid (%)	Time (min)
S2.5	2	75	1	18.2
S2.1	2	75	0.11	45
S2.7	2	75	1	45
S2.2	2	75	1.89	45

2.3. Method Description

Extraction: Weigh 2.00 g of homogeneous test material into a 50 mL centrifuge tube. Pipette 100 µL of an internal standard solution containing TEA-$^{13}C_2$ at 2500 ng/mL concentration, ALT-d_6 at 1000 ng/mL concentration and AOH-d_3, TEN-d_3 and AME-d_3 at 500 ng/mL concentration into the sample tube. Add 15 mL of extraction solvent (85/14/1 methanol/water/acetic acid, *v/v/v*) to the solid samples (wheat and sunflower seeds) and 14 mL to the liquid test materials (tomato products). Extract the sample for 45 min at room temperature in a wrist-shaker at maximum speed. Centrifuge the sample for 10 min at approximately 3200× *g* and transfer 7.5 mL of the upper layer to a new 50 mL centrifuge tube. Dilute this extract with 7.5 mL of 1% (*v/v*) aqueous acetic acid solution. Homogenise the solution by vortex-mixing.

SPE enrichment and clean up: Condition a Strata-XL cartridge (or equivalent) with 7 mL of methanol, 7 mL of water and 4 mL of 1% (*v/v*) acetic acid solution. Close the tap under the cartridge and pipette 3 mL of 1% (*v/v*) acetic acid solution into the SPE cartridge. Attach a reservoir and load the diluted sample on it. Wash the tube that contained the diluted sample with 4 mL of 1% (*v/v*) acetic acid solution and load it into the reservoir. Percolate the sample through the SPE cartridge at approximately 1 drop/s. Remove the reservoir and wash the cartridge with 4 mL of solution 2% (*v/v*) Tween 20 followed by 4 mL of 1% (*v/v*) aqueous acetic acid solution. Dry the adsorbent thoroughly with vacuum.

Extract elution: Add 7 mL of methanol/ethyl acetate (75/25, *v/v*) into the SPE cartridge and collect the eluate drop-wise into a glass test tube. Evaporate the eluate to dryness at 50 °C under a gentle stream of nitrogen. Reconstitute the extract with 400 µL of methanol followed by 600 µL of HPLC mobile phase A. Filter the extract through a PTFE syringe filter into a HPLC vial.

Chromatographic analysis: the sample extract (5 µL) is analysed by LC-MS/MS using a Waters XSelect HSS T3 column or equivalent (100 mm × 2.1 mm ID with 2.5 µm particle size) with the respective pre-column kept in an oven at 30 °C. The ATs are separated using a mobile phase gradient composed of (A) 5 mM ammonium acetate buffer at pH ~8.0 and (B) methanol, infused at 0.3 mL/min and following this program: 10% B for 1 min, raise B to 100% until 10 min and hold for 2 min, return B to 10% in 0.2 min and let the column stabilise until 16 min.

The ionisation of the analytes and respective isotopologues is performed in negative ion electrospray ionisation mode (ESI^-). The ESI source of the QTrap system is operated under the following conditions: curtain gas—20, collision gas—high, ion spray voltage: −4000 V, temperature—600 °C, ion spray gas 1–30, ion spray gas 2–30. The two most intense and selective parent-to-product ion transitions of the analytes (Table 2) are monitored where the first (bold) is used as the quantification trace and the second for identity confirmation (qualifier).

Table 2. Instrumental parameters used in the QTrap 6500 LC-MS/MS system for the detection of the ATs and the respective isotopologues.

Analyte	Time (min)	Precursor Ion $[M-H]^-$ (*m*/*z*)	Product Ion (*m*/*z*)	DP (V) *	EP (V) *	CE (V) *	CXP (V) *
TEA	3.9	196.0	111.8	−55	−10	−32	−9
			139.0	−55	−10	−26	−7
TEA-$^{13}C_2$		197.9	113.9	−20	−10	−34	−13
			140.8	−20	−10	−28	−17
ALT	7.6	290.9	**185.9**	−80	−10	−34	−11
			214.1	−80	−10	−28	−13
ALT-d_6		297.0	**189.0**	−90	−10	−38	−9
			216.9	−90	−10	−28	−13
AOH	8.4	256.9	212.0	−65	−10	−38	−9
			214.9	−65	−10	−38	−19
AOH-d_3		260.0	215.0	−65	−10	−40	−13
			217.9	−65	−10	−38	−25
TEN	8.75	413.1	140.8	−65	−10	−24	−9
			271.1	−65	−10	−20	−15
TEN-d_3		416.1	141.0	−60	−10	−26	−9
			274.0	−60	−10	−22	−17
AME	9.8	270.9	227.8	−60	−10	−38	−13
			255.9	−60	−10	−28	−19
AME-d_3		274.0	231.1	−60	−10	−38	−15
			258.8	−60	−10	−30	−27

