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Collaborative Study Report: Determination of *Alternaria* toxins in cereals, tomato juice and sunflower seeds by liquid chromatography tandem mass spectrometry

Ádám Tölgyesi
Joerg Stroka

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Acknowledgements

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Table 1: The laboratories participated in the pre-trial and the trial.

Organisation	Country
ROMER Labs Diagnostic GmbH	Austria
University of Natural Resources and Life Sciences Vienna	Austria
University of Vienna	Austria
Ghent University	Belgium
Agriculture and Agri-Food Canada	Canada
STATE GENERAL LABORATORY	Cyprus
Finnish Customs Laboratory	Finland
Laboratoire SCL de Rennes	France
Bundesanstalt für Materialforschung und –prüfung (BAM)	Germany
Chemisches und Veterinäruntersuchungsamt Sigmaringen	Germany
PhytoLab GmbH & Co. KG	Germany
Westfälische Wilhelms-Universität Münster	Germany
Public Analyst's Laboratory	Ireland
Barilla G.R. F.lli SpA	Italy
GMO and Mycotoxin Unit Veterinary Public Health and Food Safety Department	Italy
Institute of Sciences of Food Production (ISPA)	Italy
Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche	Italy
National Research Council (CNR)	Italy
National Food and Veterinary Risk Assessment Institute	Lithuania
RIKILT	Netherlands
National Veterinary Research Institute	Poland
National Veterinary Institute (SVA)	Sweden
National Institute for Health, Environment and Food	Slovenia
UL Veterinary Faculty, National Veterinary Institute	Slovenia
National Center for Food	Spain
Sanitary Veterinary and Food Safety Laboratory	Romania
Fera Science Ltd	UK

Abstract

The Institute for Reference Materials and Measurements of the Joint Research Centre, a Directorate-General of the European Commission, organised a method validation study to evaluate the performance of a method for the simultaneous determination of five *Alternaria* toxins in cereals, tomato juice and sunflower seed samples.
The method validation study was conducted according to the International Union for Pure and Applied Chemistry harmonised protocol. The method was used for the determination of altenuene, alternariol, alternariol monomethyl ether, tentoxin and tenuazonic acid in both naturally contaminated and fortified samples. It was based on the extraction of the test materials with an acidified methanol – water mixture, followed by solid phase extraction clean-up. The determination was carried out by reversed phase high performance liquid chromatography coupled to a triple quadrupole mass spectrometric detector. The trial involved 16 participants representing a cross-section of research, private and official control laboratories from 11 EU Member States and Canada. The selection of collaborators was based on the performance in the pre-trial that was organised prior to the collaborative trial with participation of 25 laboratories.
Mean recoveries reported ranged from 53% to 107%. The sample reconstitution in a water-based injection solution is thought to be responsible for the low recovery obtained for alternariol monomethyl ether, which is the least polar compound from the toxins of interest. The relative standard deviation for repeatability (RSD_r) ranged from 2.0 to 34.8%. The relative standard deviation for reproducibility (RSD_R) ranged from 7.7 to 49.6%, reflecting HorRat values from 0.5 to 2.4 according to the Horwitz function modified by Thompson. A correction for recovery with the data generated by spiking experiments partially improve the reproducibility performance of the method.
The results highlight that the performance characteristics strongly depend on the matrix analysed, despite that fact that matrix matched calibration was used. These matrix effects can be compensated using stable isotope labelled internal standards; however, stable isotope analogues for the analysed compounds are not commercially available so far.

The outcome of this study however underpins its fitness-for-purpose, which is a requirement for its formal standardisation by the European Committee for Standardization (CEN).

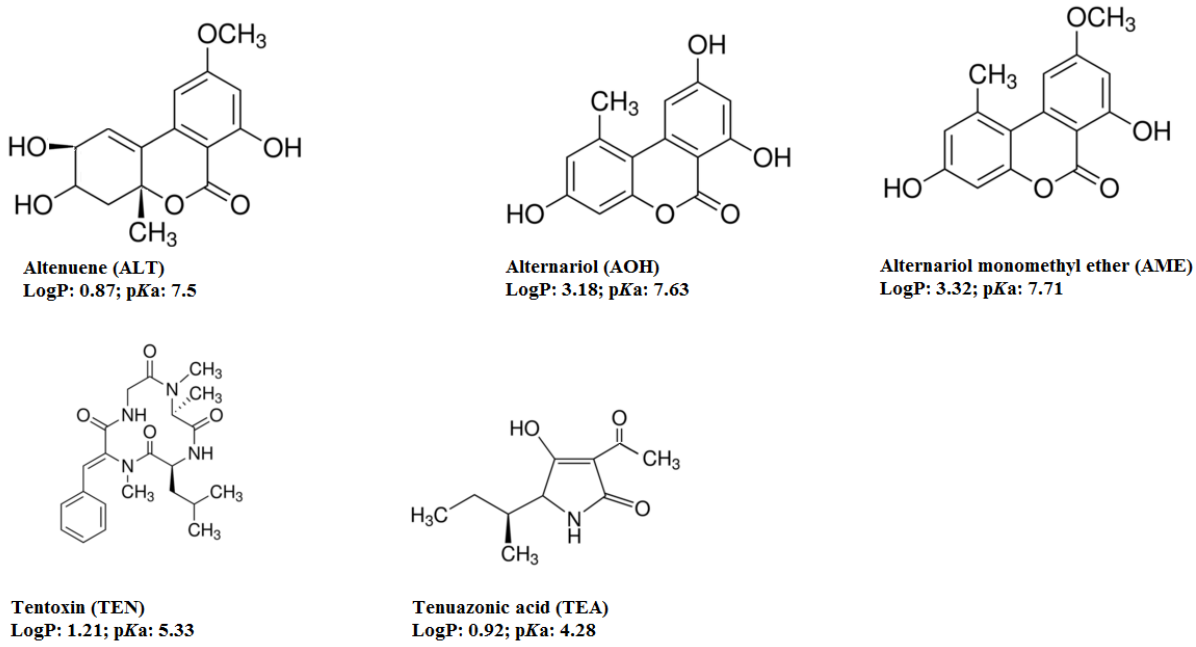
1. Introduction

Alternaria species (e.g. *Alternaria alternata*) produce more than seventy secondary metabolites, but only a few of them have been structurally identified and reported as toxic. Among these *Alternaria* toxins altenuene (ALT), alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN) and tenuazonic acid (TEA) are the main toxins of concern [**1-2**].

In the European Union (EU) maximum levels (ML) for a number of mycotoxins in food and feed are in force [**3**]; however not for *Alternaria* toxins. In 2011 the European Food Safety Authority (EFSA) has published a scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food [**2**]. In this opinion the need for "certified reference materials and defined performance criteria for the analysis of Alternaria toxins in various foods and feeds" was highlighted. The EFSA also concluded that "several chromatography based techniques are suitable for Alternaria toxin quantification in foods and feeds and liquid chromatography coupled to (tandem) mass spectrometry (LC-MS) has become the method of choice due to its sensitivity, selectivity and specificity". Furthermore, the opinion states that "representative occurrence data on *Alternaria* toxins in food and feed across the European countries are required to refine exposure assessment". Such assessments will be best performed with validated LC-MS methods.

Alternaria species can occur in vegetables, cereals, fruits and oilseeds and the continuous consumption of food infected by *Alternaria* mycotoxins can cause fetotoxic and teratogenic effects. Moreover, AOH and AME showed mutagenic and genotoxic properties [**2**]. ML for *Alternaria* toxins in food are currently under consideration by the European Commission (EC) based on the available data on their toxicity, hazard and occurrence. According to EFSA, agricultural commodities in Europe frequently contain ALT (73% of the analysed samples, maximum 41 µg/kg in wheat grains), AOH (31% of the analysed samples, maximum 1840 µg/kg in sunflower seeds), AME (6% of the analysed samples, maximum 184 µg/kg in cereals) and TEA (15% of the analysed samples, maximum 4310 µg/kg in oats). Foods often contaminated with TEN are legumes, nuts and oilseeds. The average concentration detected for TEN is 50 µg/kg in these samples (maximum 880 µg/kg) [**2**].

Figure 1: Structure of *Alternaria* toxins



As mentioned before for the determination of *Alternaria* toxins at levels in the low µg/kg range, only chromatographic methods are appropriate [**4**]. *Alternaria* toxins have weak acidic property (p*K*a 3.55 – 7.71), except TEN (**Figure 1**). The polarity of *Alternaria* toxins varies from polar to medium polar or non-polar). Most of them show adequate liquid chromatographic (LC) separation on reversed phase stationary phases, and their detection can be carried out using optical or mass spectrometric (MS) detectors [**2,4**]. TEA in its native form has an ability to form some tautomers and rotamers [**5**] that makes an adequate chromatographic separation of TEA difficult. A pre-column derivatisation for TEA with 2,4-dinitrophenylhydrazine (DNPH) as a derivatization agent has been introduced in the past to improve the HPLC separation and MS sensitivity of TEA [**6**]. In addition, the derivatisation also results in a shift of retention time as result of the less polar derivative. In the present validation TEA iwa determined in its native form without chemical derivatisation as members of the European Committee for Standardization Technical Committee 275, Working group 5 (CEN/TC 275/WG 5) opposed the need for such derivatisation upon own experience showing that a derivatisation is not necessary.

The Joint Research Centre's Institute for Reference Materials and Measurements (JRC-IRMM) hosts the European Union Reference Laboratory for Mycotoxins (EURL Mycotoxin). The main activities of the EURL are to organise proficiency test (PT) and to provide fully validated analytical methods for the network of National Reference Laboratories (NRLs). In 2010 and 2011 collaborative studies were conducted at JRC-IRMM to validate an analytical method for the determination of ochratoxin A in liquorice, paprika and chilli [**7-8**]. In 2015, an interlaboratory validation was carried out at JRC-IRMM for the determination of multi-toxins in feed [**9**].

Recently, a new liquid chromatography tandem mass spectrometric (LC-MS/MS) method was developed for *Alternaria* toxins by the EURL Mycotoxins [**10**]. The method was successfully in-house validated for various tomato samples and applied to tomato juice in an international PT organised by the Federal Institute for Risk Assessment (BfR, Berlin, Germany). The method was submitted to CEN under a standardization mandate issued by the European Commission. Upon request of CEN TC 275 / WG 5 the chemical

derivatisation was excluded from the original method proposal and a modified version was adopted. The validation study started with a preliminary validation (pre-trial) to introduce the method in those laboratories that were interested in participating in the trial.

Previous collaborative studies have shown that, with care and attention to detail during the organisation of a collaborative trial, it is possible to achieve impressive method performance characteristics even at low analyte levels close to the limits of detection (LOD). Due to the complexity of the matrices, particular care was taken during preparation of the test materials (blending of relevant matrix constituents and extensive homogenisation) and in demonstrating between-unit homogeneity before undertaking the study.

2. Scope

This method validation study (MVS) aimed at evaluating the recovery and precision characteristics of an analytical method for the determination of *Alternaria* toxins in cereals, tomato juice and sunflower seed samples. The validation ranges suggested by CEN were 1 to 10 µg/kg for ALT, AOH and AME; and 10 to 1000 µg/kg for TEN and TEA.

A test portion is extracted with a mixture of methanol and water and acetic acid. The extract is centrifuged and an aliquot of the upper layer is diluted with 1% (v/v) acetic acid in water. Then, the sample is cleaned-up on a polymeric based solid phase extraction cartridge. The toxins in the purified extract are quantified by LC-MS/MS.

The study was designed and evaluated according to the International Union for Pure and Applied Chemistry (IUPAC) Harmonised Protocol [**11**]. Statistical analyses were performed along the lines of ISO 5725 [**12**] using the ProLab software [**13**].

3. Design of the study

3.1 The pre-trial

The collaborative pre-trial was conducted for the identification and quantification of the five mentioned *Alternaria* toxins in the range of 1.02 to 403 µg/kg in tomato juice samples using the LC-MS/MS method provided by the EURL Mycotoxin. NRLs, Official Control Laboratories (OCLs), research and private mycotoxin laboratories were invited to participate in the pre-trial of the MVS. The aim of the pre-trial was to allow laboratories to familiarise with the method, to optimise instrument parameters where needed and, most important, to check the detection capability of laboratories' instruments in view of the anticipated working range.

The pre-trial was organised in two turns between March and July 2015. Three tomato juice test samples (pre-trial sample A, B and C) and one blank tomato juice (40 mL) were sent together with the working standard mixture solutions for matrix-matched calibration in dry ice to 25 participants. Only three laboratories out of twenty-five had experience with *Alternaria* toxin analysis. Consequently individually stock solutions were also provided for the laboratories to tune the LC-MS/MS instruments for these compounds. Initially, samples were dispatched to fifteen laboratories in March and the left over samples were sent to ten laboratories after the stability test in May. Collaborators were kindly requested to send back their results within two months after receipt of the samples.

Statistical analysis was performed along the lines of ISO 5725; the outliers and the non-compliant results were excluded from the evaluation. Results are summarised in (**Table 2**). Finally, those laboratories that could analyse all mycotoxins at least at the medium levels were invited to take part in the MVS. Sixteen participants registered for the full collaborative validation of the method including two laboratories that could not participate in the pre-trial, but they had experience in *Alternaria* LC-MS/MS analysis and had taken part in a recent proficiency test on *Alternaria* toxins in tomato juice.

Table 2: The pre-trial results.

	ALT			AOH			AME			TEN			TEA		
Sample	Pre-trial A	Pre-trial B	Pre-trial C	Pre-trial A	Pre-trial B	Pre-trial C	Pre-trial A	Pre-trial B	Pre-trial C	Pre-trial A	Pre-trial B	Pre-trial C	Pre-trial A	Pre-trial B	Pre-trial C
Mean value (µg/kg)	1.02	48.8	5.36	1.54	46.02	7.18	1.17	38.0	5.15	45.8	4.55	403	5.03	< 1	47.3
Repeatability RSD_r %	5.24	5.64	4.70	22.3	20.9	8.02	14.0	20.9	14.4	4.37	6.82	4.56	7.55		6.35
Reproducibility RSD_R %	15.6	20.3	11.8	29.2	21.8	24.2	21.1	20.9	22.7	19.4	23.2	24.5	27.6		26.0

3.2 Time frame

The pre-trial was open to all types of laboratories dealing with mycotoxin determination and capable to apply the method as described. The pre-trial and the MVS were first announced at the annual EURL/NRL workshop in October 2014. In addition, an announcement was sent to the NRLs and research laboratories by email after the workshop. Then, the MVS was published on the website of JRC. Those laboratories that demonstrated sufficient instrument detection capability in the pre-trial were invited to take part in the MVS. Laboratories were requested to register online using a link provided by the EURL Mycotoxin.

After preparing the test materials (**Table 3** and **Annex 1**) for the MVS the outline of the study (**Annex 2**) and the subscription form (**Annex 3**) were sent out to the invited laboratories on 1st of September with a deadline set on 18th of September 2015. In total 16 laboratories registered to the MVS in September 2015.

The test items were dispatched on 6th of October 2015. The reporting deadline was 24th of November 2015.

3.3 Materials and documents

Each participant received:

- An accompanying letter with instructions (**Annex 4**).
- Eighteen units of coded samples in plastic containers (six test samples per matrix) with unknown identity to the participants.
- One blank sample per matrix for matrix-matched calibration.
- Two working standard mixture solutions in methanol for matrix-matched calibration.
- Two samples per matrix labelled "Spike I-II" and "Spike III-IV" for spiking experiments with unknown content of *Alternaria* toxins to the participants.
- Four standard mixture solutions in methanol for spiking experiments with unknown content of *Alternaria* toxins to the participants.
- Dimethyl sulfoxide (8 mL) for sample reconstitution.
- A materials receipt form (**Annex 5**)
- A spiking protocol (**Annex 6**)
- Laboratory specific files with the extension "*.LAB" and "*.LA2", which were generated by the ProLab software, were provided to each laboratory individually (personal files) by email upon dispatch. These files were needed for reporting results and filling out the questionnaire (**Annex 7** and **8**).
- A standard operation procedure (**Annex 9**)
- The critical steps (**Annex 10**)
- Eighty pieces of solid phase extraction cartridges (Strata-XL)
- Eighty pieces of syringe filters (Phenex PTFE)

3.4 Organisation

The 16 laboratories that enrolled in the collaborative trial represented a cross-section of research, private and OCLs from 11 EU Member States and Canada.

Participants had to fill in a questionnaire (**Annex 8**) where they were asked to report any deviations from the standard operation procedure they might have applied. This information was used to identify non compliances.

4. Test Materials

4.1 Description

Test materials were obtained from various sources and some cereal and sunflower seed materials were surplus materials from previous projects. Naturally contaminated tomato juice test samples were additionally spiked to obtain relevant concentration levels of all toxins in the method scope. The cereal and sunflower seed test samples were all naturally contaminated. Test items were remixed where necessary to meet the target levels and coded to maintain an unknown identity to the participants. Each of the contaminated samples and the samples for spiking were analysed as blind duplicates. Additionally one blank sample per matrix was also sent to each participant.

Table 3: The samples analysed in the MVS. Results are obtained from homogeneity test.

Sample description	Test Material	Design	ALT (µg/kg)	AOH (µg/kg)	AME (µg/kg)	TEN (µg/kg)	TEA (µg/kg)
Sample A and B	Sorghum	Blind replicates	< 1	< 1	< 0.1	34.5	185
Sample C and D	Triticale	Blind replicates	< 1	116	10.6	9.79	67.7
Sample E and F	Wheat	Blind replicates	12.8	17.8	40.7	< 3	146
Sample G and H	Tomato juice	Blind replicates	5.49	5.70	6.04	141	171
Sample I and J	Tomato juice	Blind replicates	11.0	11.0	10.7	216	258
Sample K and L	Tomato juice	Blind replicates	20.9	20.6	18.7	523	597
Sample M and N	Unpeeled sunflower seeds	Blind replicates	< 1	23.1	1.88	22.9	615
Sample P and Q	Unpeeled sunflower seeds	Blind replicates	< 1	154	7.37	43.3	1370
Sample R and T	Sunflower mixture	Blind replicates	< 1	55.9	2.69	5.83	363
Spike I-II for cereals	Wheat	For spiking	< 1	< 1	< 0.1	< 1	< 1
Spike III-IV for cereals	Wheat	For spiking	< 1	< 1	< 0.1	< 1	< 1
Spike I-II for tomato	Tomato juice	For spiking	< 1	< 1	< 0.1	< 1	< 1

juice							
Spike III-IV for tomato juice	Tomato juice	For spiking	< 1	< 1	< 0.1	< 1	< 1
Spike I-II for sunflower	Peeled sunflower seeds	For spiking	< 1	< 1	< 0.5	4	20
Spike III-IV for sunflower	Peeled sunflower seeds	For spiking	< 1	< 1	< 0.5	4	20
Blank	Wheat	For calibration	< 1	< 1	< 0.1	< 1	< 1
Blank	Tomato juice	For calibration	< 1	< 1	< 0.1	< 1	< 1
Blank	Peeled sunflower seeds	For calibration	< 1	< 1	< 0.5	4	20

4.2 Preparation

4.2.1 Test samples

Cereal test items were milled using a centrifugal mill (ZM 200, Retsch, Haan, DE) with a 250 µm sieve. The milled material was further homogenized for 4 hours in a Lödige laboratory mixer (Model L20, Paderborn, Germany).

