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International standardization of an LC-MS/MS based food analytical method: development of a generally accepted test procedure for Alternaria toxins

Keywords: mycotoxin, isotope dilution mass spectrometry (LC-IDMS), standardization, citrinin, validation, HorRat value, Horwitz-Thompson equation

1. SUMMARY

There are more than seventy varieties of Alternaria toxins, but researchers have so far identified only a few of them structurally. The objective of this paper is to present a nearly ten-year process, during which an international standard for the simultaneous analysis of five Alternaria toxins in food samples was developed. This long process includes the development of the need for the standard and, in addition to the preparation and evaluation of the standardization tender, the development of the method, its validation and documentation. The paper focuses mainly on the development and validation of the analytical method, which is the longest and most labor-intensive part of the process, but in order to understand the overall picture, it is also necessary to emphasize the first and final steps. The development of a standard is a task of great responsibility for both the preparers of the standard and those involved in the validation and documentation of the standard, as the use of standardized methods is expected by the customers of the laboratories. On the other hand, laboratories that choose unique, self-developed methods can ascertain the accuracy and precision of their procedure by comparing them with the standard method. In this process that went on for nearly ten years, the original analytical method underwent several changes; the goal of these improvements was to make the procedure as simple and reproducible as possible. This is how the use isotope dilution mass spectrometry was reached through derivatization. It is important to emphasize that one of the goals of standardization is to have an appropriate analytical method available to authority laboratories for the testing of legally prescribed food contaminants, which procedure is available to any laboratory, however, it is questionable, whether the cost of the test covers its application. Consequently, it is not necessarily the most cost-effective analysis which is recommended by the standard, which may be the cause of conflict between the professional and economic managers of a laboratory in the case of private laboratories. The final form of the liquid chromatography/isotope dilution mass spectrometry (LC-IDMS) standard method developed for Alternaria toxins is likely to be approved and published by the European Committee for Standardization (CEN) in the end of 2021 (the standard has been issued since the article was submitted: CEN EN 17521:2021 Foodstuffs - Determination of Alternaria toxins in tomato, wheat and sunflower seeds by SPE clean-up and HPLC-MS/MS. The Editor). The standard will contain the determination of tenuazonic acid (TEA), altenuane (ALT), alternariol (AOH), tentoxin (TEN) and alternariol monomethyl ether (AME).

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2. Introduction

Legislation on natural (such as plant toxins) or artificial (such as residual substances) contaminants in foods is strictest in the European Union (EU) worldwide, regulating maximum allowable levels and limit values for contaminants in foods and feeds of plant and animal origin. Commission Regulation (EC) 1881/2006 **[1]** contains the so-called mycotoxin limit values in foods from byproducts of the secondary metabolism of molds in agricultural crops. The regulation is expanded constantly: while initially it only contained „classical" toxins such as deoxynivalenol (DON), aflatoxins (B1, G1, B2, G2, M1), fumonisins (B1 and B2) or patulin, by 2013 T-2, HT-2, and by 2016 citrinin were also included in the toxin regulation. The range of components is expanded constantly; the process is preceded by a scientific opinion formulated by the European Food Safety Authority (EFSA), as well as other impact studies. They take into account both the economic points of view of producers and the short- and long-term health risks of the toxins. *Alternaria* toxins are not yet regulated, the permissible limit values are expected in the 1-10 µg/kg range for ALT, AOH and AME, and in the 10-1,000 µg/kg range for TEA and TEN. The foodstuffs concerned are cereals (primarily wheat), tomato-based foods (tomato juice or puree) and products made from sunflower seeds and similar raw materials **[2]**.

The EFSA report on *Alternaria* toxins titled *„Scientific Opinion on the risks for animal and public health related to the presence of Alternaria toxins in feed and food"* was published in 2011 **[2]**, and it discusses their presence in various foods, human and animal health studies and their potential risks over 97 pages. A further goal of the report is to draw attention to future regulations and to the development of a uniform analytical method. Accordingly, the analysis of *Alternaria* toxins in wheat, tomato and sunflower seeds by liquid chromatography tandem mass spectrometry (LC-MS/MS) was published as a standardization procedure in the mycotoxin standardization tender announced by CEN in spring 2013.

3. Initial (intra-laboratory) analytical method

According to the basic requirement of the tender, the aspirant laboratory must have a valid accredited status according to standard ISO 17043, which applies to the organization of proficiency tests and means a well-defined test protocol that meets the analytical performance characteristics for single laboratory validation **[3].** Lacking this, the laboratory must have a procedure previously certified by inter-laboratory validation. Due to its cost implications, the latter is a rarer case, but it is much more efficient in demonstrating the true reproducibility of the method than the requirements of standard ISO 17043, whereas the former validation only shows the in-laboratory reproducibility (intermediate precision) of the analysis.