* DP—declustering potential; EP—entrance potential; CE—collision energy; CXP—collision cell exit potential. Acquisition time window in scheduled MRM—90 s; bold—quantification product ion

The quantification is based on an internal standard calibration. TEA is calibrated in the range 10 to 1000 µg/L; ALT, AOH and AME in the range 1 to 100 µg/L and TEN in the range 5 to 250 µg/L at 5 levels, adding 50 µL of the internal standard solution mentioned above (to 1 mL). The concentration of the analytes in the sample interpolated in these calibration curves can be expressed directly in µg/kg.

2.4. In-House Validation Experiments

The fined-tuned method was in-house validated to assess the selectivity, linearity, limits of detection (LOD) and of quantification (LOQ), matrix effects, trueness (recovery), precision and robustness, as agreed under the standardisation mandate M/520 of the European Commission. The method, fitness-for-purpose, was then evaluated.

The selectivity was verified by checking the absence of interferences in the expected retention times of the analytes when analysing blank tomato puree, wheat and sunflower seeds. The most appropriate MRM transitions (in terms of selectivity or sensitivity) were chosen for quantification (Table 2). The linearity was tested applying the Mandel test to the calibration data. For a matter of simplicity, the LODs and LOQs were estimated as the amount of analyte in neat solvent and matrix-matched standards that generate a chromatographic peak with a signal-to-noise (S/N) ratio of three and ten, respectively. The S/N ratios were gathered from the three lowest calibration standards. The matrix effects on the ESI efficiency (ion suppression or enhancement) were assessed by comparing the slopes of the calibration curves of matrix-matched standards with the slopes of the calibration with standards in neat solvent (in %).

The precision of the method was evaluated as repeatability and intermediate precision analysing blank wheat, tomato and (near blank) sunflower matrices spiked at the following levels: 100 μg/kg for TeA; 10 μg/kg for ALT, AOH and AME; and 50 μg/kg for TEN. A nested experimental design was run consisting of fully independent triplicate analyses by two operators in three days. The data were evaluated by ANOVA extracting the variance components of the variables: day, operator and random. The random component of variability (within groups variance) was considered the repeatability of the method (s_r) whereas the total variability due to the components day, operator, and random (within + between groups variance) was considered the intermediate precision (s_{ip}). The recoveries (yield) of the method were determined by running triplicate analyses of blank tomato, wheat and (near blank) sunflower seeds spiked at low and high levels: 30 and 100 μg/kg for TEA; 3 and 10 μg/kg for ALT, AOH and AME; and 15 and 50 μg/kg for TEN. The extraction and clean-up yield was then computed comparing the response observed in the spiked matrices with the expected signal intensity, assuming no losses during the sample preparation (spiked extracts).

The robustness of the method was assessed introducing deliberate variations in the parameters that were deemed to be the most influential on its accuracy. A fractional factorial design ($2^{(4-1)}$, resolution IV) was deployed in which the variables: extraction solvent volume (setting 15 mL as optimised value), extraction time (setting 45 min), sample volume loaded onto the SPE cartridge (setting 7.5 mL) and elution solvent volume (setting 7 mL) were tested at 2 levels (±2.5% of the optimised setting). The extraction solvent composition has to be controlled precisely. Tomato puree, wheat flour and sunflower seeds flour contaminated at 100 μg/kg for TeA; 10 μg/kg for ALT, AOH and AME; and 50 μg/kg for TEN were processed according to the experimental design and analysed. The significance of the effects was evaluated at 95% confidence level using Statistica v.8 (Statsoft, Tulsa, OK, USA).