Sunflower seeds were cryo-milled in liquid nitrogen. Test items were packed into plastic containers and labelled with a letter and three digit codes (**Table 3**). The amount of material in each container was about 20 g.

The tomato juice test samples (3 batches, each 2 L) were spiked with standard solutions to obtain the desired levels and homogenised individually with an Ultra Turrax T25 (Janke & Kunkel GmbH, Staufen, Germany) for 1 h at 13000 min^{-1} speed. Then, they were transferred into glass bottles and shaken for 3 h at 200 min^{-1} using a reciprocating shaker (Labortechnik GmbH, Burgwedel, Germany). In order to avoid the possibility of segregation of solids in the juice, aliquots of 10 mL were taken and filled into 15 mL plastic bottles, while the bulk test material was kept on a magnetic stirrer at 600 min^{-1}. Samples were labelled with a letter and three digit codes (**Table 3**). One hundred samples per batch were made. All samples were stored at -18 °C until dispatch.

Blank samples for each matrix were also provided for matrix-matched calibration. These blanks were also filled into the containers labelled "Spike I-II" and Spike "III-IV" (**Table 3**).

4.2.2 Common calibrants

The standard solutions supplied to participants were prepared from the following calibrants:

Altenuene (Toronto Research Chemicals Inc., cat: A575740, lot: 889101-41-1, 98%)

Alternariol (Sigma-Aldrich, cat: A1312, lot: 084M4167V, 97%)

Alternariol monomethyl ether (Sigma-Aldrich, cat: A3171, lot: 045M4017V, 99%)

Tentoxin (Sigma-Aldrich, cat: T8019, lot: 081M4101V, 99.2%)

Tenuazonic acid copper salt (Sigma-Aldrich, cat: T3408, lot: 015M4052V, 99%)

TEA stock solution was prepared from its copper salt and the concentration was checked according to Asam et al. [**6**]. The concentration of TEA stock solution was spectrophotometrically verified applying **Equation 1** below:

$$C_{TEA} = \frac{A_{\max}}{\varepsilon \times l} \qquad \textbf{Equation 1.}$$

where

C_{TEA} is the concentration of stock solution in mol/L;

$A_{\max}$ is the absorption determined on wavelength of 277 nm;

ε is the molar absorption coefficient of TEA in methanol (1.298×10^4 L mol^{-1} cm^{-1})

l is the optical path length of the quartz cell (1 cm).

Working standard mixture solutions (1 and 2), each with all *Alternaria* toxins covered in the MVS were supplied for calibration:

Working standard mixture solution 1:

Mixture of each 100 ng/mL of ALT, AOH and AME, 500 ng/mL of TEN and 1000 ng/mL of TEA in neat methanol.

Working standard mixture solution 2:

Mixture of each 500 ng/mL of ALT, AOH and AME, 2000 ng/mL of TEN and 5000 ng/mL of TEA in neat methanol..

About 50 vials (5 mL) were labelled each with either "Working standard solution 1" or "Working standard solution 2", subsequently filled with 4 mL of the respective solution and crimp-capped. The vials were stored at -18 °C until dispatch. Each participant received two vials (working standard solution 1 and 2). These solutions were used for matrix-matched calibration according to the SOP (**Annex 9**).

4.2.3 Spiking solutions and levels

Four mixed standard solutions containing all *Alternaria* toxins (toxins were the same as mentioned in section 4.2.2) in methanol labelled as "spiking solution A", "spiking solution B", "spiking solution C" and "spiking solution D" were supplied for fortification experiment. The spiking solutions A and B as well as C and D contained an identical solution. Therefore, the spiking experiment was performed at two different levels as blind duplicates.

Spiking solutions A and B:

Neat methanol solution containing each 40 ng/mL of ALT, AOH and AME as well as 1000 ng/mL of each TEN and TEA.

Spiking solutions C and D:

Neat methanol solution containing each 160 ng/mL of ALT, AOH and AME as well as 4000 ng/mL of each TEN and TEA.

Aliquots of 1.5 mL of the mixed standard solutions were filled in labelled HPLC vials and stored at -18 °C until dispatch.

Participants were asked to fortify the samples labelled as "Spike I-II" and "Spiked III-IV" following the spiking protocol provided (**Annex 6**). The spiking volume was 100 µL. The following spiking levels were set (**Table 4**):

Table 4: The contamination levels obtained after spiking.

Matrix	Level	ALT (µg/kg)	AOH (µg/kg)	AME (µg/kg)	TEN (µg/kg)	TEA (µg/kg)
Cereal (wheat)	A - B	2.0	2.0	2.0	50	50
	C - D	8.0	8.0	8.0	200	200
Tomato juice	A - B	2.0	2.0	2.0	50	50
	C - D	8.0	8.0	8.0	200	200
Sunflower seed	A - B	2.0	2.0	2.0	54	70
	C - D	8.0	8.0	8.0	204	220

Sunflower test material for spiking contained naturally 4 µg/kg TEN and 20 µg/kg TEA, resulting in final levels of 54 respectively 204 µg/kg TEN and 70 respectively 220 µg/kg TEA.

4.3 Homogeneity

Homogeneities of the test materials were evaluated according to chapter 3.11.2 of the Harmonised Protocol [**11**]. Ten units were randomly selected. The content of each unit was split and the two sub-samples were randomly analysed for all toxins by LC-MS/MS. No trend was observed during the analysis sequence and samples were found to be homogeneous (**Annex 1**). Sufficient homogeneity was assumed for the calibration and spiking solutions after mixing.

The target standard deviation was calculated using the Horwitz equation modified by Thompson [**14**]:

for analyte concentrations < 120 µg/kg

$$\sigma_p = 0.22 \cdot c$$ **Equation 2.**

for analyte concentrations ≥ 120 µg/kg

$$\sigma_p = 0.02 \cdot c^{0.8495}$$ **Equation 3.**

where:
c is concentration of the measurand expressed as a dimensionless mass ratio, e.g. 1 ppb = 10^{-9}, 1 ppm = 10^{-6}.

In such a case sampling variance should be:

$$\sigma^2_{sam} \leq 0.3\sigma_p$$ **Equation 4.**

or for analyte concentrations < 120 µg/kg:

$$\sigma^2_{sam} \leq 0.07c$$ **Equation 5.**

and for analyte concentrations ≥ 120 µg/kg:

$$\sigma^2_{sam} \leq 0.006 \cdot c^{0.8495}$$ **Equation 6.**

where:
σ^2_{sam}: sampling variance

All test samples passed the criteria.

4.4 Stability

The samples were dispatched in Styropor containers with dry ice to maintain a temperature below 0 °C during shipping. Laboratories were requested to store the test materials at -18 °C upon arrival until analysis.

Test materials that remained at IRMM for stability testing were stored at +4 °C and -18 °C to verify stability, including -70°C as reference temperature. The amounts of mycotoxins in the test materials were monitored (n=2) over a period of eight weeks (from 06/10/2015 until 01/12/2015) with an isochronous stability test as it is suggested in the Harmonised Protocol [**15**]. No significant differences in the results of analysis for the tested dates (06/10/2015; 03/11/2015; 01/12/2015) were found. The materials proved to be adequately stable at +4 °C and -18 °C for the period between dispatch and the deadline for submission of results. This is in agreement with the finding of a recent proficiency test on *Alternaria* toxins in tomato juice [**16**], where test materials were considered stable at -18 °C for at least 4 months.

5. Results and discussions

5.1 General

Each participant reported the analytical results as listed in **Annex 11**. The results were subjected to statistical analysis including outlier removal using ProLab [**13**] and the performance characteristics were calculated as shown in (**Table 5-9**).

The Horwitz ratio (HorRat) values were derived from the Horwitz function modified by Thompson, leading to a constant target standard deviation of 22% for analyte levels below 120 µg/kg [**14**]. A HorRat value between 0.5 and 2.0 is considered to be satisfactory.

Nine naturally contaminated test materials (3 samples per matrix in blind duplicates) were analysed. Furthermore, 2 spiked samples per matrix (low and high levels) in blind duplicates were also measured. Participant answers were checked for deviations from the method protocol. The protocol required blind duplicate analyses, therefore single results were considered non-compliant. Also, the submitted chromatograms were checked to identify results not meeting the identification criteria (i.e. ion ratio error, low chromatographic resolution) set in the SOP. Non-compliant data were removed prior to statistical evaluation. The remaining results underwent statistical data analysis (Grubbs tests applied to single and then multiple suspect mean measurement values and Cochran test applied to any suspect repeatability variances). Statistical analyses were performed along the lines of ISO 5725 [**12, 14**]. The functional relationships between the repeatability/reproducibility standard deviation and the measured value were calculated as described in ISO 5725 Part 2 [**12**] by ProLab software. The results showed that the repeatability for most of the cases was adequate at the validation levels (< 20%), but the reproducibility was higher than 30% for some particular compounds. This suggests that the different LC-MS/MS instruments, in particular their ion sources, used in this study were differently affected by matrix components.

Recoveries were obtained from the values reported for the spiked samples (low and high levels) by applying **Equation 7**. The satisfactory range for recovery was set between 70% and 110%.

$$R\% = \frac{C_{toxin,recovered}}{C_{toxin,spiked}} x100 \qquad \textbf{Equation 7.}$$

5.2 Evaluation of questionnaire – deviations from the method description

All answers to the questionnaire were compiled in the tables in **Annex 13**. All participants were familiar with most of the steps performed during the analysis. According to the collaborators the sample reconstitution step before injection into the LC-MS/MS may be improved. However, the high differences in the polarity of toxins do not allow reconstitution after evaporation to dryness. As CEN/TC 275/WG 5 delegates required the determination of underivatised a suitable procedure had to be implemented allowing the complete dissolution of all *Alternaria* toxins. Taking into account the high polarity and mass fraction range of TEA a polar solvent is favourable for TEA. Furthermore, the chromatographic separation required a polar injection solution (90% water), in order to not deteriorate completely the peak shape of TEA. For the non-polar toxins like AME reconstitution with pure methanol would be favourable, as it was demonstrated in [**10**], but cannot be used because of the short-comings mentioned

above for TEA. Therefore, DMSO was used to aid the reconstitution of the lesser polar toxins, inhibiting a complete dryness state during evaporation.

This sample reconstitution detailed in the SOP was found to be better than the complete evaporation of the eluate after the clean-up during method development. The sample reconstitution has then been done in a 90% water-based solvent, because the mobile phase has the same composition when the sample injection takes place.

Critical points considered for possible non-compliance were significant deviations from the method description and problems/abnormalities reported by the participants (**Annex 13**). Each laboratory followed the provided standard operation procedure step by step, but some problems occurred for participants.

Laboratory 603 reported a problem with the instrumental analysis of sunflower seed and tomato juice samples (**Annex 13**). None of the tomato juice samples could be analysed. The results for sunflower seed samples were rather different to those obtained by other laboratories. This could have been caused due to the HPLC separation problem reported, therefore only the results for cereals were considered for laboratory 603.

Laboratory 612 reported that they did not follow the sample reconstitution step outlined in the SOP (**Annex 13**). This was only for tomato juice samples that were analysed on the first day. The other samples were manipulated appropriately. Consequently, the results on tomato juices were not considered for laboratory 612.

Laboratory 613 reported that the operator who participated in the pre-trial was not available to perform the analysis. Therefore measurements were done by different staff members. It appeared from the results that sometimes the parallel samples were swapped (i.e. Sample P, Q, R, T) or compound identification problems occurred. Hence, clarification on the results was requested, but no answer was returned. The questionnaire that offered room for explaining these problems was not filled out by laboratory 613 (**Annex 13**). Therefore, all the results of laboratory 613 were considered as non-compliant.

Laboratory 614, which did not participate in the pre-trial, reported ion ratio problems in several samples, mainly for ALT, AME and TEN at both low and high concentration levels. In addition, the concentrations detected in the parallel samples showed big differences for some particular compounds. This suggested that the instrument used for analysis was not optimised appropriately, and also unknown matrix interference could play a role. These are evidenced by the chromatograms submitted. Consequently, the results of laboratory 614 were considered as non-compliant.

5.3 Evaluation of chromatograms

Participants were requested to send chromatograms for the analysed samples. They were checked by the study organiser for sufficient resolution between the analyte peaks and neighbouring peaks. Moreover, chromatograms were checked for consistency in the retention time of the *Alternaria* toxin peaks and for sufficient peak intensity.

In the case of laboratory 615 matrix interferences could be seen on the chromatogram of ALT in cereal samples. In addition, the chromatogram of ALT in sunflower showed poor peak intensity for this compound. However, the determination of ALT in tomato juice sample was not compromised by other matrix peaks. The results of laboratory 615 for ALT were considered as non-compliant in cereal and sunflower seed samples due to the non-selective separation and poor peak intensity.

5.4 Evaluation of results

Table 5: Performance characteristics for ALT calculated for each sample analysed during the collaborative trial study.

Sample description	**Sample A, B**	**Sample C, D**	**Sample E, F**	**Spiked**	**Spiked**	**Sample G, H**	**Sample I, J**	**Sample K, L**	**Spiked**	**Spiked**	**Sample M, N**	**Sample P, Q**	**Sample R, T**	**Spiked**	**Spiked**
Matrix	**Cereals**					**Tomato juice**					**Sunflower seeds**				
Sample	Sorghum	Triticale	Wheat	Low level	High level	Tomato juice	Tomato juice	Tomato juice	Low level	High level	Unpeeled	Unpeeled	Mixture	Low level	High level
Number of reported results			16	12	15	15	15	15	12	15				12	15
Number of laboratories considered as non-compliant			3	2	3	3	2	3	3	3				3	5
Number of outliers (laboratories)			0	0	0	1	1	1	0	1				1	0
Number of accepted (quantitative) results			13	10	12	11	12	11	9	11				9	10
Mean value, $\bar{x}$, µg/kg	< 1	< 1	19.5	1.62	6.1	7.67	11.9	24.3	2.15	7.81	< 1	< 1	< 1	1.52	6.71
Repeatability standard deviation s_r, µg/kg			3.47	0.22	0.37	0.45	1.6	1.31	0.3	0.84				0.24	0.53
Repeatability relative standard deviation, RSD_r, %			17.8	13.5	6.13	5.83	13.4	5.39	13.8	10.8				15.8	7.9
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg			9.6	0.61	1.03	1.24	4.42	3.63	0.82	2.33				0.67	1.47
Reproducibility standard deviation s_R, µg/kg			5.5	0.56	1.63	1.13	1.89	4.32	0.42	1.69				0.65	1.67
Reproducibility relative standard deviation, RSD_R, %			28.2	34.8	26.8	14.7	15.9	17.8	19.4	21.6				43.0	24.9
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg			15.2	1.56	4.52	3.13	5.24	12	1.15	4.68				1.81	4.62
Recovery%			n.a.	81	76	n.a.	n.a.	n.a.	107	98				76	84
Relative target standard deviation %			22	22	22	22	22	22	22	22				22	22
HorRat value			1.3	1.6	1.2	0.7	0.7	0.8	0.9	1.0				2.0	1.1

n.a.: not applicable

Table 6: Performance characteristics for AOH calculated for each sample analysed during the collaborative trial study.

Sample description	**Sample A, B**	**Sample C, D**	**Sample E, F**	**Spiked**	**Spiked**	**Sample G, H**	**Sample I, J**	**Sample K, L**	**Spiked**	**Spiked**	**Sample M, N**	**Sample P, Q**	**Sample R, T**	**Spiked**	**Spiked**
Matrix	**Cereals**					**Tomato juice**					**Sunflower seeds**				
Sample	Sorghum	Triticale	Wheat	Low level	High level	Tomato juice	Tomato juice	Tomato juice	Low level	High level	Unpeeled	Unpeeled	Mixture	Low level	High level
Number of reported results		15	16	16	15	15	15	15	14	15	16	16	16	14	16
Number of laboratories considered as non-compliant		2	2	2	3	3	5	4	3	3	3	3	4	3	3
Number of outliers (laboratories)		1	1	2	0	1	0	1	1	0	0	0	0	1	0
Number of accepted (quantitative) results		12	13	12	12	11	10	10	10	12	13	13	12	10	13
Mean value, $\bar{X}$, µg/kg	<1	95.9	13.4	1.84	6.03	5.61	8.77	18.6	2.07	7.17	22.9	139	46.8	1.86	6.05
Repeatability standard deviation s_r, µg/kg		16.7	2.67	0.31	0.70	1.52	1.9	2.52	0.26	0.69	2.55	10.1	2.57	0.3	0.59
Repeatability relative standard deviation, RSD_r, %		17.4	20	16.9	11.5	27.1	21.7	13.6	12.3	9.67	11.1	7.25	5.48	16.1	9.72
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg		46.2	7.39	0.86	1.93	4.21	5.26	6.98	0.71	1.92	7.08	28.1	7.11	0.83	1.63
Reproducibility standard deviation s_R, µg/kg		37.7	3.97	0.49	1.82	2.15	2.37	6.8	0.92	1.11	7.17	41.5	12.3	0.62	1.10
Reproducibility relative standard deviation, RSD_R, %		39.4	29.8	26.7	30.2	38.3	27	36.6	44.3	15.5	31.3	29.7	26.2	33.1	18.2
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg		104	11	1.36	5.1	5.96	6.57	18.8	2.54	3.07	19.9	114	33.9	1.71	3.06
Recovery%		n.a.	n.a.	92	75	n.a.	n.a.	n.a.	103	90	n.a.	n.a.	n.a.	93	76
Relative target standard deviation %		22	22	22	22	22	22	22	22	22	22	21.5	22	22	22
HorRat value		1.8	1.4	1.2	1.4	1.7	1.2	1.7	2.0	0.7	1.4	1.4	1.2	1.5	0.8

n.a.: not applicable

Table 7: Performance characteristics for AME calculated for each sample analysed during the collaborative trial study.