The European Commission Joint Research Centre (JRC, Geel, Belgium) is a joint research center within the EU, which until 2017 included the EU Reference Laboratory for Mycotoxins (EU-RL for Mycotoxins). In 2013, an LC-MS/MS method was developed as an EU-RL method for wheat, tomato juice and sunflower seeds for the following five main *Alternaria* toxins (***Figure 1***): tenuazonic acid (TEA), altenuane (ALT), alternariol (AOH), tentoxin (TEN) and alternariol monomethyl ether (AME) **[4]**. Of the five toxins, TEA has the most different structure and physicochemical properties (chelating properties) from the other toxins **[5]**. Accordingly, previous literature has focused on the determination of TEA **[5]**, or the other toxins **[6]**, less attention has been paid to their simultaneous analysis. Our goal was a five-component simultaneous analysis, which was achieved by chemical derivatization. The structure of TEA contains an aldehyde functional group that is highly reactive with 2,4-dinitrophenylhydrazine (DNPH), and the physicochemical properties (e.g., Log *P*, octanol-water distribution) of the resulting TEA hydrazone are much closer to those of the other *Alternaria* toxins from a chromatography point of view **[5]**. In the derivatized form, it loses its chelating properties. DNPH reacts only with TEA among the target components (***Figure 2***), it does not interfere with the determination of the others **[7]**. The extraction procedure arrived at in the method was developed using an experimental design with a sample of rye naturally contaminated with the toxins. In addition to *Alternaria* toxins, citrinin was also included in the method. The main characteristics of the method developed in this way are the following **[4]**, **[7]**:

- Analysis of six components (TEA, ALT, AOH, TEN, AME and citrinin);
- Matrices: cereals, tomato juice, peeled sunflower seeds;
- Sample weight for liquid samples: 1.0 g;
- Extraction solvent for liquid samples: 5 mL of methanol;
- Sample weight for solid samples: 2.0 g;
- Extraction solvent for solid samples:15 mL of methanol-water (70/30, v/v) mixture;
- Derivatizing agent: 0.58% DNPH in aqueous hydrochloric acid;
- Stop reagent: 5% (v/v) undecanal in methanol;
- Sample purification: polymer-based solid phase extraction (SPE);
- Sample evaporation and redissolution in methanol;