3. Results and Discussion

3.1. Optimum Chromatographic Separation

For the determination of TeA in the native form (underivatised), the pH of the mobile phase has to be judiciously adjusted, as TeA can form pH-dependent tautomers and rotamers making its chromatographic separation challenging [36–38]. TeA is easily ionisable and polar (pka 4.28 and log k_{ow} of 0.92) [3]. In addition, the target ATs span over a wide polarity scale with log k_{ow} values ranging from 0.87 (ALT) to 3.32 (AME). The best TeA peak shape under reverse-phase chromatography is obtained when TeA is fully ionised (alkaline pH). However, its retention in a conventional C18 stationary phase is weak. Therefore, the eluotropic strength (organic solvent composition) of the injected solution (sample extract) has to be low, so as to not to distort the TeA peak. On the other hand, AME is very non-polar and requires high eluotropic strength to be fully soluble. Tölgyesi et al. postulated that the insufficient solubility of AME in the aqueous injection solution (originally 10% methanol in water) was responsible for its low recoveries [34].

Therefore, we decided to test different analytical columns specially engineered to analyse polar substances (T3 family from Waters®), aiming at selecting the one with the best retention of TeA. In turn, this would enable us to inject a sample extract with higher organic solvent content. The mobile phase composition and the gradient elution program were kept as in the former method, except the pH of the aqueous mobile phase (A) that was lowered to 8.0, instead of 8.7, so as to not to exceed the upper working limit of the selected columns.

The Atlantis T3 column provided good retention of the target analytes, but it can only be operated routinely, up to a pH of 7. Therefore, it was disfavoured (see Figure 1). The Cortecs T3 column offered weaker retention of all analytes (>0.5 min shorter retention times). The analytical columns XSelect HSS T3 and Acquity HSS T3 enabled a similarly good resolution but the latter requires the use of ultra-performance liquid chromatography (UPLC) instruments. Compared with the Gemini NX-C18 column, the XSelect HSS T3

column offered an equivalent capacity factor (k′) for TeA (3.49 vs. 3.51), a longer retention time (RT, 3.76 vs. 3.35 min), a better asymmetry factor (As, 1.39 vs. 2.13) and higher number of theoretical plates (N, 6890 vs. 2300). Although the Gemini NX-C18 column provided better performance parameters than other columns frequently used in the analysis of mycotoxins (e.g., Acquity BEH), and may be a valid alternative, the XSelect HSS T3 column outperformed it in the analysis of TeA. The latter also provided enhanced retention of TeA in comparison with the Ascentis Express C18 fused-core column suggested before [34] (the k′ value of TeA improved by 60%). Hence, the XSelect HSS T3 column was selected for all further experiments. Table 3 displays the chromatographic parameters (RT, k′, As and N) obtained with the three best performing columns, while Figure 1 depicts a chromatogram generated using the XSelect HSS T3 column.