Sample description	**Sample A, B**	**Sample C, D**	**Sample E, F**	**Spiked**	**Spiked**	**Sample G, H**	**Sample I, J**	**Sample K, L**	**Spiked**	**Spiked**	**Sample M, N**	**Sample P, Q**	**Sample R, T**	**Spiked**	**Spiked**
Matrix	**Cereals**					**Tomato juice**					**Sunflower seeds**				
Sample	Sorghum	Triticale	Wheat	Low level	High level	Tomato juice	Tomato juice	Tomato juice	Low level	High level	Unpeeled	Unpeeled	Mixture	Low level	High level
Number of reported results		15	16	15	15	15	15	15	13	14	14	15	15	15	16
Number of laboratories considered as non-compliant		1	3	2	4	3	4	3	3	4	2	1	3	4	2
Number of outliers (laboratories)		0	0	0	0	1	1	1	1	0	3	2	2	1	1
Number of accepted (quantitative) results		14	13	13	11	11	10	11	9	10	9	12	10	10	13
Mean value, $\bar{X}$, µg/kg	< 0.1	8.66	38.1	1.45	4.96	4.78	7.36	14.2	1.93	5.38	1.58	6.77	1.61	1.49	4.24
Repeatability standard deviation s_r, µg/kg		1.35	13.3	0.25	0.91	1.39	2.04	2.29	0.37	0.68	0.48	1.20	0.19	0.26	0.55
Repeatability relative standard deviation, RSD_r, %		15.5	34.8	17.6	18.3	29.1	27.7	16.2	19	12.7	30.2	17.7	11.9	17.6	13.0
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg		3.73	36.7	0.70	2.51	3.85	5.64	6.35	1.02	1.89	1.32	3.32	0.53	0.73	1.52
Reproducibility standard deviation s_R, µg/kg		3.32	16.6	0.52	1.90	1.39	2.86	5.40	0.96	2.12	0.68	2.62	0.46	0.58	1.65
Reproducibility relative standard deviation, RSD_R, %		38.4	43.7	36	38.2	29.1	38.9	38.1	49.6	39.3	43	38.7	28.7	39.1	38.9
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg		9.21	46.1	1.45	5.26	3.85	7.93	15.0	2.65	5.86	1.88	7.25	1.28	1.62	4.56
Recovery%		n.a.	n.a.	72	62	n.a.	n.a.	n.a.	97	67	n.a.	n.a.	n.a.	75	53
Relative target standard deviation %		22	22	22	22	22	22	22	22	22	22	22	22	22	22
HorRat value		1.7	2.0	1.6	1.7	1.3	1.8	1.7	2.3	1.8	2.0	1.8	1.3	1.8	1.8

n.a.: not applicable

Table 8: Performance characteristics for TEN calculated for each sample analysed during the collaborative trial study.

Sample description	**Sample A, B**	**Sample C, D**	**Sample E, F**	**Spiked**	**Spiked**	**Sample G, H**	**Sample I, J**	**Sample K, L**	**Spiked**	**Spiked**	**Sample M, N**	**Sample P, Q**	**Sample R, T**	**Spiked**	**Spiked**
Matrix	**Cereals**					**Tomato juice**					**Sunflower seeds**				
Sample	Sorghum	Triticale	Wheat	Low level	High level	Tomato juice	Tomato juice	Tomato juice	Low level	High level	Unpeeled	Unpeeled	Mixture	Low level	High level
Number of reported results	15	16		16	14	15	15	15	15	15	16	16	16	16	16
Number of laboratories considered as non-compliant	2	4		2	1	3	3	3	3	4	5	4	4	4	3
Number of outliers (laboratories)	1	0		0	0	1	1	1	1	1	0	0	2	1	0
Number of accepted (quantitative) results	12	12		14	13	11	11	11	11	10	11	12	10	11	13
Mean value, $\bar{x}$, µg/kg	51.4	10.4	< 3	40.8	162	152	232	465	48.7	185	36.3	63.5	10.0	45.2	180
Repeatability standard deviation s_r, µg/kg	3.12	0.84		3.65	7.93	9.81	8.44	20.9	2.76	5.23	4.89	7.18	0.49	3.28	11.3
Repeatability relative standard deviation, RSD_r, %	6.07	8.09		8.94	4.9	6.45	3.64	4.49	5.67	2.82	13.5	11.3	4.85	7.25	6.28
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	8.65	2.34		10.1	22.0	27.2	23.4	57.8	7.65	14.5	13.5	19.9	1.35	9.09	31.4
Reproducibility standard deviation s_R, µg/kg	19.2	2.9		10.3	30.5	20.8	17.9	72.8	8.56	20.7	10.1	20.8	3.69	5.12	22.0
Reproducibility relative standard deviation, RSD_R, %	37.3	27.9		25.2	18.8	13.7	7.71	15.7	17.6	11.2	27.7	32.8	36.8	11.3	12.2
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	53.2	8.04		28.4	84.4	57.5	49.6	202	23.7	57.3	28.0	57.7	10.2	14.2	60.9
Recovery%	n.a.	n.a.		82	81	n.a.	n.a.	n.a.	90	91	n.a.	n.a.	n.a.	90	90
Relative target standard deviation %	22	22		22	21	21.2	19.9	18	22	20.6	22	22	22	22	20.7
HorRat value	1.7	1.3		1.2	0.9	0.6	0.4	0.9	0.8	0.5	1.3	1.5	1.7	0.5	0.6

n.a.: not applicable

Table 9: Performance characteristics for TEA calculated for each sample analysed during the collaborative trial study.

Sample description	**Sample A, B**	**Sample C, D**	**Sample E, F**	**Spiked**	**Spiked**	**Sample G, H**	**Sample I, J**	**Sample K, L**	**Spiked**	**Spiked**	**Sample M, N**	**Sample P, Q**	**Sample R, T**	**Spiked**	**Spiked**
Matrix	**Cereals**					**Tomato juice**					**Sunflower seeds**				
Sample	Sorghum	Triticale	Wheat	Low level	High level	Tomato juice	Tomato juice	Tomato juice	Low level	High level	Unpeeled	Unpeeled	Mixture	Low level	High level
Number of reported results	16	16	16	16	15	15	15	15	15	15	15	15	16	16	16
Number of laboratories considered as non-compliant	3	4	2	2	3	3	3	4	3	3	5	4	5	5	4
Number of outliers (laboratories)	0	0	0	0	0	2	2	3	2	2	0	0	0	0	0
Number of accepted (quantitative) results	13	12	14	14	12	10	10	8	10	10	10	11	11	11	12
Mean value, $\bar{x}$, µg/kg	206	57.1	125	46.9	165	168	259	563	51.5	186	804	1102	452	53	153
Repeatability standard deviation s_r, µg/kg	33.8	7.27	14.2	3.91	12.7	11.3	8.44	11.2	2.26	4.35	151	164	68.9	5.49	17.7
Repeatability relative standard deviation, RSD_r, %	16.4	12.7	11.4	8.32	7.7	6.76	3.26	2.0	4.39	2.33	18.8	14.9	15.3	10.4	11.6
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	93.6	20.1	39.3	10.8	35.2	31.4	23.4	31.0	6.27	12.0	418	454	191	15.2	49.0
Reproducibility standard deviation s_R, µg/kg	57.3	21.8	27.9	9.09	21.8	25.8	31.8	76.2	7.15	19.8	318	422	198	18.9	39.4
Reproducibility relative standard deviation, RSD_R, %	27.9	38.2	22.4	19.4	13.2	15.4	12.3	13.5	13.9	10.6	39.5	38.3	43.7	35.7	25.8
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	159	60.4	77.2	25.1	60.5	71.6	88.0	211	19.8	54.9	880	1170	547	52.4	109
Recovery, %	n.a.	n.a.	n.a.	94	82	n.a.	n.a.	n.a.	74	85	n.a.	n.a.	n.a.	76	70
Relative target standard deviation %	20.3	22	21.9	22	21	20.9	19.6	17.4	22	20.6	16.5	15.8	18	22	21.2
HorRat value	1.4	1.7	1.0	0.9	0.6	0.7	0.6	0.8	0.6	0.5	2.4	2.4	2.4	1.6	1.2

n.a.: not applicable

As EU legislation for food requires to consider analyte recovery for accepting or rejection of lots in official food control, the principle of recovery correction was applied for the calculation of method performance in this study. As a result, the data sets of the analytical results from naturally contaminated materials were corrected with the mean recovery value of the recovery experiments (two duplicates). The result of this treatment on the calculated method performance is shown in **Annex 12**. A correction for recovery with the data generated by spiking experiments did not change to a significant extent the reproducibility of the method.

6. Interpretation of the results and conclusions

In total, 15 samples (five samples per matrix) had to be analysed as blind duplicates during the MVS. The applicable range was found to be 1.61 to 1102 µg/kg across different *Alternaria* toxins in the naturally contaminated samples. The required range was 1 to 1000 µg/kg.

The repeatability was below 20% for ALT, TEN and TEA, but exceeded 20% for AOH and AME in two and three samples, respectively. Overall, the repeatability varied between 2.0% and 34.8%.

The reproducibility ranged from 7.7% to 49.6%, reflecting HorRat values from 0.5 to 2.4 according to the Horwitz function modified by Thompson. . HorRat values were between 0.4 and 2.0 for ALT, AOH and TEN. HorRat values higher than 2 were calculated for AME and TEA in one and three samples, respectively. In the case of AME a HorRat value of 2.3 was computed for spiked tomato juice. A HorRat value of 2.4 was calculated for TEA in all naturally contaminated sunflowers, while HorRat values of 1.2 and 1.6 were obtained for TEA in spiked sunflower samples. HorRat values calculated for TEN in spiked sunflower samples were three times better than those obtained in naturally contaminated sunflower samples. These are thought to be because the matrix matched calibration as well as the spiking experiments were done using peeled sunflower material. Unpeeled sunflower seeds could not be used for matrix matched calibration, because all available materials of that kind contained high levels of *Alternaria* toxins. However, unpeeled sunflower seeds were needed to obtain test material with sufficient levels of natural contamination. This represents a more complex matrix than the peeled sunflower seeds. These differences in performance characteristics seen between peeled and unpeeled sunflower seeds demonstrate that matrix effects influence the determination of *Alternaria* toxins.
These matrix effects have been compensated using stable isotope labelled internal standards in studies on other mycotoxins in complex matrices [**9**]. However, the stable labelled standards needed for this MVS are currently not commercially available for *Alternatria* toxins.

Recoveries for *Alternaria* toxins were between 70% and 110%, with the exception of AME. This is due to the low solubility of AME in the water based injection solution and to the high matrix effect in the ion source caused by the co-eluting matrix compounds. The recoveries for AME varied from 53% to 67% at the higher level (8 µg/kg), while the recoveries were above 70% for AME at the lower level (2 µg/kg). Overall, mean absolute recoveries ranged from 53% to 107%.

Low precision and recovery were due to the strong matrix effects caused by the co-eluting matrix solutes, in agreement with what has recently been found by Walravens et al. [**10]** and Tölgyesi et al. [**17**]. In addition, the wide polarity difference (**Figure 1**) between *Alternaria* toxins also influenced the performance characteristics. The sample reconstitution in water-based injection solution is prone to lead to low recoveries for AME, the most non-polar compound in this study.

In order to allow the injection of TEA onto the HPLC system with injection solutions containing higher amounts of organic solvent, a pentafluorophenyl (F5) column was tested. These column types are alternatives to standard C-18 reversed phase columns, generally providing a good separation of both polar and non-polar compounds, thus offering improved peak parameters – especially for TEA – as well as better resolution for the remaining toxins of interest. However, this approach failed to give an acceptable peak shape for TEA, in both, acidic and alkaline mobile phases (**Annex 14**). As a result, the method protocol will include a standard C-18 HPLC column, while the organic solvent fraction in the injection solvent could not be increased to a level of >10% and required the use of DMSO. This means that injecting underivatised TEA together with the lesser polar toxins remained a critical compromise between a sufficient peak shape for TEA on

one hand and the quantitative re-dissolution of the less polar toxins, such as AOH and AME on the other hand, especially taking into account the desired measurement capacity.

As a result the method will be submitted to CEN TC 275 / WG 5 for consideration as basis for a future CEN standard.

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List of abbreviations and definitions

ALT	Altenuene
AME	Alternariol monomethyl ether
AOH	Alternariol
CEN	European Committee for Standardisation
EC	European Commission
EU	European Union
EURL	European Union Reference Laboratory
F5 column	Pentafluorophenyl column
HPLC	High-performance liquid chromatography
IRMM	Institute for Reference Materials and Measurements
ISO	International Organisation for Standardisation
IUPAC	International Union for Pure and Applied Chemistry
JRC	Joint Research Centre
LC-MS/MS	Liquid chromatography tandem mass spectrometry
TEA	Tenuazonic acid
TEN	Tentoxin

Repeatability: Precision under repeatability conditions, i.e. conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time. (ISO 3534-1)

Reproducibility: Precision under reproducibility conditions, i.e. conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment. (ISO 3534-1)

HorRat value: ratio of the reproducibility relative standard deviation to the target standard deviation (calculated by Horwitz equation modified by Thompson for the concentration below 120 ppb)

Cochran test: removal of laboratories showing significantly greater variability among replicate (within-laboratory) analyses than the other laboratories for a given material

Grubbs test: removal of laboratories with extreme averages

7. Annexes

7.1 Annex 1 – Homogeneity

Concentrations given here may be different from the consensus values of results of participants. These are rough estimates of concentrations obtained with other calibration solution. All data below is given in [µg/kg].

Sample A and B

Homogeneity according to IUPAC	Analyte				
	ALT	AOH	AME	TEN	TEA
Mean	< 1	< 1	< 0.1	34.5	185
$\hat{\sigma}$				7.60	40.8
σ^2_{all}				5.19	150
σ^2_{an}				4.14	149
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)				13.9	431
σ^2_{sam}				3.71	34.3
σ^2_{sam} < critical	n.a	n.a	n.a.	Passed	Passed

n.a.: not applicable

Sample C and D

Homogeneity according to IUPAC	Analyte				
	ALT	AOH	AME	TEN	TEA
Mean	< 1	116	10.6	9.79	67.4
$\hat{\sigma}$		46.5	4.1	2.15	14.8
σ^2_{all}		193	1.53	0.417	19.8
σ^2_{an}		102	1.50	0.769	71.8
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)		470	3.09	1.56	109.8
σ^2_{sam}		192	1.50	0.025	0.0
σ^2_{sam} < critical	n.a	Passed	Passed	Passed	Passed

n.a.: not applicable

Sample E and F

Homogeneity according to IUPAC	Analyte				
	ALT	AOH	AME	TEN	TEA
Mean	12.8	17.8	40.7	< 2	146
$\hat{\sigma}$	2.81	3.92	8.95		35.1
σ^2_{all}	0.707	1.38	7.20		111
σ^2_{an}	0.915	0.961	26.3		261
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)	2.25	3.57	40.1		472
σ^2_{sam}	0.137	0.467	0.0		107
σ^2_{sam} < critical	Passed	Passed	Passed	n.a	Passed

n.a.: not applicable

Sample G and H

Homogeneity according to IUPAC	Analyte				
	ALT	AOH	AME	TEN	TEA
Mean	5.49	5.70	6.04	141	171
$\hat{\sigma}$	1.21	1.25	1.33	31.0	37.6
σ^2_{all}	0.131	0.141	0.159	86.2	127
σ^2_{an}	0.334	0.164	0.459	48.4	173
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)	0.58	0.43	0.76	211	415
σ^2_{sam}	0.019	0.0	0.0	5.58	0.0
σ^2_{sam} < critical	Passed	Passed	Passed	Passed	Passed

Sample I and J

Homogeneity according to IUPAC	Analyte				
	ALT	AOH	AME	TEN	TEA
Mean	11.0	11.0	10.7	216	258
$\hat{\sigma}$	2.43	2.43	2.35	47.6	56.8
σ^2_{all}	0.530	0.530	0.496	204	291
σ^2_{an}	0.308	0.30	1.87	194	540
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)	1.31	1.30	2.82	579	1092
σ^2_{sam}	0.317	0.158	0.0	71.5	0.0
σ^2_{sam} < critical	Passed	Passed	Passed	Passed	Passed

Sample K and L

Homogeneity according to IUPAC	Analyte				
	ALT	AOH	AME	TEN	TEA
Mean	20.9	20.6	18.7	523	597
$\hat{\sigma}$	4.59	4.52	4.11	115	131
σ^2_{all}	1.89	1.84	1.52	1190	1550
σ^2_{an}	3.42	3.038	3.16	1005	2132
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)	7.02	6.53	6.05	3253	5069
σ^2_{sam}	0.0	0.0	0.684	415	276
σ^2_{sam} < critical	Passed	Passed	Passed	Passed	Passed

Sample M and N

Homogeneity according to IUPAC	Analyte				
	ALT	AOH	AME	TEN	TEA
Mean	< 1	23.1	1.88	22.9	615
$\hat{\sigma}$		5.082	0.414	5.049	148
σ^2_{all}		2.32	0.015	2.29	1963
σ^2_{an}		5.82	0.1312	25.5	12902
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)		10.3	0.16	30.1	16724
σ^2_{sam}		0.019	0.011	0.34	0.0
σ^2_{sam} < critical	n.a	Passed	Passed	Passed	Passed

n.a.: not applicable

Sample P and Q

Homogeneity according to IUPAC	Analyte				
	ALT	AOH	AME	TEN	TEA
Mean	< 1	154	7.37	43.3	1370
$\hat{\sigma}$		49.3	2.21	9.52	301
σ^2_{all}		656	0.44	8.15	8176
σ^2_{an}		123	1.12	18.33	9334
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)		536	1.97	33.8	24800
σ^2_{sam}		207	0.373	26.3	10095
σ^2_{sam} < critical	n.a	Passed	Passed	Passed	Passed

n.a.: not applicable

Sample R and T

Homogeneity according to IUPAC	Analyte				
	ALT	AOH	AME	TEN	TEA
Mean	< 1	55.9	2.69	5.83	363
$\hat{\sigma}$		12.3	0.591	1.34	79.9
σ^2_{all}		13.6	0.031	0.162	574
σ^2_{an}		40.9	0.397	0.508	1701
critical value ($F_1\ \sigma^2_{all} + F_2\ \sigma^2_{an}$)		67	0.46	0.82	2798
σ^2_{sam}		8.67	0.0	0.155	1818
σ^2_{sam} < critical	n.a	Passed	Passed	Passed	Passed

n.a.: not applicable

7.2 Annex 2 – Outline of the study

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
European Union Reference Laboratory for Mycotoxins

EURL European Union Reference Laboratory Mycotoxins

Geel, 22 September 2015

Method validation study on the determination of *Alternaria* toxins in wheat, tomato juice and sunflower seeds by solid phase extraction clean-up and liquid chromatography with tandem mass spectrometric detection

Dear Participant,

The EU-RL Mycotoxins organises a method validation study (by inter-laboratory comparison) on the determination of *Alternaria* toxins in wheat, tomato juice and sunflower seeds. The study is foreseen to take place in **October 2015**.