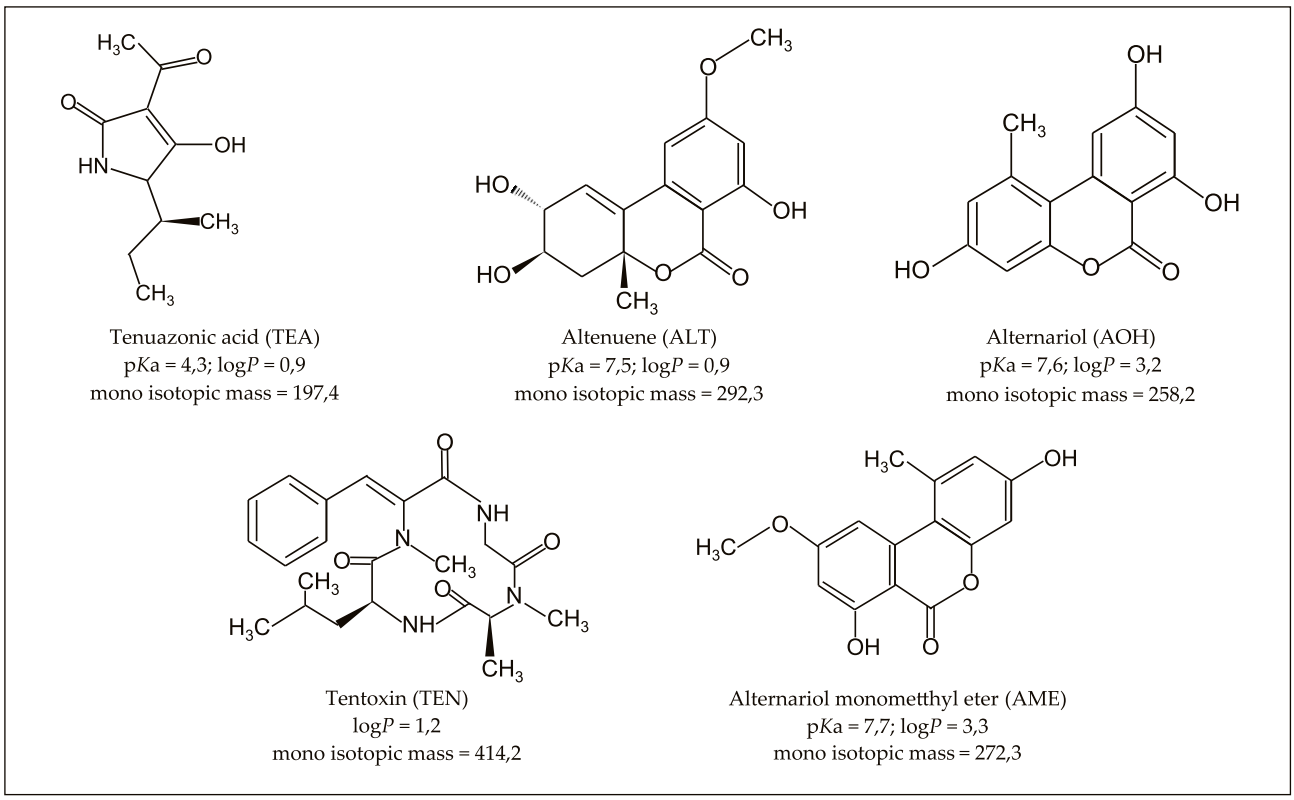

Figure 1. The structure of Alternaria toxins and their most important property

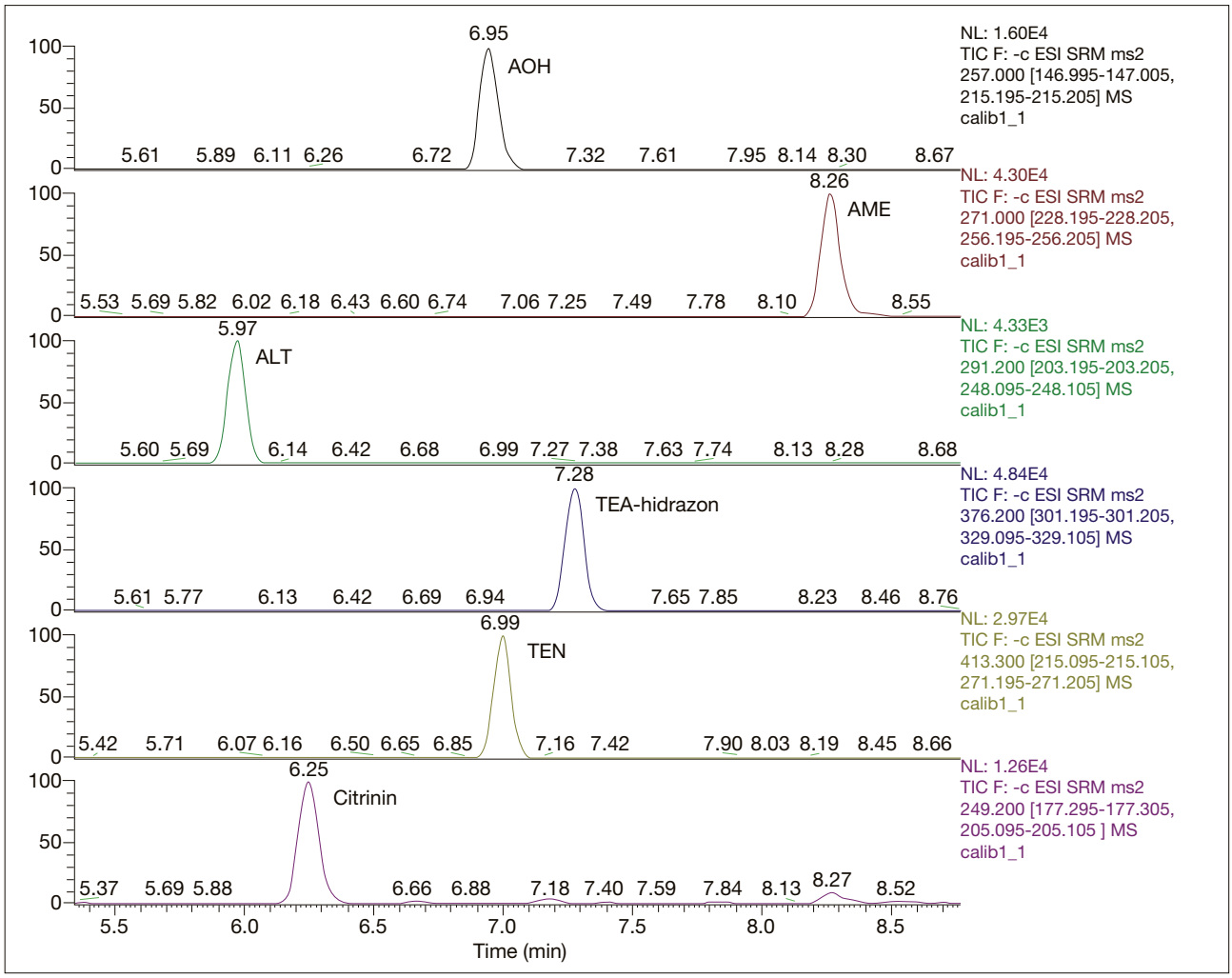

Figure 2. LC-MS/MS chromatograms of Alternaria toxins and the citrinin (10 µg/kg); (the TEA was in derivatisation form)

- Syringe filtration on PTFE filter;
- LC-MS/MS separation: acidic eluent, C-18 stationary phase and ESI negative ionization (***Table 1***);
- Syringe filtration on hydrophilic PTFE filter;
- Calibration: matrix-matched calibration without isotope-labelled internal standard.