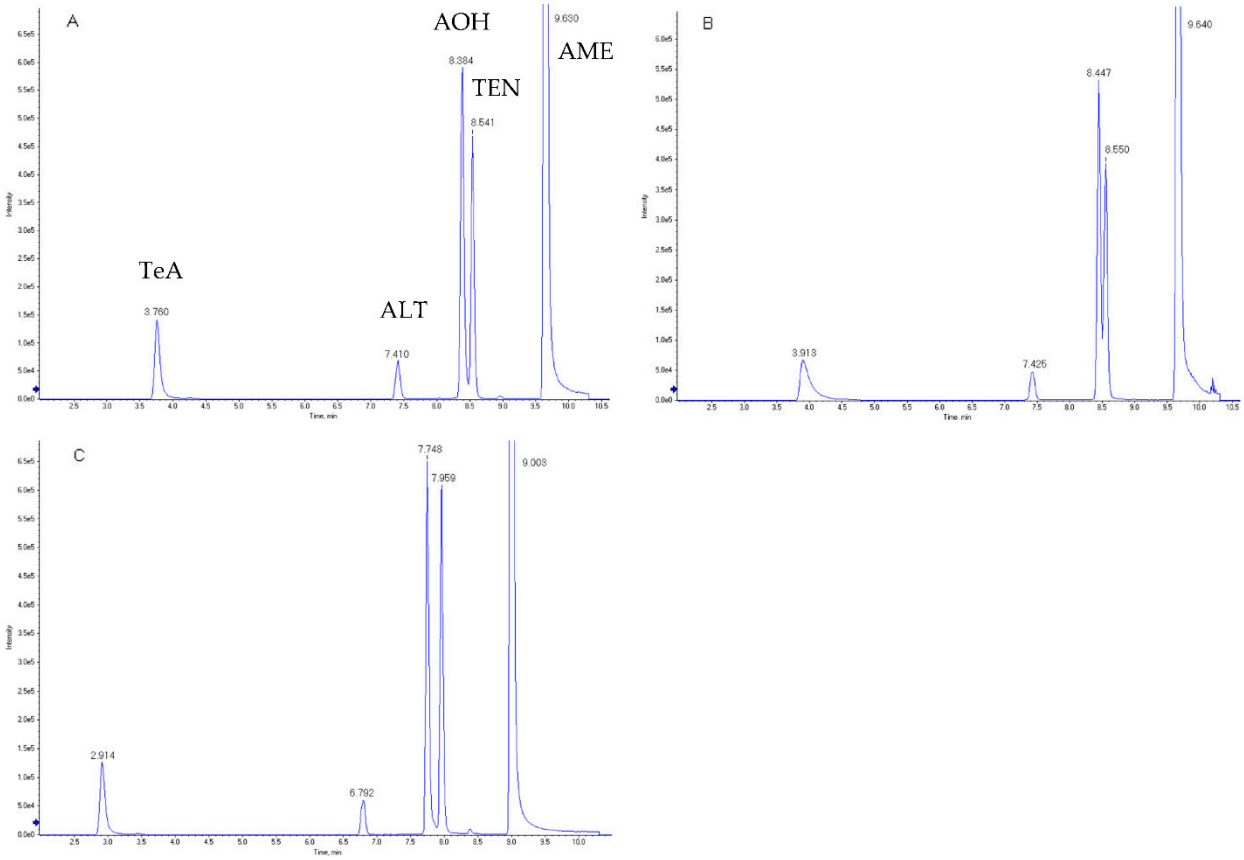

Figure 1. Chromatograms of a solution of *Alternaria* toxins at 50 µg/L obtained with the analytical columns: (**A**) XSelect HSS T3; (**B**) Atlantis T3; and (**C**) Cortex T3.

Table 3. Chromatographic parameters obtained on the separation of *Alternaria* toxins using the three most suitable columns (attached with a pre-column).

	XSelect HSS T3				Acquity HSS T3				Gemini NX-C18			
	RT	**k′**	**As**	**N**	**RT**	**k′**	**As**	**N**	**RT**	**k′**	**As**	**N**
TeA	3.83	3.49	1.39	6892	3.96	3.81	1.53	5911	3.35	3.51	2.13	2333
ALT	7.42	7.73	1.13	59,663	7.56	8.16	1.08	65,387	7.11	8.58	1.11	48,759
AOH	8.36	8.82	0.92	26,851	8.46	9.24	0.71	42,726	7.94	9.68	0.96	48,017
TEN	8.58	9.09	1.03	54,859	8.67	9.50	1.03	67,865	8.22	10.06	0.91	51,902
AME	9.70	10.40	1.08	83,869	9.85	10.93	1.09	88,546	9.34	11.57	0.99	74,041

RT: retention time; k′ capacity factor; As: asymmetry factor; N: number of theoretical plates.

A gradient of aqueous ammonium acetate buffered at pH 8.0 (mobile phase A) and methanol (mobile phase B) enabled the separation of all analytes at 10% peak height, and TeA eluted at a gradient composition of about 40% of methanol. Therefore, the injection solution can be prepared with 60% mobile phase A and 40% mobile phase B. This preserves the peak shape of TeA and ensures complete dissolution of AME. The 60/40 composition also facilitates the re-dissolution of matrix components in the sample extract. With the adopted elution gradient the five ATs were determined in a chromatographic run of 16 min.

3.2. Extraction of the Sample

The analysis of ATs in dry matrices (e.g., cereals and oil seeds flour) requires a wisely selected set of interconnected parameters to achieve a quantitative and reliable analysis. TeA is a polar and ionisable substance, while AOH and AME are non-polar; hence, the optimum extraction medium must reflect the hydrophilic and lipophilic properties of the ATs and allow a near quantitative and robust extraction.