Please read the following information carefully.

Timing

Participants will receive **two weeks** before the starting of the exercise a **preannouncement** of the sample dispatch.
A second reminder will be sent the **day before dispatch** of samples and participants will receive a dispatch note containing all data for tracking the shipment.

We ask you to report results back within **six weeks;** including the modalities which will be detailed in following communications.

Materials supplied for the study

Participants will receive **two** parcels: one parcel will be sent at room temperature, the other will be dispatched in dry ice.

First parcel will contain the following items:

1. The necessary **solid phase extraction columns** and **syringe filters** (taking also into account possible repetition of a failed analysis).
2. The standard operating procedure (**SOP**) to be applied for the analysis of the test samples and the spiked samples.
3. A manual on the **critical steps**.
4. The **spiking protocol.**
5. A "**Material receipt form**". If the materials/consumables have been received damaged, immediately request a new one (the materials and standard solutions will be shipped in dry ice; store the samples at **-18 °C** until subjected to analysis).

Second parcel will contain the following items:

1. Two ***Alternaria* working standard mixture** solutions (1 and 2) to be used for preparing the **calibration** solutions.
2. Four ***Alternaria* spiking standard mixture** solutions (A, B, C and D) to be used for fortifying the samples.
3. **Dimethyl sulfoxide (DMSO)** for sample reconstitution (store the DMSO at **room temperature**).
4. A set of samples, comprising:
   a. **Six test materials/matrix** for single analysis with different content levels of *Alternaria* toxins.
   b. **One blank sample/matrix** to be used for matrix-matched calibration and for blank.
   c. **Two samples/matrix** to be used for preparing the spiked samples.

Participants will also receive by email, after dispatch of samples, instructions how to **report results** and fill in the **questionnaire** using **ProLab** software.

Participants will be asked to analyse each sample once and to report the requested results **in µg/kg** for both test materials and spiked samples.
They will be also asked to send to the organiser the chromatograms of calibration solutions and samples as specified the SOP.

In case of questions please do not hesitate to contact us at the following address:

Adám Tölgyesi
Institute for Reference Materials and Measurements (IRMM)
EU-RL Mycotoxins
Retieseweg 111
B-2440 Geel, Belgium
Tel: +32-14-571 313
FAX: +32-14-573 015
E-mail: jrc-irmm-eurl-mycotox@ec.europa.eu

With kind regards,

Adám Tölgyesi

7.3 Annex 3 – Subscription form

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
European Union reference laboratory for mycotoxins

EURL European Union Reference Laboratory Mycotoxins

Geel, 22 September 2015

Subscription questionnaire for inter-laboratory study

Determination of ***Alternaria*** **toxins in wheat, tomato juice and sunflower seeds** by solid phase extraction clean-up and liquid chromatography with tandem mass spectrometric detection

Participants data (contact person and affiliation details):

Title:

Name + SURNAME:

Institute:

Department:

Street, number:

City:

Post code:

Country:

Phone:

Fax:

e-mail:

Please read carefully the following before signing

Retieseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://irmm.jrc.ec.europa.eu
Telephone: direct line (32-14) 571 313. Fax: (32-14) 573015.

E-mail: jrc-irmm-crl-mycotox@ec.europa.eu

1. Having read the attached method and the outline of the study, we understand that:
   a. All essential apparatus, chemicals and other requirements specified in the method protocol attached to this form must be available in our laboratory when the programme begins;
   b. Timing requirements, such as starting date, order of testing specimens and time for reporting will be respected and possible delay communicated in due time;
   c. The method must be strictly followed;
   d. Samples must be handled according to instructions;
   e. A qualified operator must perform the measurements;

2. Comments you wish to address before participation:

3. Our Laboratory is interested to participate in this method validation study (*collaborative trial*).

YES **NO**

Signature:_____

Once you filled-in the form, print it, sign the hardcopy and email it to: JRC-IRMM SFB, Ádám Tölgyesi, Retieseweg 111, B-2440 Geel, Belgium; E-mail: jrc-irmm-eurl-mycotox@ec.europa.eu

7.4 Annex 4 – Instructions to the participants

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
EU reference laboratory for mycotoxins

EURL
European Union Reference Laboratory
Mycotoxins

Dear Participant,

On behalf of the EU-RL for Mycotoxins, I announce the opening of the inter-laboratory validation of the method for the determination of *Alternaria* toxins in wheat, tomato juice and sunflower seeds.

I thank you for joining the study and ask you, in order to obtain consistent results, to please follow all instructions included in the documents you received.

In particular, you should note the following:

1. Please check that the content of the parcels is **complete and undamaged** (and fill out and e-mail the enclosed receipt form).
2. Please store goods at appropriate conditions (**-18 °C for standard solutions and test materials, room temperature for dimethyl sulfoxide (DMSO) and consumables**) until the analysis. Let materials reach ambient temperature before use.
3. Your participation code will be randomly generated by ProLab software and sent by email.
4. Read **all** accompanying documents before starting the analysis. **THE METHOD PROTOCOL MUST BE FOLLOWED.** In particular the following points should be remarked:
   - Analyse only **one matrix per day** and apply matrix-matched calibration outlined in the method protocol.
   - The amount of sample to be extracted (**2.00** g) should not deviate from the one indicated in the method protocol. This is of crucial importance due to the material homogeneity requirements.
   - All samples should be homogenised before taking the test portion for performing the analysis.
5. Make sure that all required instruments and consumables are at hand before starting the analysis.
6. **Analyse each sample only once**. In case you encounter any problem during the analysis, please contact us for a replacement of the lost sample.

Retieseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://irmm.jrc.ec.europa.eu
Telephone: direct line (32-14) 571 229. Fax: (32-14) 571783.

E-mail: jrc-irmm-crl-mycotox@ec.europa.eu

7. **Reporting the results:**

The data generated by the participants will be collected by using RingDat software, supplementary to ProLab software.
You will receive by mail two files for reporting results, please follow these instructions:

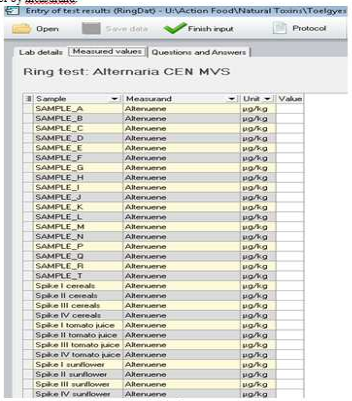
a. Download a simple data entry program RingDat from the QuoData web page using the following link: http://quodata.de/ringdat_en.php. You do not have to register there, just use the username and password provided:

Username: *ringdat*
Password: *prolabdata*

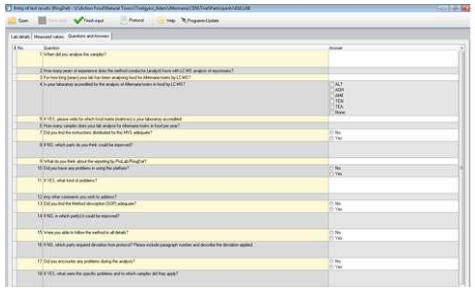
Save the file on your computer.

b. To the same folder where you have saved the RingDat file, save the two lab specific files with the extension **"*.LAB"** and **"*.LA2"**, that were generated by the ProLab software and provided to each laboratory individually (personal files) with this mail.

c. Start the RingDat.exe program and open the **"*.LAB"** file for reporting the results. A table will appear with cells for every measurand/sample combination
- The **"*.LA2"** file contains information about the participant – laboratory name and laboratory code;
- The **"*.LAB"** file is unique to each laboratory (personal) and contains information about the samples and measurands that have to be analysed and reported.
- The first tab contains general information about the laboratory.
- The second tab contains a table for entering the results. You can sort the entries by sample or by measurand:



- The third tab contains a general questionnaire:



d. Fill in the result table with your data. **DO NOT** CORRECT ANY RESULTS FOR RECOVERY. Please report only **ONE** final value per measurand per sample in **µg/kg**.

e. Afterwards, please fill in the questionnaire on the next tab.

f. After finishing the input, save the file using the button on the top menu of the window. You can still change the inputs after saving the file as long as you have not pushed the "Finish input" button. At the end finalise the data entry by pushing the "Finish input" button.

g. Send both the **"*.LAB"** and **"*.LA"** files back to us by e-mail on our functional mail box - JRC-IRMM-EURL-MYCOTOX@ec.europa.eu

h. If you want **to correct some of your entries after finishing the input, you shall overwrite the *.LA2 file with original one** downloaded from the mail. This will allow you to modify the data but you will have to fill the questionnaire out, again. Then, you need to finish input, again.

8. Please also send back the **chromatogram of the third calibration level** for each matrix. They can be sent back by e-mail (jrc-irmm-eurl-mycotox@ec.europa.eu).

The deadline for this collaborative trial is **24/11/2015** which gives a time period of **six weeks** for all experiments. We are looking forward to hear from you and hope the method suits your needs for future use.

A detailed outline of the study is included in the MVS sample parcel together with the spiking protocol and the method protocol (SOP); in addition, in this document you find further details. Anyhow we would like to encourage you to contact us, in case you seek further clarification, at the following address: jrc-irmm-eurl-mycotox@ec.europa.eu

With kind regards,

7.5 Annex 5 – Materials receipt form

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Mycotoxins

EURL
European Union Reference Laboratory
Mycotoxins

RECEIPT FORM

Name of Participant	
Affiliation	
Lab code	
Country	

NOTE: UPON RECEIPT STORE THE CONSUMABLES AND DIMETHYL SULFOXIDE AT ROOM TEMPERATURE AND THE WORKING/SPIKING SOLUTIONS AND THE MATERIALS IN A FREEZER (AT -18 °C)

You will receive **two** parcels, please fill in the material receipt form after receiving both parcels. Please ensure that the items listed below have been received undamaged, and then check the relevant statement in the **table at next page**:

Contents of parcels

a) A copy of the instructions
b) The SOP of the method
c) The critical steps (operation manual)
d) The spiking protocol
e) Three test materials identified for blank (one blank/matrix for matrix-matched calibration)
f) Eighteen coded test materials for direct analysis (six sample/matrix)
g) Five test materials identified for spiking (2-2 samples for cereals and tomato juice and 1 sample for sunflower)
h) Two working standard solutions (1 and 2) for calibration
i) Four spiking solutions (A, B, C and D)
j) Dimethyl sulfoxide (DMSO)
k) 80 Strata-XL SPE cartridges and 80 syringe filters

Date of the receipt of the test materials/consumables	
All items have been received undamaged	YES / NO
If NO, please list damaged items according to the letters associated at each item in the list above Please write one item per row	
Items are missing	YES / NO
If YES, please list missing items according to the letters associated at each item in the list above Please write one item per row	
Serial numbers of the test samples you received	Sample A __
	Sample B __
	Sample C __
	Sample D __
	Sample E __
	Sample F __
	Sample G __
	Sample H __
	Sample I __
	Sample J __
	Sample K __
	Sample L __
	Sample M __
	Sample N __
	Sample P __
	Sample Q __
	Sample R __
	Sample T __
Samples to be spiked	Cereals for SPIKE I-II
	Cereals for SPIKE III-IV
	Tomato juice for SPIKE I-II
	Tomato juice for SPIKE III-IV
	Sunflower for SPIKE I-II and SPIKE III-IV
Working standard solution	1 and 2
Spiking solutions	A, B, C and D
Dimethyl sulfoxide (DMSO)	

SIGNATURE: _____

Please email the completed form to:

Ádám Tölgyesi
European Commission - DG Joint Research Centre
Institute for Reference Materials and Measurements
B-2440 Geel, Belgium
Email: jrc-irmm-eurl-mycotox@ec.europa.eu

7.6 Annex 6 – Spiking protocol

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

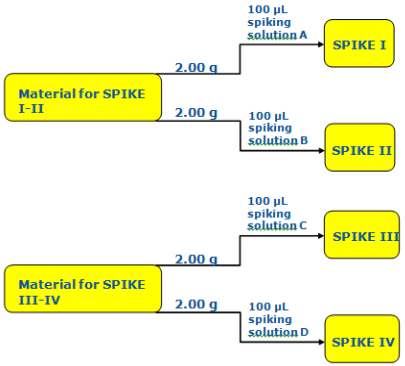
Institute for reference materials and measurements
Community reference laboratory for mycotoxins

EURL
European Union Reference Laboratory
Mycotoxins

SPIKING PROTOCOL FOR THE VALIDATION STUDY ON THE DETERMINATION OF *ALTERNARIA* TOXINS IN WHEAT, TOMATO JUICE AND SUNFLOWER SEEDS

The cooled box contains four vials labelled Spiking solution **A**, **B**, **C** and **D**. Materials to be spiked are also included in the parcel. In the case of **cereal** and **tomato juice** materials are labelled **"for SPIKE I-II"** or **"for SPIKE III-IV"**. For **sunflower** only **one** material is provided to be spiked and it is labelled **"for SPIKE I-II** and **SPIKE III-IV"**. Prepare **four** spiked samples for each matrix using the materials and spiking solutions provided. The solvent of the *Alternaria* spiking solutions is methanol.

For spiking experiments, proceed as follows:

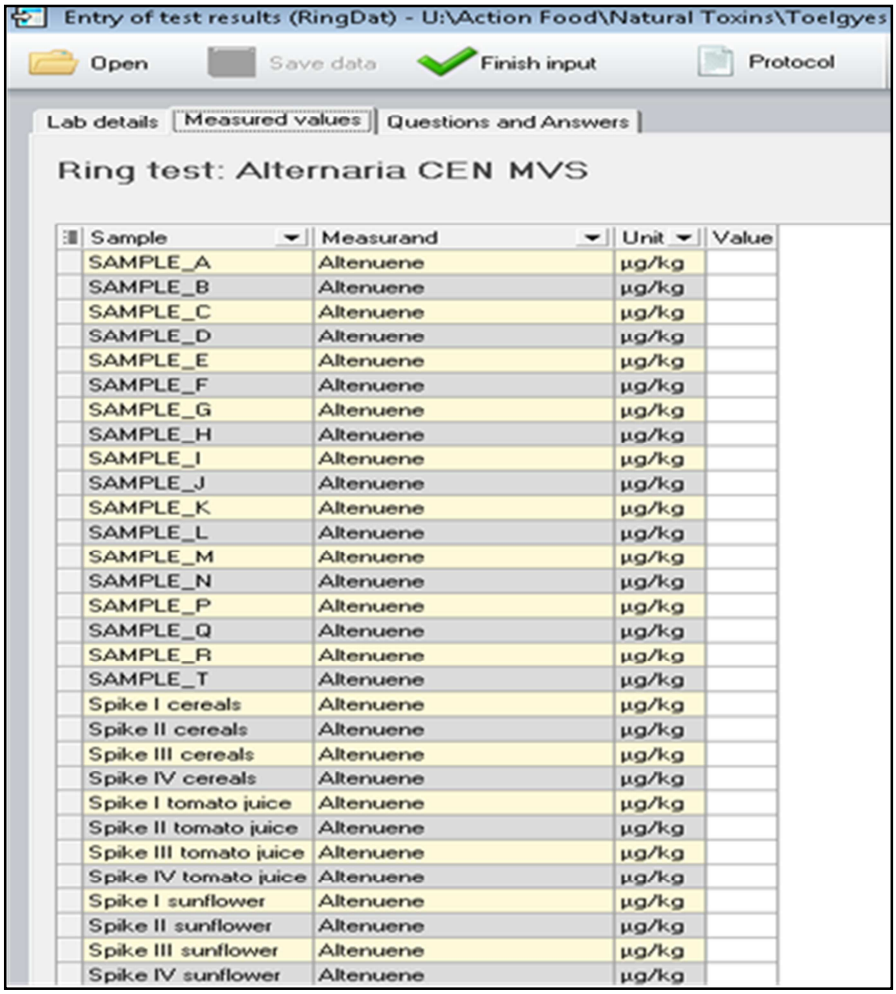


Retieseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://irmm.jrc.ec.europa.eu
Telephone: direct line (32-14) 571 229. Fax: (32-14) 571783.

E-mail: jrc-irmm-eurl-mycotox@ec.europa.eu

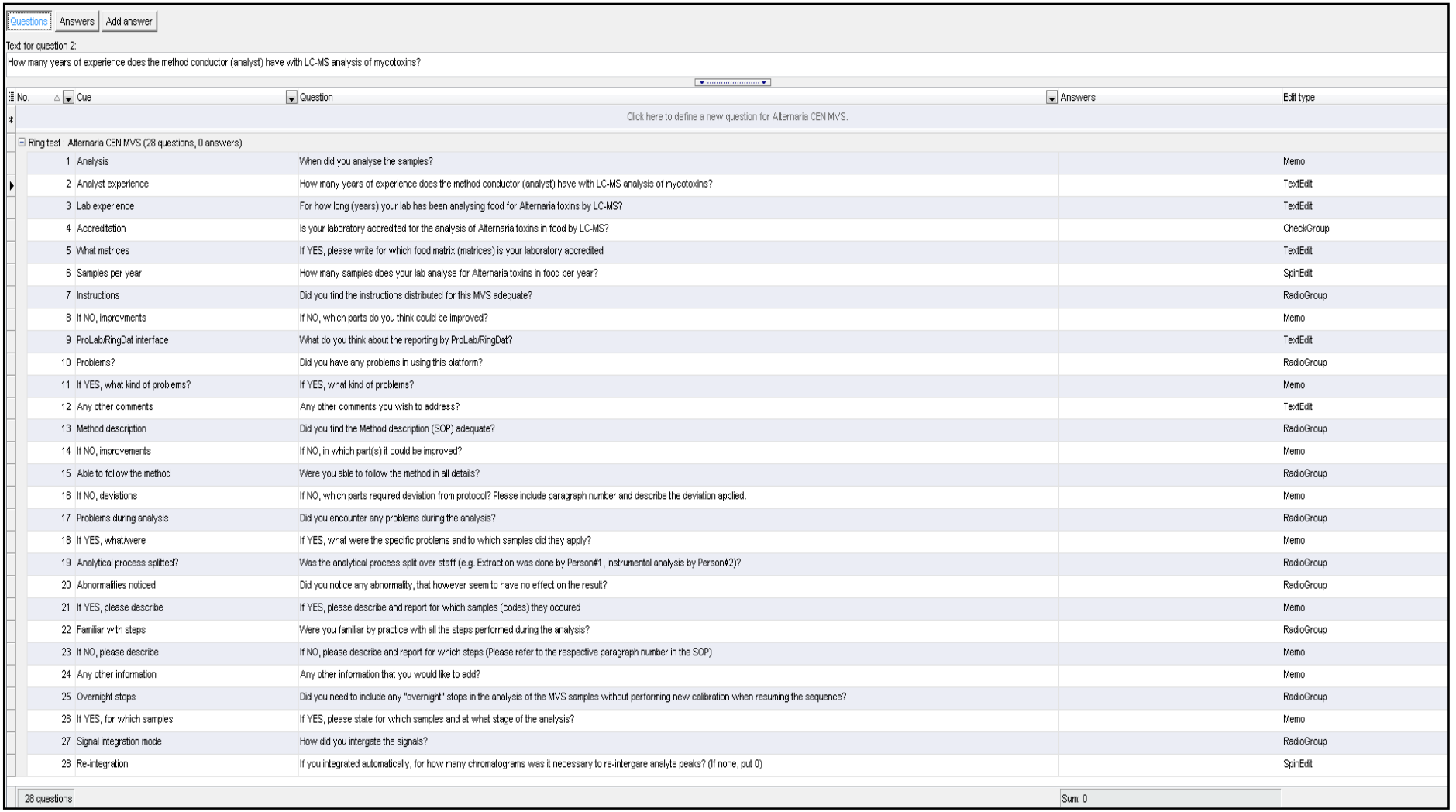
- Weigh **2.00 g** "material for **SPIKE I-II**" in 50 mL centrifuge tube **twice** and label the first material as **SPIKE I** and label the other material as **SPIKE II.**
- Fortify them with **different** spiking solutions (**A** and **B**).
- Add **exactly 100 µL** of "**Spiking solution A**" to the sample labelled **SPIKE I**.
- Add **exactly 100 µL** of "**Spiking solution B**" to the sample labelled **SPIKE II**.
- Weigh **2.00 g** "material for **SPIKE III-IV**" in 50 mL centrifuge tube **twice** and label the first material as **SPIKE III** and label the other material as **SPIKE IV.**
- Add **exactly 100 µL** of "**Spiking solution C**" to the sample labelled **SPIKE III**.
- Add **exactly 100 µL** of "**Spiking solution D**" to the sample labelled **SPIKE IV**.
- Let the samples stand for at least 1 h at room temperature to allow the solvent of the spiking solution to evaporate and the toxins to migrate into the matrix.
- Analyse the spiked samples according to the method protocol.

7.7 Annex 7 – Results form



Sample	Measurand	Unit	Value
SAMPLE_A	Altenuene	µg/kg	
SAMPLE_B	Altenuene	µg/kg	
SAMPLE_C	Altenuene	µg/kg	
SAMPLE_D	Altenuene	µg/kg	
SAMPLE_E	Altenuene	µg/kg	
SAMPLE_F	Altenuene	µg/kg	
SAMPLE_G	Altenuene	µg/kg	
SAMPLE_H	Altenuene	µg/kg	
SAMPLE_I	Altenuene	µg/kg	
SAMPLE_J	Altenuene	µg/kg	
SAMPLE_K	Altenuene	µg/kg	
SAMPLE_L	Altenuene	µg/kg	
SAMPLE_M	Altenuene	µg/kg	
SAMPLE_N	Altenuene	µg/kg	
SAMPLE_P	Altenuene	µg/kg	
SAMPLE_Q	Altenuene	µg/kg	
SAMPLE_R	Altenuene	µg/kg	
SAMPLE_T	Altenuene	µg/kg	
Spike I cereals	Altenuene	µg/kg	
Spike II cereals	Altenuene	µg/kg	
Spike III cereals	Altenuene	µg/kg	
Spike IV cereals	Altenuene	µg/kg	
Spike I tomato juice	Altenuene	µg/kg	
Spike II tomato juice	Altenuene	µg/kg	
Spike III tomato juice	Altenuene	µg/kg	
Spike IV tomato juice	Altenuene	µg/kg	
Spike I sunflower	Altenuene	µg/kg	
Spike II sunflower	Altenuene	µg/kg	
Spike III sunflower	Altenuene	µg/kg	
Spike IV sunflower	Altenuene	µg/kg	

7.8 Annex 8 – Questionnaire



Questions | Answers | Add answer

Text for question 2:

How many years of experience does the method conductor (analyst) have with LC-MS analysis of mycotoxins?

No.	Cue	Question	Answers	Edit type
*		Click here to define a new question for Alternaria CEN MVS.		
	Ring test : Alternaria CEN MVS (28 questions, 0 answers)			
1	Analysis	When did you analyse the samples?		Memo
2	Analyst experience	How many years of experience does the method conductor (analyst) have with LC-MS analysis of mycotoxins?		TextEdit
3	Lab experience	For how long (years) your lab has been analysing food for Alternaria toxins by LC-MS?		TextEdit
4	Accreditation	Is your laboratory accredited for the analysis of Alternaria toxins in food by LC-MS?		CheckGroup
5	What matrices	If YES, please write for which food matrix (matrices) is your laboratory accredited		TextEdit
6	Samples per year	How many samples does your lab analyse for Alternaria toxins in food per year?		SpinEdit
7	Instructions	Did you find the instructions distributed for this MVS adequate?		RadioGroup
8	If NO, improvments	If NO, which parts do you think could be improved?		Memo
9	ProLab/RingDat interface	What do you think about the reporting by ProLab/RingDat?		TextEdit
10	Problems?	Did you have any problems in using this platform?		RadioGroup
11	If YES, what kind of problems?	If YES, what kind of problems?		Memo
12	Any other comments	Any other comments you wish to address?		TextEdit
13	Method description	Did you find the Method description (SOP) adequate?		RadioGroup
14	If NO, improvements	If NO, in which part(s) it could be improved?		Memo
15	Able to follow the method	Were you able to follow the method in all details?		RadioGroup
16	If NO, deviations	If NO, which parts required deviation from protocol? Please include paragraph number and describe the deviation applied.		Memo
17	Problems during analysis	Did you encounter any problems during the analysis?		RadioGroup
18	If YES, what/were	If YES, what were the specific problems and to which samples did they apply?		Memo
19	Analytical process splitted?	Was the analytical process split over staff (e.g. Extraction was done by Person#1, instrumental analysis by Person#2)?		RadioGroup
20	Abnormalities noticed	Did you notice any abnormality, that however seem to have no effect on the result?		RadioGroup
21	If YES, please describe	If YES, please describe and report for which samples (codes) they occured		Memo
22	Familiar with steps	Were you familiar by practice with all the steps performed during the analysis?		RadioGroup
23	If NO, please describe	If NO, please describe and report for which steps (Please refer to the respective paragraph number in the SOP)		Memo
24	Any other information	Any other information that you would like to add?		Memo
25	Overnight stops	Did you need to include any "overnight" stops in the analysis of the MVS samples without performing new calibration when resuming the sequence?		RadioGroup
26	If YES, for which samples	If YES, please state for which samples and at what stage of the analysis?		Memo
27	Signal integration mode	How did you intergate the signals?		RadioGroup
28	Re-integration	If you integrated automatically, for how many chromatograms was it necessary to re-intergare analyte peaks? (If none, put 0)		SpinEdit

28 questions | Sum: 0

7.9 Annex 9 – Standard operation procedure

Determination of *Alternaria* toxins in tomato, wheat and sunflower seeds by liquid chromatography tandem mass spectrometry

Standard operation procedure for the method validation study of SA/CEN/ENTR/520/2013-17 project

Page 1 of 14

Contents

Foreword

THIS IS A STUDY FOR THE EVALUATION OF THE METHOD, NOT FOR ASSESSING THE PERFORMANCE OF THE LABORATORY. THEREFORE THE METHOD MUST BE FOLLOWED AS GIVEN IN THE SOP. ANY DEVIATIONS FROM THE METHOD AS DESCRIBED, NO MATTER HOW TRIVIAL THEY MAY SEEM, MUST BE NOTED ON THE REPORT FORM.

WARNING — the use of this protocol involves hazardous materials, operations and equipment. This protocol does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the compatibility with regulatory limitations prior to use.

1 Scope

This protocol specifies a method for the determination of five *Alternaria* toxins in wheat, tomato juice and sunflower seed samples by liquid chromatography tandem mass spectrometry (LC-MS/MS). The method includes the analysis of Altenuene (ALT), Alternariol (AOH), Alternariol monomethyl ether (AME) in the range of 1 – 100 µg/kg, and Tentoxin (TEN) in the range of 5 – 500 µg/kg, and Tenuazonic acid (TEA) in the range of 10 – 1000 µg/kg.

2 Normative reference

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)*.

ISO 1042/1998, *Laboratory glassware - One-mark volumetric flasks.*

Commission regulation (EC) No 401/2006, *of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (Text with EEA relevance)*.

3 Principle

A test portion is extracted with methanol – water – acetic acid mixture. The sample is centrifuged and an aliquot of the upper layer is collected. Then, the extract is diluted with an equal volume of 1% aqueous acetic acid solution, and purified on a polymeric based solid-phase extraction (SPE) cartridge. The purified sample is eluted with methanol from the SPE column and the resulting eluate is evaporated, reconstituted and filtered through PTFE syringe filter prior analysed by LC-MS/MS.

4 Reagents

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696, unless otherwise specified. Solvents shall be of quality for LC-MS analysis, unless otherwise specified. Commercially available solutions with equivalent properties to those listed may be used.

4.1 Nitrogen purified compressed gas (purity equivalent to 99.99% or better)

4.2 MILLI-Q water, HPLC grade

4.3 CHROMASOLV® Water, LC-MS grade

4.4 Methanol, LC-MS grade

4.5 Dimethyl sulfoxide HPLC grade

4.6 n-Hexane, HPLC grade

4.7 25% ammonium hydroxide, LC-MS grade

4.8 Acetic acid, ACS reagent, ≥99.7%

4.9 Ammonium acetate, (CH_3COONH_4), LC-MS grade

4.10 1 M ammonium acetate solution

Dissolve 77.08 g ammonium acetate (4.9) in 1 L water (4.2).

4.11 Extraction solvent

Methanol – water – acetic acid (80/19/1, v/v/v) mixture.

Mix 800 mL methanol (4.4) with 190 mL water (4.2) and with 10 mL acetic acid (4.8).

4.12 1% (v/v) acetic acid in water

Mix 990 mL water (4.2) with 10 mL acetic acid (4.8) and homogenise it.

4.13 HPLC mobile phase A

5 mM ammonium acetate buffer at pH ~8.7.

Mix 5 mL 1 M ammonium acetate solution (4.10) and 110 µL 25% ammonium hydroxide (4.7) with 900 mL LC-MS grade water (4.3). Adjust the volume with LC-MS grade water (4.3) to 1 L and homogenise it.

Note: Check the pH of eluent with pH meter or pH stick (5.1), pH shall be between 8.6 and 8.7.

4.14 HPLC mobile phase B

100% methanol (4.4).

4.15 Standards as powder or dried film

Altenuene (ALT), 1 mg at least 98%,

Alternariol (AOH), 5 mg at least 96%

Alternariol monomethyl ether (AME), 5 mg at least 98%

Tentoxin (TEN), 1 mg at least 99%

Tenuazonic acid (TEA), 100.0 µg at least 99%

WARNING – Protective clothing, gloves and safety glasses should be worn at all times, and all standard and sample preparation stages should be carried out in a fume cupboard.

4.16 Working standard solution 1

A methanolic standard mixture that contains ALT, AOH, AME in 100 ng/mL concentration and TEN in 500 ng/mL concentration and TEA in 1000 ng/mL concentration.

4.17 Working standard solution 2

A methanolic standard mixture that contains ALT, AOH, AME in 500 ng/mL concentration and TEN in 2500 ng/mL concentration and TEA in 5000 ng/mL concentration.

4.18 Spiking solution A

A methanolic standard mixture that contains ALT, AOH, AME TEN, and TEA standards.

4.19 Spiking solution B

A methanolic standard mixture that contains ALT, AOH, AME TEN, and TEA standards.

4.20 Spiking solution C

A methanolic standard mixture that contains ALT, AOH, AME TEN, and TEA standards.

4.21 Spiking solution D

A methanolic standard mixture that contains ALT, AOH, AME TEN, and TEA standards.

Note: Store the working and spiking standard solutions in a freezer at approximately -18 °C for up to three months. Let the mixture reach room temperature and vortex-mix it before use.

5 Apparatus

General
Usual laboratory glassware (graduated cylinders, glass funnels, beakers, pipettes, screw-cap, screw-cap amber vials, etc.) and equipment and, in particular, the following:

5.1 pH stick or pH meter

5.2 50 mL polypropylene (PP) centrifuge tube with scale on it

5.3 Laboratory balance, with a mass resolution of 0,01 g

5.4 Analytical balance, with a mass resolution of 0,0001 g

5.5 Adjustable hand shaker

5.6 Centrifuge, with temperature control and at least 2773 x *g* speed

5.7 Calibrated volumetric pipettes

5.8 Hamilton syringe, with 5 µL, 10 µL, 100 µL, and 1000 µL capacity

5.9 Displacement pipettes, of 100 µl and 1000 µl capacity, with appropriate tips

5.10 Solid-phase extraction (SPE) cartridge with hydrophilic modified styrene polymer with 6 mL reservoir capacity, 200 mg adsorbent mass and 100 µL particle size

NOTE: Phenomenex Strata-XL, hydrophilic modified styrene polymer with 6 mL reservoir capacity, 200 mg adsorbent mass and 100 µL particle size have shown to meet this specifications.

5.11 PP reservoirs (approx. 25 mL), fit to SPE columns.

5.12 Polytetrafluoroethylene (PTFE) syringe filter, 13 mm or 15 mm and 0.2 µm.

5.13 1 mL syringe with needle

5.14 Vacuum manifold for SPE clean-up, with taps

5.15 Vortex mixer

5.16 Sample concentrator, with temperature control and gas supply

5.17 Glass receiving tubes for sample elution/evaporation

5.18 Glass vials, ~ 1.5 ml capacity with **insert** and crimp caps or equivalent

5.19 250 mL beaker

5.20 10 mL, 25 mL and 50 mL volumetric flasks

5.21 LC-MS/MS apparatus, comprising the following:

5.21.1 Mobile phase pump, gradient, capable of maintaining a volume flow rate of 0.3 mL/min pulse free

5.21.2 Degasser, optional, for degassing HPLC mobile phases

5.21.3 Injector system, capable of injecting e.g. 10 µL

5.21.4 Column oven, capable to operate at 30 °C ± 1 °C

5.21.5 Triple quadrupole detector equipped with electrospray interface

5.21.6 Recorder, integrator or computer based data processing system

5.22 HPLC reversed phase column

NOTE: Supelco Ascentis Express C-18 with column dimension of 100 mm x 2.1 mm and 2.7 µm particle size has shown to give acceptable results.

5.23 Pre-column, with the same stationary phase material as the analytical column (5.22).

6 Procedure

Analyse only **one** matrix (e.g. cereal) per **day**. Weigh **2.00** g of test portion in a 50 mL centrifuge tube (5.2) for **single** analysis.

In the case of **tomato juices**, one day before analysis, take the test, spike and blank samples into the fridge and allow them to melt completely at around +4 °C overnight (~16 h). After the complete melting of the samples homogenize them by hand shaking or vortex-mixing for 1 min. Take the remaining samples back to the freezer at -18 °C and used them later if the analysis has to be repeated.

In the case of **sunflower** agglomerates can appear in the containers due to the fatty material. Therefore, it is very important to homogenise the samples in the containers before weighing them in.

6.1 Extraction

Weigh **2.00** g of test portion in a 50 mL centrifuge tube (5.2).

Add **15** mL of extraction solvent (4.11) and cap the tube. Vortex-mix it for 10 s to obtain a homogeneous suspension and then shake it for 45 min at room temperature at 600 1/min speed using a hand shaker (5.5).

Note: Small part of the sunflower samples may stick to the wall of the tube during extraction. Therefore, every 10 min the tube shall be taken out from the shaker and the tube shall be shaken by hand for 2 s to remove the material from the wall of the tube. Afterwards, the tube can be taken back into the shaker.

Then, centrifuge the sample at approximately 22 °C for 10 min at 2773 x *g* (4000 rpm) speed and transfer an aliquot of upper layer (equal to **1.0 g** sample, see below) into a new 50 mL centrifuge tube (5.2).

In the case of **cereals** and **sunflower** seeds **7.5** mL upper layer (equal to **1.0 g** sample) has to be collected in a new 50 mL tube.

For **tomato juice 8.0** mL of upper layer (equal to **1.0 g** sample) has to be transferred into a new 50 mL tube.

6.2 Dilution

Dilute the collected upper layer in the tube with equal volume of 1% (v/v) aqueous acetic acid solution (4.12). Then, homogenize the sample by vortex-mixing for 5 s.

7.5 mL of upper layer is diluted with **7.5 mL** 1% aqueous acetic acid for **cereals** and **sunflower seeds** in the tube.

8.0 mL of upper layer is diluted with **8.0 mL** 1% aqueous acetic acid for **tomato juice** in the tube.