A systematic optimisation was done by employing a central composite design, where the methanol content, the amount of acetic acid and the extraction time were varied. The Pareto charts of main effects indicate that the methanol content in the extraction solution is the most influential variable for the dibenzo-α-pyrones (ALT, AOH and AME), with a significant effect ($p < 0.05$) for AME. The quadratic coefficients of the response surface function for these compounds were negative, indicating a surface with a maximum within the investigated experimental domain (see Figure 2). A methanol amount of about 80% yielded the maximum extractability of AOH and AME (Figure 2b,c). This is in agreement with the fact that AOH and AME are weakly acidic and non-polar substances (pKa 7.63 and 7.71, respectively). Therefore, the amount of organic solvent has a major effect, compared to the pH of the extraction medium.

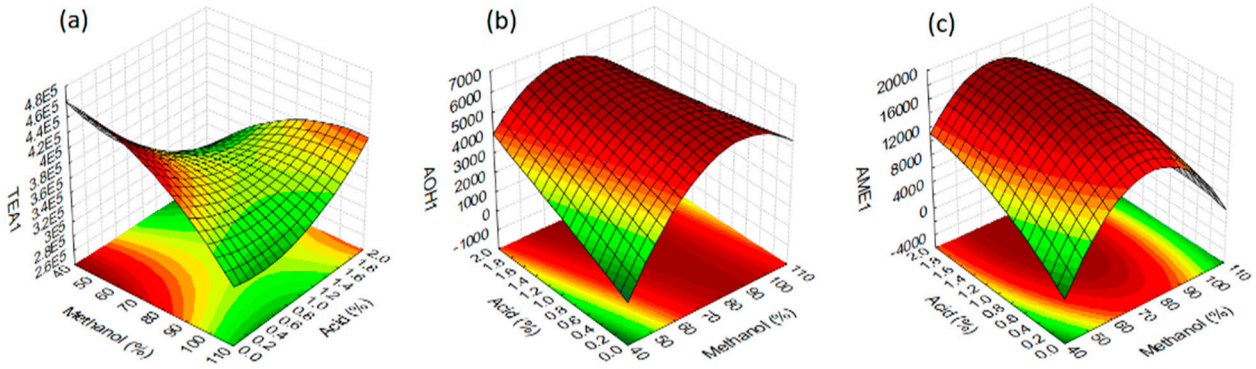

Figure 2. Response surfaces for TeA (**a**), AOH (**b**) and AME (**c**), as a function of the methanol and acid percentage compositions derived from a central composite design (slice at the extraction time of 45 min).

The extractability of TeA is influenced simultaneously by the amount of acetic acid and the amount of methanol. The respective response surface (Figure 2a) clearly shows an interaction between both parameters. This is in agreement with the fact that TeA is a strongly acidic and polar molecule, which can be extracted by a predominantly aqueous or organic solvent mixture, depending on its ionisation state (driven by the amount of acetic acid).

Based on the response surfaces presented, amounts of 1% acetic acid and 80% methanol in the extraction solution were adopted. As the extraction time did not significantly affect the extractability of the ATs in the tested range, 45 min were considered sufficient.

Later fine-tuning experiments indicated that to obtain balanced recoveries for TeA and AME (both >70%) in sunflower seeds, a slightly higher methanol content (85%) was more appropriate. The extraction recoveries of AME in tomato, wheat and sunflower seeds improved by an average of 3% at the expense of an average 5% decrease in the recoveries

of TeA. This increase was particularly noticeable in sunflower seeds, tackling the extraction limitations formerly reported by Tölgyesi et al. [34]. The remaining analytes were not significantly affected. In conclusion, a mixture of methanol + water + acetic acid (85/14/1, *v/v/v*, pH 2) was finally chosen as the extraction solvent.

It was also observed that neat methanol was not capable of completely eluting AME from the SPE cartridge. A noticeable improvement was obtained when ethyl acetate (more apolar) was added to the elution solution at the 25% level, compared to both neat methanol and 50% ethyl acetate in methanol. Section 2.3 provided a detailed description of the improved analytical method.