6.3 SPE clean-up

Connect the SPE cartridge (5.10) to the vacuum manifold (5.14). Condition the cartridge with 7 mL methanol (4.4), followed by 7 mL water (4.2) and 3 mL 1% (v/v) acetic acid solution (4.12).

After the 3 mL 1% (v/v) acetic acid solution (4.12) passed through, close the tap under the cartridge and pipette 3 mL 1% (v/v) acetic acid solution (4.12) into the SPE column, again. Then, attach a reservoir (5.11) onto the SPE column.

Load the diluted sample (6.2) into the reservoir and open the tap. Wash the 50 mL PP tube, which contained the diluted sample (6.2), with 3 mL 1% (v/v) acetic acid solution (4.12) and load it into the reservoir. Pass the sample through slowly (approximately 1 drop/s). Then, remove the reservoir and wash the cartridge with 7 mL 1% (v/v) aqueous acetic acid solution (4.12). Dry the cartridge with vacuum for half min before washing it with 7 mL n-hexane (4.6). Then, dry the cartridge with vacuum for 1 min before sample elution.

6.4 Sample elution

Pipette 100 µL dimethyl sulfoxide (4.5) into a glass receiving tube (5.17), and take the tube into the vacuum manifold for sample elution. Elute the sample with **6 mL methanol** (4.4) into the glass receiving tube that contains 100 µL dimethyl sulfoxide (4.5). After the methanol passed through, dry the cartridge for 10 s with vacuum.

NOTE: if the n-hexane and the methanol used for washing and elution, respectively, do not want to start dropping, a gentle vacuum can be applied in order to start the elution. After the first drop passed through the cartridge, the vacuum is not needed.

6.5 Sample evaporation

Evaporate the methanolic eluate (6.4) in the glass receiving tubes (5.17) to 100 µL at 50 °C using the sample concentrator (5.16) and a gentle stream of nitrogen (4.1).

NOTE: The dimethyl sulfoxide will not evaporate, therefore it prevents the complete evaporation of solvent. If the solvent volume does not change, it means that only dimethyl sulfoxide (approx. 100 µL) remains in the tube and the evaporation step is complete.

6.6 Sample reconstitution

Vortex-mix the evaporated sample (approx. 100 µL) for at least 15 s to re-dissolve the purified sample residues. Afterwards, adjust the volume of sample to 1.0 mL with water (4.2) and vortex-mix it for at least 30 s. Filter the sample through PTFE syringe filter (5.12) and transfer the sample into HPLC vial containing **insert** (5.18).

Note: As syringe filters will adsorb approximately 500 µL sample, it is recommended to take an insert into the vial to allow sufficient solvent levels in the vial.

7 LC-MS/MS analysis

7.1 LC-MS/MS operating conditions

When the column specified in 5.22 and the mobile phases A and B specified in 4.13 and 4.14 were used, the following settings were found to be appropriate:

Table 1: gradient conditions

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	90	10
1.00	90	10
10.00	0	100
18.00	0	100
18.50	90	10
23.00	90	10

Flow rate: 0.3 mL/min.

Column oven temperature (including the guard column): 30 °C;

Autosampler temperature: 25 °C;

Injection volume: 10 µL;

Analysis time: 23 min;

Ion source: electrospray;

Ionization mode: negative.

Ion source temperatures (i.e. vaporizer, drying gas), gas flows, and voltages (i.e. capillary) depend on the instruments used for analysis and shall be optimized for every laboratory. Optimized ion source settings for Ultima PT and Thermo TSQ Quantum Ultra systems are the following:

Ultima PT: source temperature 125 °C, desolvation temperature 370 °C, drying gas flow 902 L/Hr, cone gas flow 76 L/Hr, and capillary voltage -2.8 kV.

TSQ Quantum Ultra: sheath gas pressure 30 arbitrary unit (Arb), ion sweep gas pressure 10 Arb, aux gas pressure 5 Arb, vaporizer and capillary temperatures 325 °C, capillary voltage -3.0 kV.

Alternaria toxins can be ionized in negative mode resulting in $[M-H]^-$ precursor ions. Typical ion transitions are reported in **Table 2**. The corresponding voltages (i.e. cone, tube lens, collision energy), dwell times and segment times depend on the instruments used for analysis and shall be optimized for every laboratory. Optimized settings for Ultima PT and Thermo TSQ Quantum Ultra systems are the following (quantifier ion transition is highlighted with bold):

Table 2: Ion transitions set on Ultima PT and TSQ Quantum Ultra instruments

Compounds	Detector time segment (min)	Precursor ion $[M-H]^-$ (m/z)	Product ions (m/z)	Cone / tube lens voltage (V)	Collision energies (eV)	Dwell time (s)
Ultima PT						
TEA	2-7.5	196.1	**139.0** 111.9	70	15 20	0.250 0.250
AOH	7.5-9.3	257.1	**215.0** 146.7	50	20 20	0.170 0.170
ALT	7.5-9.3	291.2	**248.1** 202.9	50	20 30	0.170 0.170
TEN	9.3-10.3	413.5	**271.2** 214.8	50	15 15	0.250 0.250
AME	10.3-11.3	271.1	**256.2** 228.2	50	20 20	0.250 0.250
Thermo TSQ						
TEA	0-4	196.1	**139.1** 112.2	80	20 15	0.200 0.200
AOH	4-7	257.0	**215.2** 147.0	120	25 30	0.150 0.150
ALT	4-7	291.2	**248.1** 203.2	130	25 30	0.150 0.150
TEN	7-8	413.3	**271.2** 215.1	120	15 20	0.200 0.200
AME	8-20	271.0	**256.2** 228.2	100	25 30	0.200 0.200

7.2 Preparation of blank and matrix-matched calibration solutions for LC-MS/MS analysis

Prepare one blank and five matrix-matched calibration solutions:

Weigh **2.00** g of **blank** sample in 50 mL centrifuge tubes six times. Extract (6.1), dilute (6.2), and clean-up (6.3) the samples. Before sample elution (6.4) pipette **different volumes of working standard solutions** (4.16 and 4.17) into five glass receiving tubes (5.17) to which the calibration samples will be eluted. Leave the sixth tube without fortification and use it as blank. Add also 100 µL dimethyl sulfoxide (4.5) into the tubes (5.17) and take them into the vacuum manifold for sample elution. Elute the samples (6.4).

Evaporate the eluates using the above mentioned process (6.5) and re-dissolve the samples using the procedure mentioned in section 6.6.

Fortification volumes and concentrations obtained are detailed in **Table 3.**

Note: The blank tomato juice sample is free of *Alternaria* toxins, but the blank cereal and sunflower samples contain a low naturally contamination. This is important for the calculation, because the peak area of toxins obtained from the chromatogram of blank sample must be deducted from the peak areas obtained from the chromatogram of matrix-matched calibration solutions.

Table 3: Preparation of matrix-matched calibration solutions. These volumes of working standard solutions shall be pipetted into the glass receiving tubes before sample elution in order to obtain the matrix-matched calibration solutions.

			ALT	AOH	AME	TEN	TEA
Calibration samples	**Working standard solution 1 (µL) (4.16)**	**Working standard solution 2 (µL) (4.17)**	Concentration **in µg/kg**				
Blank	-	-					
CAL 1	10	-	1	1	1	5	10
CAL 2	50	-	5	5	5	25	50
CAL 3	100	-	10	10	10	50	100
CAL 4	-	50	25	25	25	125	250
CAL 5	-	200	100	100	100	500	1000

7.3 Calibration curve

Prepare a calibration curve by injecting the matrix-matched calibration solutions (7.2) at the beginning of the sequence. Plot the peak areas against the concentrations in the injected matrix-matched calibration solutions (**Table 3**) and check the curve for linearity.

7.4 Determination of *Alternaria* toxins in spiked and test samples

Inject the solvent, the blank, the matrix-matched calibration solutions, the spiked and the test solutions into the LC-MS/MS system. The injection of calibration, spiked and test sample solutions is performed with **single** injection.

Note: Before starting the sequence the instrument shall be equilibrated by injecting matrix-free solvents and matrix-free standard solutions.

The sequence shall follow the order reported below:

- Methanol (4.4)
- Blank (7.2)
- The five matrix-matched calibration solutions (7.2) from the lowest to the highest level with **single** injection.
- Methanol (4.4)
- Test solutions from spiking experiments (Spike I, II, III, and IV according to the spiking protocol) with **single** injection.
- Methanol (4.4)
- Test solutions from samples with **single** injection.

7.5 Peak identification

Identify the *Alternaria* toxins of the test solutions by comparing the **retention time** of toxins obtained from the chromatogram of test solutions to the retention time obtained from the chromatogram of matrix-matched calibration solutions. Also, calculate the **ion ratios** of qualifier and quantifier ion transitions (relative intensity). The ion ratios obtained from test solutions should be in the permitted tolerance intervals. Tolerance intervals can be calculated from the ion ratios obtained from matrix-matched calibration solutions in accordance with **Table 4**. **Report only those compounds that meet the peak identification criteria**.

Table 4: Calculation of permitted tolerance of ion ratios

Ion ratio in calibration solution (relative intensity)	Permitted tolerance in test solution
> 50%	± 20%
20% - 50%	± 25%
10% - 20%	± 30%
≤ 10%	± 50%

8 Preparation of spiked samples

Prepare **four** spiked samples for each matrix according to the spiking protocol.

9 Calculation

9.1 Preparation of the calibration graph

If no blank sample is available for matrix-matched calibration, the initial contamination of toxins of interest must be taken into account. For that the peak area of toxin obtained from the chromatogram of the unfortified sample (**blank**) must be deducted from the peak areas obtained from the chromatogram of matrix-matched calibration solutions according to **Equation 1**.

Equation 1 $$A_{toxin} = A_{toxin,calib} - A_{toxin,blank}$$

Where:

A_{toxin} is the peak area of mycotoxin obtained from the chromatogram of the matrix-matched calibration solution after the correction with the peak area of mycotoxin obtained from the chromatogram of the unfortified sample;

$A_{toxin,calib}$ is the peak area of mycotoxin obtained from the chromatogram of the matrix-matched calibration solution (7.2);

$A_{toxin,blank}$ is the peak area of mycotoxin obtained from the chromatogram of the unfortified sample;

Plot the peak areas (y-axis) against the concentrations in matrix-matched calibration solutions (7.2) [µg/kg] (x-axis) and calculate the calibration curve using linear regression. Add the calibration curve and display the resulting function ($A_{toxin} = a*C_{toxin,calib} + b$) and the r-squared value on chart.

Where:

A_{toxin} is the peak area of mycotoxin obtained from the chromatogram of the matrix-matched calibration solution after the correction with the peak area of mycotoxin obtained from the chromatogram of the unfortified sample;

a is the value of the slope of the linear function;

$C_{toxin,\ calib}$ is the concentration of mycotoxin, in microgram per kilogram, in the matrix-matched calibration solution (7.2);

b is the value where the calibration function intercepts the y-axis.

9.2 Calculation of *Alternaria* toxin content in the test and spiked samples

Calculate the concentration of mycotoxins, expressed in µg/kg, in the test and spiked samples using the resulting function ($A_{toxin,sample} = a*C_{toxin,sample} + b$) and **Equation 2**.

Equation 2 $$C_{toxin,sample} = \frac{A_{toxin,sample} - b}{a}$$

Where:

$C_{toxin,sample}$ is the concentration of mycotoxin, in microgram per kilogram, in the test or spiked sample;

$A_{toxin,sample}$ is the peak area of mycotoxin obtained from the chromatogram of the test solution;

a is the value of the slope of the linear function (9.1);

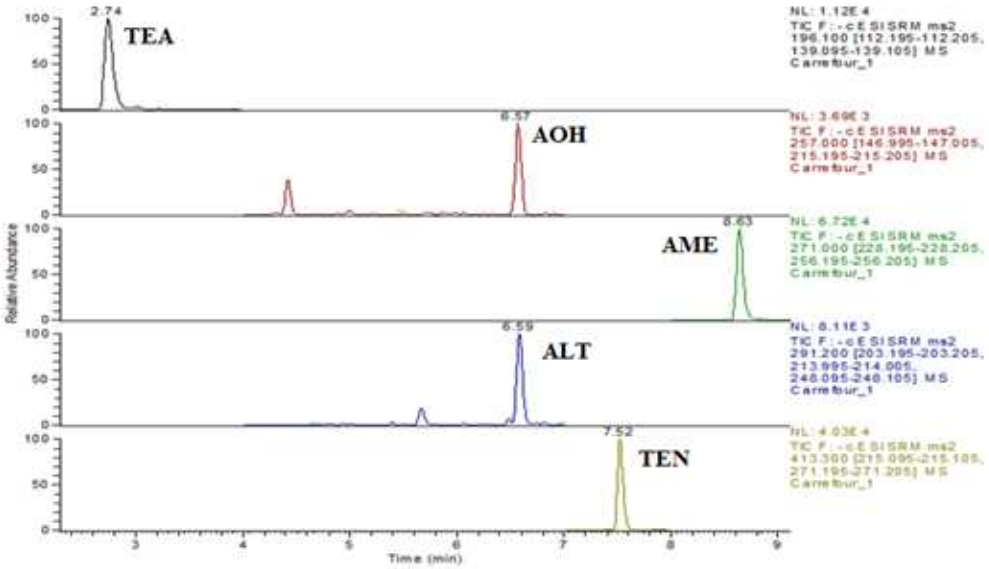
b is the value where the calibration function intercepts the y-axis (9.1).

If the peak areas obtained from the chromatogram of the test or spiked samples are between the first and third calibration levels, the fourth and fifth calibration levels have to be excluded from the calibration.

Annex A
(informative)

Typical chromatogram

Figure 1



Key:

Figure 1 Total ion chromatograms (TIC) of *Alternaria* toxins in a spiked tomato sample. Analysis was performed on Thermo TSQ Quantum instrument. Spiking level was 10 µg/kg for all toxins.

Operating conditions for **Figure 1**:

Column	Supelco Ascentis Express C-18 with column dimension of 100 mm x 2.1 mm and with 2.7 µm particle size. The column is equipped with Ascentis Express C-18 pre-column with the dimension of 0.5 mm x 2.1 mm
Flow rate	0.3 mL/min
Mobile phase	HPLC gradient (4.13 and 4.14)
Column temperature	30 ° C controlled
Injection volume	10 µL
Detection	triple quadrupole MS/MS (7.1)

7.10 Annex 10 – Critical steps

Critical steps in the *Alternaria* method.

"Method validation study on the determination of *Alternaria* toxins in wheat, tomato juice and sunflower seeds by solid phase extraction clean-up and liquid chromatography with tandem mass spectrometric detection"

Sample preparation

- Weigh **2.00** g of test portion in a 50 mL polypropylene centrifuge tube for **single** analysis. All samples should be **homogenised** (mainly for **sunflower**) before taking the test portion for performing the analysis.
- Add 15 mL extraction solvent (80 parts per volume of methanol and 19 parts per volume of water and 1 parts per volume of acetic acid) (4.11).
- Vortex-mix it, then shake it for 45 minutes. Small part of the sunflower samples may stick to the wall of the tube during extraction. Therefore, every 10 min the tube shall be taken out from the shaker and the tube shall be shaken by hand for 2 s to remove the material from the wall of the tube. Afterwards, the tube can be taken back into the shaker.
- Centrifuge the sample.
- Sample dilution and solid phase extraction clean-up: **7.5** mL upper layer (equal to 1.0 g sample) + **7.5** mL 1% aqueous acetic acid for **cereals** and **sunflower** seeds; **8.0** mL upper layer (equal to 1.0 g sample) + **8.0** mL 1% aqueous acetic acid for **tomato juice**.
- Condition the cartridges: 7 mL methanol, 7 mL water and 3 mL 1% aqueous acetic acid.
- Close the tap and load 3 mL 1% aqueous acetic acid into the cartridge and attach the reservoir.
- Load the diluted sample into the reservoir and open the tap.
- Wash the 50 mL PP tube, which contained the diluted sample, with 3 mL 1% (v/v) acetic acid solution and load it into the reservoir
- Wash the cartridge with 7 mL 1% aqueous acetic acid, followed by 7 mL n-hexane.
- Dry the SPE column with vacuum for 1 min.
- Pipette **100 µL dimethyl sulfoxide (DMSO)** into the glass tube to be used for sample elution.
- Elute the sample with **6 mL methanol** into glass tube containing **100 µL DMSO**.
- Evaporate the sample at 50 °C under nitrogen stream to approximately **100 µL** (till the solvent volume does not change).
- Vortex-mix the sample for 15 s and pipette approximately 900 µL water into the tube to obtain 1.0 mL of sample volume and vortex-mix it again for 15 s.
- Filter the sample through PVDF syringe filter and transfer it into HPLC vial that contains **insert**.
  The provided Phenex syringe filter will adsorb approximately 500 µL sample, therefore it is useful to take an insert into the vial and load it with 6-8 drops of filtrate.

LC-MS/MS conditions

- Mobile phase A: 5 mM ammonium acetate in water, **pH** adjusted to **8.7** with 25% ammonia (110 µL 25% ammonium hydroxide to 1000 mL water containing 5 mM ammonium acetate)
- Mobile phase B: 100% methanol
- Flow rate: 0.3 mL/min
- Column: Ascentis Express C-18, 100 mm x 2.1 mm, 2.7 µm or other C-18 based column
- Column oven temperature: 30 °C
- Injection volume: 10 µl (higher volume may cause higher matrix effect)
- Autosampler temperature: 20 °C to 25 °C

1

- Ion source: ESI
- Ionisation mode: **negative**
- MS/MS detection in MRM (SRM) mode
- $[M-H]^-$ precursor ion with two ion transitions

Preparation of matrix-matched calibration solutions

- Weigh **2.00** g of blank sample in 50 mL centrifuge tubes six times (6 tubes).
- Extract and clean them up.
- During the SPE purification step, **before eluting the samples, add different volume of working standard solutions** according to the table below **into those glass receiving tubes to which the calibration samples will be eluted.**
- **Do not forget to add 100 µL DMSO into the** glass tubes as well.
- Elute, evaporate and reconstitute the samples according to the method protocol.

			ALT	AOH	AME	TEN	TEA
Calibration samples	**Working standard solution 1 (µL) (4.16)**	**Working standard solution 2 (µL) (4.17)**	Concentration **in µg/kg**				
Blank	-	-					
CAL 1	10	-	1	1	1	5	10
CAL 2	50	-	5	5	5	25	50
CAL 3	100	-	10	10	10	50	100
CAL 4	-	50	25	25	25	125	250
CAL 5	-	200	100	100	100	500	1000

Determination of *Alternaria* toxins in spiked and test samples

- Inject the solvent, the blank, the matrix-matched calibration solutions, the spiked and the test solutions into the LC-MS/MS system.
- Nineteen injection in total.
- The injection of calibration, spiked and test sample solutions is performed with **single** injection.
- Before starting the sequence the instrument shall be equilibrated by injecting matrix-free solvents and matrix-free standard solutions.
- The sequence shall follow the order reported below:

– Methanol (4.4)
– Blank (7.2)
– The five matrix-matched calibration solutions (7.2) from the lowest to the highest level with single injection.
– Methanol (4.4)
– Test solutions from spiking experiments (Spike I, II, III, and IV according to spiking protocol) with single injections.
– Methanol (4.4)
– Test solutions from samples with single injections.

Identification

- Identify the compounds on the chromatogram of test samples with their **retention time** and **ion ratio**.
- Due to the complexity of samples matrix peaks can appear close to the target compounds' retention time, therefore the identification shall be confirmed with the **ion ratios** according to the method protocol (Table 4 in the SOP).
- **Report only those compounds that meet the peak identification criteria.**

Calculation

- If the **blank** sample used for matrix-matched calibration **contains naturally contamination** of toxins of interest, the **peak area** of toxin obtained from the chromatogram of the **blank** sample **must be deducted** from the **peak areas** obtained from the chromatogram of the **matrix-matched calibration solutions**.
- The blank tomato juice sample is free of *Alternaria* toxins, but the blank **cereal** and **sunflower** samples contain a low naturally contamination.
- Plot the peak areas (y-axis) against the concentrations in matrix-matched calibration solutions (x-axis) (see the table above) [µg/kg] and calculate the calibration curve using linear regression. Add the calibration curve and display the **resulting function** (A = a*C+ b) and the r-squared value on chart.
- The concentration of test and spiked samples can be evaluated directly using the **resulting function**.
- If the peak areas obtained from the chromatogram of the test or spiked samples are between the **first and third** calibration levels, the fourth and fifth calibration levels have to be excluded from the calibration.
- Please **do not correct** the results for recovery.

7.11 Annex 11 – Results of laboratories

The tables show the mean of the duplicates reported by the participants. Calculations and outlier tests were performed by ProLab software. Those results that were submitted as single concentration were considered non-compliant and were excluded manually. The results of laboratory 613 and 614 were considered non-compliant in all samples and their results were excluded manually from the evaluation. Also, the results of laboratory 603 for sunflowers were not considered. The reasons for exclusion are detailed in section 5.2 and 5.3.

ALT

Laboratory	Sample E,F		Cereals, spike I-II		Cereals, spike III-IV		Sample G, H		Sample I, J		Sample K, L		Tomato juice, spike I-II		Tomato juice, spike III-IV		Sunflower, spike I-II		Sunflower, spike III-IV	
Unit	µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg	
601	10.140		1.625		4.515		8.925		12.220		21.585		2.485		9.580		1.995		6.455	
602	20.655		2.000		7.060		6.090		9.260		19.680		1.640		5.945		not tested		8.535	
603	17.823		2.539		7.988		not tested		not tested		not tested		not tested		not tested		7.764	B	12.087	D
604	22.110		not tested		4.440		8.985		10.900		23.610		not tested		12.020	C	1.500		4.120	
605	21.700		0.750		2.350		6.800		10.850		23.000		1.900		6.350		1.650		4.700	
606	20.600		0.790		not tested		7.450		14.700		34.950		2.550		6.750		not tested		not tested	
607	22.300		1.750		6.000		8.250		13.000		24.050		2.250		9.300		2.350		8.250	
608	14.885		1.560		6.900		12.740	C	10.855		14.215	C	2.240		6.795		0.745		5.060	
609	18.385		1.917		5.862		6.500		10.935		22.980		1.680		6.110		2.040		7.030	
610	17.500		1.405		6.785		6.580		11.410		22.350		2.010		6.905		1.300		8.080	
611	19.500		not tested		7.500		7.850		12.000		22.500		not tested		8.750		not tested		8.300	
612	31.350		not tested		6.250		5.900	D	11.400	C	17.700	D	0.900	D	5.700	D	0.450		5.400	D
613	37.650	D	4.915	D	16.650	D	15.300	D	24.050	D	49.200	D	2.855	D	15.500	D	3.020	D	8.885	D
614	18.300	D	not tested		15.900	D	3.145	D	3.995	D	6.870	D	not tested		3.095	D	not tested		5.990	D
615	43.650	D	6.465	D	8.070	D	9.150		12.430		23.365		2.595	D	9.755		14.455	D	17.725	D
616	16.180		1.855		7.330		7.805		14.050		29.185		2.570		9.680		1.660		6.565	
No. of laboratories after elimination of outliers type A-L except E (without laboratories that only gave states but no measured values)	**13**		**10**		**12**		**11**		**12**		**11**		**9**		**11**		**9**		**10**	

Explanation of outlier types

A: Single outlier (Grubbs)

B: Differing laboratory mean (Grubbs)

C: Excessive laboratory s.d. (Cochran)

D: Excluded manually

AOH

Laboratory	Sample C,D		Sample E,F		Cereals, spike I-II		Cereals, spike III-IV		Sample G, H		Sample I, J		Sample K, L		Tomato juice, spike I-II		Tomato juice, spike III-IV		Sample M, N		Sample P, Q		Sample R, T		Sunflower, spike I-II		Sunflower, spike III-IV	
Unit	µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg		µg/kg	
601	428.245	B	9.450		1.565		4.295		2.050		3.790	D	8.880	D	1.990		5.410		33.920		168.725		69.935		2.040		5.975	
602	107.625		12.085		1.630		4.630		7.655		10.030		11.690		1.210		5.850		14.950		81.460		29.905		2.050		5.535	
603	99.925		24.155	C	1.911		6.999		not tested		not tested		not tested		not tested		not tested		21.370	D	137.976	D	44.881	D	1.704	D	6.811	D
604	153.955		11.120		1.305		4.465		5.310		6.480		19.805		3.640		7.300		26.490		170.655		55.970		2.325	C	4.765	
605	83.500		11.850		1.350		3.800		5.250		11.000		21.150		2.000		8.550		20.200		166.050		49.250		1.500		5.600	
606	67.050		10.350		2.300		not tested		7.650		8.200		15.650		4.000	D	6.650		21.500		129.000		37.900		not tested		7.750	
607	104.900		17.450		1.600		6.300		6.800		9.750		18.250		2.750		6.600		24.600		139.400		31.600		2.300		7.650	
608	166.900		11.335		2.105		7.905		20.177	C	18.400	D	25.965	C	0.405		6.845		9.685		64.180		37.310		1.525		5.485	
609	101.710		21.595		1.825		6.140		5.730		10.695		19.525		1.530		6.845		23.520		139.080		51.085		2.555		7.320	
610	85.995		14.240		2.785		6.820		5.805		9.865		18.565		2.210		7.515		20.660		142.360		45.075		0.785		5.715	
611	220.000	D	14.000		9.500	B	3.150	D	6.950		9.450		20.500		2.150		8.550		36.000		221.500		62.500		not tested		6.000	
612	65.950		17.050		2.150		10.000		8.250	D	13.500	D	20.050	D	3.350	D	15.250	D	19.050		112.600		38.400	D	1.900		5.500	
613	194.000	D	32.500	D	5.110	D	17.250	D	15.250	D	26.800	D	49.200	D	4.020	D	19.500	D	14.750	D	75.200	D	81.700	D	2.270	D	6.695	D
614	not tested		21.850	D	4.140	D	18.400	D	2.905	D	3.165	D	3.670	D	not tested		2.040	D	28.200	D	140.500	D	48.450	D	1.845	D	6.975	D
615	42.730		10.260		2.695	C	5.460		2.445		6.990		7.705		2.875	C	7.930		24.185		120.005		41.630		2.615		6.785	
616	70.485		12.730		1.490		5.550		6.110		5.210		32.725		2.765		8.015		23.420		161.530		49.855		1.320		4.610	
No. of laboratories after elimination of outliers type A-L except E (without laboratories that only gave states but no measured values)	**12**		**13**		**12**		**12**		**11**		**10**		**10**		**10**		**12**		**13**		**13**		**12**		**10**		**13**	

Explanation of outlier types

A: Single outlier (Grubbs)

B: Differing laboratory mean (Grubbs)

C: Excessive laboratory s.d. (Cochran)

D: Excluded manually

AME

Laboratory	Sample C,D	Sample E,F	Cereals, spike I-II	Cereals, spike III-IV	Sample G, H	Sample I, J	Sample K, L	Tomato juice, spike I-II	Tomato juice, spike III-IV	Sample M, N	Sample P, Q	Sample R, T	Sunflower, spike I-II	Sunflower, spike III-IV
Unit	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
601	8.405	33.605	1.350	3.780	4.585	6.730	13.850	2.000	5.395	7.285 B	26.095 B	47.920 C	1.415	4.700
602	14.725	40.395	1.400	3.945	5.385	7.985	7.225	0.160 D	2.340 D	0.800 D	3.370	1.110	1.415	4.080
603	5.638	38.007	1.184	4.965	not tested	not tested	not tested	not tested	not tested	43.185 C	61.214 C	20.838 C	9.986 B	17.021 B
604	6.235	43.305	0.850	3.215 D	4.060	4.415	13.890	3.085	5.400	1.375	5.700	1.800	1.340 D	2.590
605	8.150	14.300 D	0.600	2.300 D	5.150	11.400	21.650	1.400	8.300	1.500	8.600	2.100	1.500	5.800
606	10.150	19.550	2.300	not tested	5.350	4.700	10.650	1.800	3.300 D	2.500	8.000	1.950	0.950	3.200
607	12.850	57.850	1.750	7.100	6.450	7.500	15.750	1.750	4.100	2.500	9.800	1.200	2.000	7.450
608	12.230	36.870	2.095	8.740	35.330 C	36.190 C	41.720 C	7.020 B	8.730	1.030	2.685	0.835	0.880	3.275
609	7.875	71.185	1.225	4.110	5.275	10.365	19.445	0.925	5.030	1.610	7.270	2.010	1.510	5.675
610	6.175	35.065	1.620	5.805	5.315	9.695	19.925	2.210	6.210	1.075	4.440	1.390	1.170	4.515
611	12.000	23.000	not tested	3.750	3.400	5.600	11.400	not tested	3.850	1.700	8.400	1.850 D	0.550 D	2.450
612	6.500	35.750	1.400	4.350	6.700 D	5.750 D	12.650 D	2.500 D	8.150 D	2.200 D	6.950	1.950	1.300	3.350
613	64.800 D	175.000 D	4.815 D	18.550 D	13.100 D	31.300 D	30.300 D	3.160 D	10.520 D	not tested	1.620 D	1.965 D	0.050 D	2.420 D
614	not tested	18.400 D	6.595 D	20.700 D	2.495 D	2.490 D	2.085 D	not tested	not tested	not tested	not tested	not tested	not tested	4.125 D
615	5.070	27.205	1.970	5.690	3.820	5.225	5.635	3.510	5.130	4.810 B	10.510	3.625 D	2.785	5.845
616	5.250	33.075	1.085	2.355	3.785	2.330 D	16.260	0.690	1.695	0.905	5.500	1.725	0.545 D	2.155
No. of laboratories after elimination of outliers type A-L except E (without laboratories that only gave states but no measured values)	**14**	**13**	**13**	**11**	**11**	**10**	**11**	**9**	**10**	**9**	**12**	**10**	**10**	**13**

Explanation of outlier types

A: Single outlier (Grubbs)

B: Differing laboratory mean (Grubbs)

C: Excessive laboratory s.d. (Cochran)

D: Excluded manually

TEN

Laboratory	Sample A,B	Sample C,D	Cereals, spike I-II	Cereals, spike III-IV	Sample G, H	Sample I, J	Sample K, L	Tomato juice, spike I-II	Tomato juice, spike III-IV	Sample M, N	Sample P, Q	Sample R, T	Sunflower, spike I-II	Sunflower, spike III-IV
Unit	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
601	68.795	9.650	25.375	92.675	152.015	205.420	337.310	46.225	200.245	78.825 D	105.625	205.700 C	48.570	191.170
602	61.770	11.515	41.140	164.345	145.015	226.820	442.370	41.450	177.480	27.895	41.125	9.755	43.205	159.635
603	77.613	16.063	51.196	179.745	not tested	not tested	not tested	not tested	not tested	29.113 D	40.968 D	7.399 D	47.593 D	177.024 D
604	28.350	7.795	30.310	152.615	196.340	238.800 C	481.325	40.735	229.815 C	40.850	84.645	15.645	37.610	158.470
605	25.700	5.800	24.950	143.150	165.750	257.450	472.100	43.900	189.700	23.200	47.800	9.500	42.000	148.400
606	49.150 C	11.950	47.500	not tested	161.000	255.500	504.500	49.450	186.500	33.750	64.650	4.400	41.900	173.500
607	111.150 D	12.850	58.150	190.600	153.250	243.000	504.650	60.450	204.000	37.800	59.150	8.050	61.600 D	212.800
608	34.035	1.465 D	45.010	197.400	286.350 C	232.350	298.950 C	65.270	217.650	104.750 D	126.950 D	14.005	64.090 C	193.850
609	83.160	11.705	48.670	182.330	156.250	234.280	428.745	40.150	169.645	33.025	40.645	5.780	47.210	187.955
610	37.955	8.045	45.315	185.650	129.550	234.885	615.325	52.260	184.195 D	30.115	41.800	3.675 D	44.890	192.355
611	42.500	12.500	35.000	162.000	150.000	238.000	498.000	53.000	194.500	37.000	56.500	13.500 C	45.500	163.500
612	57.200	2.900 D	33.400	122.450	185.550 D	270.550 D	474.950 D	53.900 D	256.450 D	40.150	72.500	8.450	53.400	205.050
613	91.100 D	28.950 D	108.500 D	420.500 D	314.000 D	487.000 D	949.000 D	90.200 D	383.000 D	22.200 D	27.250 D	28.195 D	47.200 D	193.000 D
614	not tested	513.000 D	64.750 D	not tested	29.850 D	31.450 D	120.850 D	4.210 D	19.950 D	4.800 D	8.505 D	2.340 D	7.700 D	38.050 D
615	42.785	8.710	37.935	148.380	121.765	213.420	408.250	36.750 C	149.610	59.935	82.855	14.220	51.030	196.345
616	57.390	8.410	46.660	182.015	141.755	212.090	417.310	43.050	164.600	35.850	64.945	10.625	42.190	161.510
No. of laboratories after elimination of outliers type A-L except E (without laboratories that only gave states but no measured values)	**12**	**12**	**14**	**13**	**11**	**11**	**11**	**11**	**10**	**11**	**12**	**10**	**11**	**13**

Explanation of outlier types

A: Single outlier (Grubbs)

B: Differing laboratory mean (Grubbs)

C: Excessive laboratory s.d. (Cochran)

D: Excluded manually

TEA

Laboratory	Sample A,B	Sample C,D	Sample E,F	Cereals, spike I-II	Cereals, spike III-IV	Sample G, H	Sample I, J	Sample K, L	Tomato juice, spike I-II	Tomato juice, spike III-IV	Sample M, N	Sample P, Q	Sample R, T	Sunflower, spike I-II	Sunflower, spike III-IV
Unit	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg	µg/kg
601	255.780	50.950	103.880	48.465	153.975	137.125	197.545	409.355	44.995	150.020	838.435	914.765	794.800 D	50.975	124.145
602	189.535	59.295	123.435	48.370	164.695	142.240	231.910	510.890	42.205	163.555	310.500	572.735	202.935	62.815	165.600
603	187.444	53.783	104.886	51.697	166.858	not tested	not tested	not tested	not tested	not tested	21.986 D	67.612 D	21.386 D	15.996 D	47.360 D
604	177.265	22.250	102.920	29.575	111.385 D	210.930	258.490 C	623.990	58.000	228.685 C	120.200 D	269.935 D	166.885 D	28.840	124.700 D
605	160.100	72.400	135.750	61.500	163.650	179.400	287.400	568.100	46.000	186.500	1063.150	1784.600	598.300	73.300 D	214.300
606	118.550	10.150 D	150.500	44.850	not tested	167.500	273.500	601.500	54.700	195.500	387.000	573.000	337.000	27.600	134.500
607	186.950	62.550	145.200	56.500	189.900	175.000	266.650	571.050	56.050	181.600	1134.700	1361.050	569.000	71.000	187.250
608	81.375 D	31.830	91.650	45.090	136.350	286.350 C	232.350	298.950 C	65.270	217.650	915.850	1441.000	246.200	20.525 D	83.605
609	327.875	100.720 D	184.115	51.365	185.740	183.950	300.440	655.505	48.360	188.730	not tested	not tested	665.225	78.945	186.345
610	227.185	26.875	90.765	30.345	138.085	148.870	285.775	639.755 C	49.750	205.025	744.245 D	970.420	285.945	24.825	113.675
611	246.000	86.000	123.500	42.000	202.500	189.500	262.000	590.000 D	50.000	196.000	813.000	1187.000	709.000	69.500	189.000
612	170.350	80.700	145.050	50.050	152.350	125.450 D	176.750 D	404.550 D	48.900 D	144.850 D	532.800	661.200	281.350	54.950	144.750
613	419.000 D	155.500 D	328.000 D	113.500 D	446.500 D	440.500 D	684.500 D	1465.000 D	99.350 D	440.000 D	285.000 D	367.000 D	389.500 D	34.050 D	118.500 D
614	215.500 D	78.400 D	118.000 D	53.850 D	177.500 D	156.500 D	243.000 D	503.000 D	50.150 D	156.500 D	519.500 D	1026.500 D	397.500 D	58.850 D	150.500 D
615	224.455	78.585	128.440	47.760	157.010	141.275	254.195	564.505	44.130 C	177.900	895.645	1126.365	411.725	55.095	150.470
616	201.865	59.985	115.845	49.415	168.250	131.225 C	200.775 C	63.930 B	16.905 C	34.700 B	1146.375	1535.215	662.105	58.460	137.610
No. of laboratories after elimination of outliers type A-L except E (without laboratories that only gave states but no measured values)	**13**	**12**	**14**	**14**	**12**	**10**	**10**	**8**	**10**	**10**	**10**	**11**	**11**	**11**	**12**

Explanation of outlier types

A: Single outlier (Grubbs)

B: Differing laboratory mean (Grubbs)

C: Excessive laboratory s.d. (Cochran)

D: Excluded manually

7.12 Annex 12 – Characteristics after recovery correction

Precision estimates for ALT, AOH and AME calculated for naturally contaminated materials after recovery correction of results.