3.3. Results of the In-House Validation

For the detection of the ATs, the most intense multiple reaction monitoring (MRM) transitions coincided with the most selective ones, except for ALT (in bold in Table 2). The fragmentation of ALT produces multiple product ions of which the most intense *m/z* 229, 247 and 248 display very noisy traces or interferences in the analysis of food samples (of wheat and, especially, of sunflower). The absence of interferences in the signal of the internal standard also has to be taken into account. As a compromise, the slightly less intense MRM transition *m/z* 291 > 186 (*m/z* 297 > 189 for ALT-d_6) was chosen for quantification, instead of the transition *m/z* 291 > 214 (*m/z* 297 > 217 for ALT-d_6), ensuring better selectivity and sensitivity.

The statistical analysis of the calibration data according to Mandel's test indicated that the calibrations of all ATs were suitably described by linear least squares regressions. Particular attention was devoted to the amount of TeA-$^{13}C_2$ added to the calibrant/sample to ensure that the natural occurrence of ^{13}C did not contribute significantly to the signal of the internal standard, which would result in loss of linearity. Given the high amount of TeA often found in the samples, a concentration of TeA-$^{13}C_2$ equivalent to 125 µg/kg was adopted.

The estimated LOQs of the method varied between 0.1 and 2.2 µg/kg in tomato, between 0.2 and 1.4 µg/kg in wheat and between 0.1 and 2.6 µg/kg in sunflower seeds (Table 4). The LOQs of all analytes were below the lowest levels of interest in this study (10 µg/kg for TeA, 1 µg/kg for AOH, ALT and AME and 5 µg/kg for TEN) except for ALT, which were marginally above 1.0 µg/kg.

Losses in the sample preparation (extraction and clean-up) were evaluated by means of recovery calculation (trueness) [39] and the influence of the matrix on the ESI efficiency was also investigated (matrix effect). The recoveries calculated at two spiking levels are displayed in Table 4. Overall, the recovery range was very similar in the low (74–112%) and high (75–105%) spiking levels. Moreover, the recoveries across the spiking levels were similar in wheat (74–109%) and sunflower (75–112%), whereas in tomato they were slightly higher (82–111%). The average recoveries across the three matrices were satisfactory: TeA (90%), ALT (99%), AOH (96%), TEN (102%) and AME (84%). One of the major challenges on the analysis of ATs is reflected in the recovery figures. Whereas the recoveries of AOH, ALT and TEN can be considered quantitative, the optimum extraction and clean-up efficiency of AME and TeA are a compromise and lie in a very narrow set of experimental conditions. A deviation from such optimal conditions may be beneficial for a given analyte while detrimental for another and, in turn, could render the recovery figures outside the acceptable range (70–120%). The recovery figures presented in Table 4 attest to the success of the optimisation strategy described in Sections 2.2.2 and 3.2

Table 4. Validation figures of merit in the analysis of tomato products, wheat and sunflower seeds.

Solvent	**LOD (µg/L)**	**LOQ (µg/L)**							
TEA	0.11	0.38							
ALT	0.05	0.16							
AOH	0.01	0.04							
TEN	0.10	0.33							
AME	0.004	0.014							
Tomato	**LOD (µg/kg)**	**LOQ (µg/kg)**	**Matrix (Effect (%)**	**Rec. (%) * low level**	**Rec. (%) * high level**	**App. Rec. * (%)**	**s_r (%)**	**s_{ip} (%)**	**Conc. tested(µg/kg)**
TEA	0.66	2.17	84.9	98	87.8	97.1	1.8	2.4	100
ALT	0.35	1.16	35.1	111	82.0	96.2	3.8	5.3	10
AOH	0.07	0.24	30.2	103	91.9	101	1.9	2.5	10
TEN	0.17	0.56	73.1	104	94.5	103	2.1	2.3	50
AME	0.03	0.10	32.0	86.9	94.5	92.8	0.9	2.6	10
Wheat									
TEA	0.32	1.05	71.8	95.1	82.8	99.7	1.5	2.2	100
ALT	0.42	1.40	25.2	109	75.1	97.2	5.7	6.6	10
AOH	0.11	0.35	26.8	99.0	91.6	100	1.8	1.8	10
TEN	0.26	0.85	48.7	107	96.6	99.5	1.9	3.8	50
AME	0.06	0.19	15.3	74.1	95.2	101	0.7	0.9	10
Sunflower									
TEA	0.78	2.58	68.9	100	75.1	97.3	3.1	3.3	100
ALT	0.37	1.22	30.9	112	105	90.9	4.7	7.0	10
AOH	0.07	0.22	33.0	100	89.6	100	2.9	3.1	10
TEN	0.15	0.51	50.5	111	99.1	99.3	2.6	2.8	50
AME	0.03	0.10	27.9	75.2	78.9	99.1	1.4	2.1	10