	Sample description	**Sample A, B**	**Sample C, D**	**Sample E, F**	**Sample G, H**	**Sample I, J**	**Sample K, L**	**Sample M, N**	**Sample P, Q**	**Sample R, T**
	Matrix	**Cereals**			**Tomato juice**			**Sunflower seeds**		
Compound	Sample	Sorghum	Triticale	Wheat	Tomato juice	Tomato juice	Tomato juice	Unpeeled	Unpeeled	Mixture
ALT	Mean value, $\bar{x}$, µg/kg	< 1	< 1	25.3	7.79	12.1	23.9	< 1	< 1	< 1
	Repeatability standard deviation s_r, µg/kg			4.02	0.43	0.45	1.15			
	Repeatability relative standard deviation, RSD_r, %			15.9	5.53	3.76	4.81			
	Reproducibility standard deviation s_R, µg/kg			9.05	0.80	1.48	4.22			
	Reproducibility relative standard deviation, RSD_R, %			35.8	10.3	12.3	17.7			
	HorRat value, recovery corrected			1.6	0.5	0.6	0.8			
AOH	Mean value, $\bar{x}$, µg/kg		145	19.6	6.50	9.49	18.4	30.9	190	64.1
	Repeatability standard deviation s_r, µg/kg		28.7	6.43	1.58	1.98	2.63	3.32	13.8	5.07
	Repeatability relative standard deviation, RSD_r, %		19.8	32.8	24.2	20.8	14.3	10.7	7.28	7.91
	Reproducibility standard deviation s_R, µg/kg		63.2	7.18	2.42	2.94	4.42	10.7	69.2	18.7
	Reproducibility relative standard deviation, RSD_R, %		43.5	36.6	37.2	31.0	24.0	34.6	36.4	29.3
	HorRat value, recovery corrected		2.0	1.7	1.7	1.4	1.1	1.6	1.7	1.3
AME	Mean value, $\bar{x}$, µg/kg		14.7	66.9	10.3	13.4	24.8	3.55	13.6	3.61
	Repeatability standard deviation s_r, µg/kg		2.18	7.04	4.86	3.48	3.44	0.91	1.90	0.46
	Repeatability relative standard deviation, RSD_r, %		14.8	10.5	47.3	26.0	13.9	25.7	13.9	12.9
	Reproducibility standard deviation s_R, µg/kg		5.27	25.4	5.94	5.94	4.67	1.94	5.11	1.42
	Reproducibility relative standard deviation, RSD_R, %		35.8	37.9	57.7	44.3	18.8	54.6	37.5	39.4
	HorRat value, recovery corrected		1.6	1.7	2.6	2.0	0.9	2.5	1.7	1.8

Precision estimates for TEN and TEA calculated for naturally contaminated materials after recovery correction of results.

	Sample description	**Sample A, B**	**Sample C, D**	**Sample E, F**	**Sample G, H**	**Sample I, J**	**Sample K, L**	**Sample M, N**	**Sample P, Q**	**Sample R, T**
	Matrix	**Cereals**			**Tomato juice**			**Sunflower seeds**		
Compound	Sample	Sorghum	Triticale	Wheat	Tomato juice	Tomato juice	Tomato juice	Unpeeled	Unpeeled	Mixture
TEN	Mean value, $\bar{X}$, µg/kg	66.4	10.8	< 3	163	245	491	40.1	73.8	11.07
	Repeatability standard deviation s_r, µg/kg	4.03	0.73		8.95	21.6	21.1	5.09	8.09	0.93
	Repeatability relative standard deviation, RSD_r, %	6.07	6.75		5.46	8.80	4.30	12.7	11.0	8.39
	Reproducibility standard deviation s_R, µg/kg	27.4	3.30		13.9	32.2	87.3	9.72	21.9	4.02
	Reproducibility relative standard deviation, RSD_R, %	41.2	30.4		8.46	13.1	17.8	24.2	29.7	36.4
	HorRat value, recovery corrected	1.9	1.4		0.4	0.7	1.0	1.1	1.3	1.7
TEA	Mean value, $\bar{X}$, µg/kg	253	73.3	153.2	181	277	589	934	1327	575
	Repeatability standard deviation s_r, µg/kg	41.9	10.6	17.9	11.0	33.6	30.9	182	183.8	84.7
	Repeatability relative standard deviation, RSD_r, %	16.5	14.4	11.7	6.02	12.1	5.25	19.5	13.8	14.7
	Reproducibility standard deviation s_R, µg/kg	73.4	24.6	28.5	14.4	35.6	79.2	446	483	190
	Reproducibility relative standard deviation, RSD_R, %	29.0	33.5	18.6	7.90	12.9	13.4	47.8	36.4	33.1
	HorRat value, recovery corrected	1.4	1.5	0.8	0.4	0.7	0.8	2.9	2.3	1.8

7.13 Annex 13 – Experimental details

When did you analyse the samples?
How many years of experience does the method conductor (analyst) have with LC-MS analysis of mycotoxins?
For how long (years) your lab has been analysing food for Alternaria toxins by LC-MS?
Is your laboratory accredited for the analysis of Alternaria toxins in food by LC-MS?
If YES, please write for which food matrix (matrices) is your laboratory accredited
How many samples does your lab analyse for Alternaria toxins in food per year?

Lab code	Sample analysis	Years of experience in mycotoxin analysis by LC-MS	Years of experience in *Alternaria* analysis by LC-MS	Accredited	Accredited matrices	Samples per year
601	9-11/01/2016	4 years	4 years	No		200
602	9 Nov 2015 (cereals)	6 years				
603	Cereals were analysed on 16 November while sunflowers were analysed on 18 November. The cereal sequence run well, while the sunflower sequence stopped during the night due to overpressure and was restarted the day after 19 November).	10 years	1 year	No		100
604	Nov 22	2	1	No		
605	16/10/215 Tomato Samples	3	1			400
606				No		
607	3-5/11/2015	0	0	No		0
608	14.-16.10.2015.	10	10	No		6000
609	2-12.11.2015'	3	0	No		
610	Sample preparation:	1 year	5 years	No	none	100
612	Tomato juice November 3	6 years	We are not analyzing alternaria toxins at the moment	No	-	0
614	November 3th, 4th and 5th	8	8+	For AOH and AME	Feed and Feed ingredients	
615	Nov 2015	5 years	5 years; group analysis of ALT, AOH and AME; not TEA and TEN	No		50
616	Tomato Juice 15/10/2015, Cereal 21-22/10/2105, Sunflower seeds 28-29/10/2015	1				

Did you find the instructions distributed for this MVS adequate?
If NO, which parts do you think could be improved?
What do you think about the reporting by ProLab/RingDat?
Did you have any problems in using this platform?
If YES, what kind of problems?
Any other comments you wish to address?

Lab code	Instructions adequate?	Improvement	Reporting	Problems with the platform	kind of problems	Any other comments
601	Yes		Good reporting platform	No		
602	Yes		I think that the reporting by ProLab/RingDat was very useful and easy to use	No		
603	Yes		Very clear and useful	No		
604	Yes		good	No		
605	Yes		Convenient and easy to handle	No		
606	Yes			Yes		
607	Yes		OK	No		NONE
608	Yes		OK	Yes	There is no option for inputting <LOD	
609	Yes		Easy to fill the tables.	No		
610	Yes		Quite ok. Copy-paste of the results works fine.	No		
612	Yes		Ok	No		-
614	Yes		not enthusiastic	Yes	time consuming	results including remarks: see Excel file
615	Yes		Inconvenient to transfer every single result from own Excel sheet into ProLab file.	Yes	Cannot open the file *.LA2	No
616	Yes		OK.	No		

Did you find the Method description (SOP) adequate?
If NO, in which part(s) it could be improved?
Were you able to follow the method in all details?
If NO, which parts required deviation from protocol? Please include paragraph number and describe the deviation applied.

Lab code	SOP adequate?	Improvement	Could you follow the method details?	Deviation from protocol
601	Yes		Yes	
602	Yes		Yes	
603	Yes		Yes	
604	Yes		No	Due to instrumentation time, all samples were processed independently on the same day.
605	Yes		No	As we did not use the same column as mentioned in the SOP (7.1) (we used a a Reprosil Gold C18 column (150 * 2 mm, 3 µm particle size, Dr. Maisch GmbH, Ammerbuch, Entringen, Germany) we had to reduce the flow rate to 0.25 mL/min due to high pressure.
606	Yes		Yes	
607	Yes		Yes	
608	Yes		No	at step 6.1. Extraction:
609	Yes		Yes	
610	Yes	The total amount of solvents and solutions required overall could be mentioned (per SPE and matrix for example).	No	6.3 SPE clean-up
612	Yes	6.4 Specify here that ehe glass receiving tubes should have volume scaling! Otherwise it is not possible to adjust the volume to 1.0 mL in step 6.6. We missed that for the first matrix analyzed	No	6.6 We had trouble filtering the extracts of all three matrices bot worst were the cereal samples. We used our own 0.45 µm filters instead.
614	No		No	6.1 Extraction: Not able to shake at 600 1/min speed. -> horizontal shaker: 200 1/min.
615	No	1) A note related to	No	Membrane

Lab code	SOP adequate?	Improvement	Could you follow the method details?	Deviation from protocol
		occuring memory effects of Alternaria toxins in HPLC analysis should be added.		filtration, see comment above. Cellulose membrane filters (0,2 µm) have to be used after centrifugation (10.000 g) of the measurement solution.
616	Yes		No	6.1 The samples were shaken on an orbital shaker, not a hand shaker.

Did you encounter any problems during the analysis?
If YES, what were the specific problems and to which samples did they apply?
Was the analytical process split over staff?
Did you notice any abnormality, that however seem to have no effect on the result?
If YES, please describe and report for which samples (codes) they occurred.

Lab code	Problem during analysis	Problem description	Process split?	Abnormality	Abnormality description
601	Yes	DMSO evaporated for the cereal matrix. Reconstitution was done in 900 µl of water anyway	No	No	
602	Yes	During the LC/MS-MS analysis of the three different matrix I noticed that the method used dirtied the chromatographic column and increased the pressure column	No	Yes	I calculated the ion ratio of all test solutions and of all spiked samples. For some samples the ion ratio was not in accordance with the tolerance intervals. In particular B192 for AOH, Spike I sunflowers and Spike II sunflowers for ALT were not in accordance with the tolerance intervals criteria.
603	Yes	Although the final purified extracts were clear, their analysis by UPLC-LC/MS gave big problems. In particular, the pressure of the colum tend to increase up to the maximum limit during the sequence and the run stopped several times. Also, the shape of the peacks get worse over the sequence. This was observed despite the column was new when it has been used for the first sequence (cereals) and had been washed repeatedly during the second	No	No	
604	No		Yes	No	
605	No		No	Yes	Some of the sunflower and cereal samples remained turbid after filtration. We did not see an increased pressure; but this could become a problem if many samples have to be analyzed.
606	No		Yes	No	
607	No		No	No	
608	No		No	No	
609	Yes	Evaluation of the volume after evaporation was impossible: all samples and calibrants were reconstituted with 900 microliter of water to 100 microliter of DMSO	Yes	No	
610	Yes	6.6 sample reconstitution	Yes	Yes	6.3 SPE clean-up
612	Yes	Equilibrium problems with the column at the high pH. Reinjected the vials for the cereal samples the next day since pressure dropped during the sequence.	No	Yes	Very cloudy extracts even after filtration for the cereals
614	No		No	No	
615	Yes	Memory effects. After each	No	No	

Lab code	Problem during analysis	Problem description	Process split?	Abnormality	Abnormality description
		sample injection two injections of methanol were applied. Sampler needle was washed 5 times with acetonitrile after each injection.			
616	No		No	No	

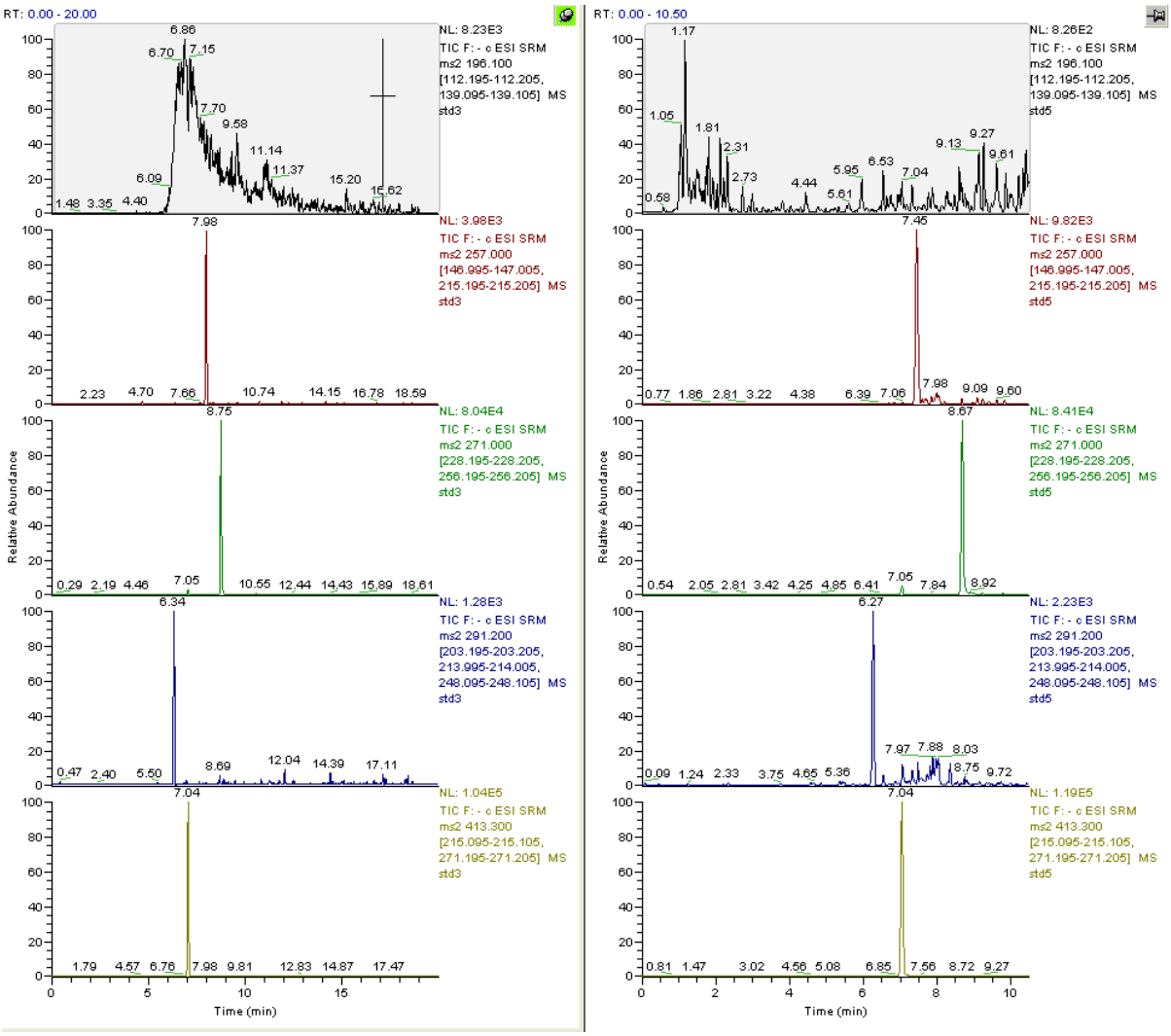
Were you familiar by practice with all the steps performed during the analysis?
If NO, please describe and report for which steps (Please refer to the respective paragraph number in the SOP)
Any other information that you would like to add?
Did you need to include any "overnight" stops in the analysis of the MVS samples without performing new calibration when resuming the sequence?
If YES, please state for which samples and at what stage of the analysis?
How did you intergate the signals?
If you integrated automatically, for how many chromatograms was it necessary to re-integrate analyte peaks? (If none, put 0)

Lab code	Familiarity	Problem description	Any other information	Overnight stop	Which sample/stage of analysis	Integration	Re-integration
601	Yes		2 internal standards were added after weighing of the samples ([2H4]-AME and [13C6,15N]-TeA)	No		Automatic with verification	0
602	Yes		During the analysis I have problems with peak shape so I prefer to chance the chromatographic column. All the samples were analysed with the same chromatographic column.	No		Manual	
603	Yes			No		Manual	
604	Yes		Samples arrived warm with no dry ice	Yes		Automatic with verification	
605	Yes			No		Manual	0
606	Yes			No		Automatic with verification	
607	Yes			No		Automatic with verification	0
608	Yes		at the 6.6. step sample reconstitution - the sample volume seems to vary across the vials	No		Automatic with verification	60
609	Yes		The method demands a lot of pipetting!	No		Manual	
610	Yes		Analyte peaks of 35 out of 390 chromatograms were re-integrated manually.	No	We had overnight stops between sample preparation (Extraction+SPE+solvent evaporation) and LC-MS	Automatic with verification	35

Lab code	Familiarity	Problem description	Any other information	Overnight stop	Which sample/stage of analysis	Integration	Re-integration
					analysis (+sample reconstitution).		
612	Yes		We mistakenly evaporated the extracts in tubes without volume scaling for the tomato juice (the first matrix we analyzed) and had to transfer the residues to new tubes. Probably poorer recovery because of this (but possibly the same poor recovery in all samples and spiked samples).	No		Automatic with verification	40
614	Yes			No		Manual	0
615	Yes		Good organization/coordination of the trial, all information regarding time schedule are available; all questions have been quickly answered by IRMM.	No		Manual	
616	Yes			No		Automatic with verification	200

7.14 Annex 14 – F5 chromatogram

A standard mixture solution containing the five *Alternaria* toxins involved in the MVS was injected onto pentafluorophenyl (F5) HPLC column. The separation was carried out at acidic (left side) and basic pH (right side). The figure shows the chromatograms of compounds. Acceptable peak shapes could be obtained for all toxins, except for TEA.



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