* Rec.—recovery; App. Rec—apparent recovery; s_r—repeatability; s_{ip}—intermediate precision

The ionisation suppression in the ESI interface was more intense for the dibenzo-α-pyrones than for TeA and TEN, and did not differ much across the matrices (Table 4). The signal intensity, expressed as a percentage of the signal observed in neat solvent standards, varied from 25% to 35% for ALT, from 27% to 33% for AOH and from 15% to 32% for AME. AME was the most affected by ionisation suppression, but it is also the one with the highest intrinsic response, hence it remains the analyte with the lowest LOQs. The analyte least affected by ionisation suppression was TeA, giving responses ranging from 69 to 85%, while TEN was in the 49 to 73 % range.

As the present method setup relies on a quantification approach based on isotope dilution—where the isotopologues are added at the beginning of the sample preparation—the matrix effects on the calibration slope were barely noticeable (apparent recoveries of 91% to 103%) [39]. This fortunate situation enables using calibrants prepared in a neat solvent for the quantification of samples, instead of matrix-matched calibrations.

The following precision figures were obtained for the investigated matrices: RSD_r (repeatability relative standard deviation) ranging from 0.7% to 5.7% and RSD_{ip} (intermediate precision relative standard deviation) ranging from 0.9% to 9.0%. The precision was best for AME and worst for ALT. The ANOVA statistics demonstrated that the random variability is the most prominent contributor, followed by the between-day variability, while the inter-operator variability was mostly non-significant.

The method proved to be robust to inaccuracies in the experimental conditions larger than those expected to happen in daily routine. None of the tested variables (extraction solvent volume, extraction time, volume of extract loaded onto the SPE cartridge and elution volume) demonstrated a significant effect ($p < 0.05$) on the response of the analytes, compared with the random error. The *p*-values were higher than 0.22 in tomato, 0.15 in wheat and 0.18 in sunflower seeds. More detailed data can be consulted in Table S1. The extraction solvent composition needs to be controlled precisely, as explained in Section 3.2.

Finally, the measurement uncertainties were estimated by combining the relative standard uncertainties of the components—calibration standards (certificate), calibration model (confidence interval) and precision (s_{ip})—using the law of error propagation. The expanded uncertainties (k = 2) varied between 5.9% and 9.5% for all ATs and matrices,

except for ALT (from 12.3–15.1%). Here, the major contributor was the uncertainty statement of the concentration of the commercial standard solution (≈10%), compared to less than 2% for the remaining analytes. The estimated uncertainties were corroborated by the reproducibility values obtained in the interlaboratory validation trial of this method [40].

The described method was used to screen for blank and naturally contaminated samples as candidate test materials for the interlaboratory validation trial on *Alternaria* toxins, to characterise the AT content in the final test materials (blends) and to assess their homogeneity and stability. This involved the analysis of more than 500 samples. Figure 3 presents some representative chromatograms of a naturally contaminated tomato puree, a wheat flour and a sunflower seeds sample. The method proved to be robust and easy to run in a large-scale sample analysis scheme. The concentrations tested ranged from the LOQ to 6000 mg/kg.

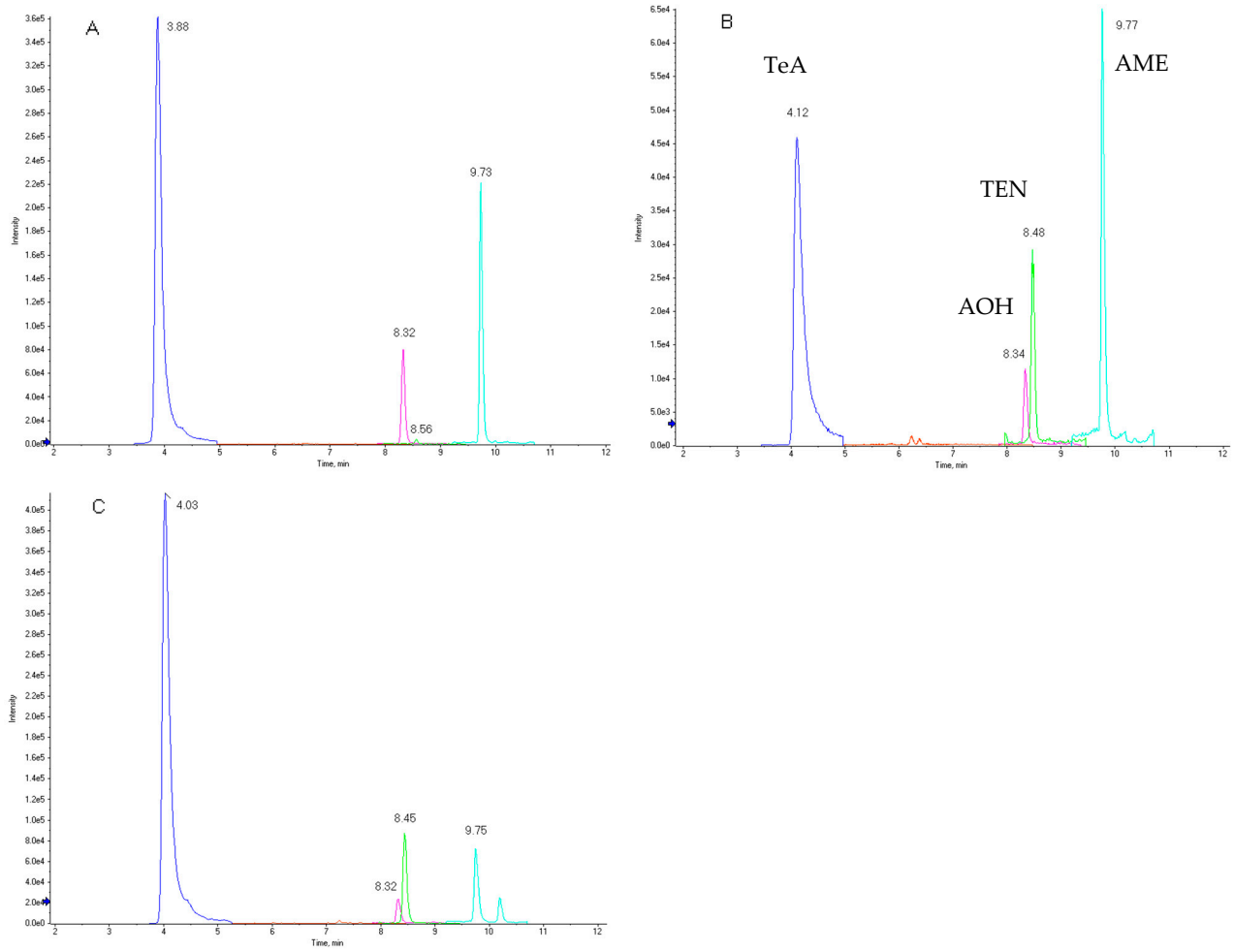

Figure 3. Representative chromatograms of the analysis of ATs in food samples. (**A**) Tomato puree: TeA 530 µg/kg, AOH 13.2 µg/kg, TEN 0.6 µg/kg (insert) and AME 5.7 µg/kg. (**B**) Wheat flour: TeA 160 µg/kg, AOH 1.7 µg/kg, TEN 5.7 µg/kg and AME 1.2 µg/kg. (**C**) Sunflower seeds: TeA 550 µg/kg, AOH 5.6 µg/kg, TEN 18.9 µg/kg and AME 4.1 µg/kg.

4. Conclusions

An analytical method was proposed for the determination of TeA, ALT, AOH, TEN and AME in tomato products, cereals and sunflower seeds at levels relevant for risk assessment (down to 1 µg/kg).

The main technical improvements introduced in the LC-MS/MS method (better chromatographic separation of TeA, balanced extraction of the ATs, efficient elution from the SPE cartridge and quantification based on isotope dilution) significantly improved the

performance, robustness and practicability of the method, rendering it suitable for official food control. The performance characteristics presented comply with the requirements set by Regulation 401/2006 [41]. Furthermore, the described method was accepted by the European Committee for Standardisation to serve as a basis for a formal interlaboratory validation study and, consequently, as a suitable candidate method for standardisation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9030070/s1. Table S1. Experimental levels and *p*-values obtained in the evaluation of the fractional factorial design implemented for assessing the robustness of the method.

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