Table 1. Ion transitions of Alternatria toxins and citrinin using ESI negative ionisation and chemical derivatisation

Component	Parent ion (m/z)	Daughter ions (m/z)	Collision energy (V)
TEA-hydrazone	376	301 329	15 15
ALT	291	248 203	20 30
AOH	257	215 147	20 20
TEN	413	271 215	15 15
AME	271	256 228	20 20
Citrinin	249	205 177	15 20

Table 2. Results of Alternaria toxins in proficiency test. The samples (tomato juice) and the standard solution were also tested after derivatization

Sample	Component	Detected concentrations (µg/kg in the sample and µg/L in the standard solution)	Assigned values (µg/kg in the samples and µg/L in the standard solution)	Z-value	Evaluation
1	TEA	51.9	53.0	-0.1	Accepted
	ALT	<LOQ (25 µg/kg)	9.48	-	Accepted
	AOH	16.5	13.9	0.8	Accepted
	TEN	9.0	8.29	0.4	Accepted
	AME	11.3	11.0	0.1	Accepted
2	TEA	28.0	27.0	0.2	Accepted
	ALT	<LOD	-	-	Accepted
	AOH	6.30	6.58	-0.2	Accepted
	TEN	<LOD	-	-	Accepted
	AME	1.64	1.56	0.2	Accepted
3	TEA	38.4	39.1	-0.1	Accepted
	ALT	30.7	30.0	0.1	Accepted
	AOH	37.5	36.3	0.1	Accepted
	TEN	31.8	27.4	0.7	Accepted
	AME	38.8	37.3	0.2	Accepted
Standard solution	TEA	7.90	11.0	-1.3	Accepted
	ALT	7.48	8.73	-0.6	Accepted
	AOH	8.46	10.2	-0.8	Accepted
	TEN	10.9	10.7	0.1	Accepted
	AME	11.8	11.4	0.1	Accepted

In addition to the in-laboratory validation of the method, we also participated in an international proficiency test organized by the Bundesinstitut für Risikobewertung (BfR, Berlin, Germany) as a National Reference Laboratory (NRL) for the determination of the five *Alternaria* toxins in tomato juice. During the analysis, the five toxins had to be determined in three samples and a standard solution **[7]**. Our results are shown in ***Table 2***. All reported values were acceptable, with Z-score values between -2 and +2. The results showed that the method recommended by us in the tender is suitable for the standardization of *Alternaria* toxins.

4. Modified analytical method

The analytical method proposed by the JRC was adopted by CEN and the mandate (mandate M/520) was given to the JRC in 2014. However, the working group (TC 275 WG 5 „Horizontal Methods for Food – Biotoxins") did not support chemical derivatization in the method on the grounds that it is an additional and time-consuming step in the method, which may reduce its precision and should be avoided. The determination of citrinin could not be included in the method either, the analysis could only contain *Alternaria* toxins.

TEA can also be analyzed in its native form, but in this case HPLC separation has to be carried out with an alkaline eluent, requiring a stationary phase that is stable up to pH 9. The method has indeed become simpler without derivatization (***Figure 3***), but this has required significant modifications to maintain the accuracy of the procedure. In addition to being time-consuming, another disadvantage of derivatization was an increase in the noise level, as many matrix-forming compounds also react with DNPH, which can co-elute with the target components, increasing the noise in the MS/MS instrument. In the modified method, essentially the HPLC separation had to be optimized and an extraction medium had to be selected which ensured the best possible recovery from each matrix.

The main characteristics of the method developed in this way **[8]**:

- Analysis of five components (TEA, ALT, AOH, TEN and AME);
- Matrices: cereals, tomato juice, sunflower seeds;
- Sample weight for liquid samples: 2.0 g;
- Extraction solvent: 15 mL methanol/water/acetic acid (80/19/1, v/v/v);
- Sample purification: polymer-based solid phase extraction (SPE);
- Sample evaporation and redissolution in 100 µL of dimethyl sulfoxide and dilution with 900 µL of water;
- Syringe filtration on hydrophilic PTFE filter;
- LC-MS/MS separation: eluent with alkaline pH (pH 8.7), C-18 stationary phase and ESI negative ionization (***Table 3***);
- Calibration: matrix-matched calibration without isotope-labelled internal standard.

This modified method was accepted by the working group and, following its in-laboratory validation, the inter-laboratory validation of the analytical method could also begin in spring 2015.

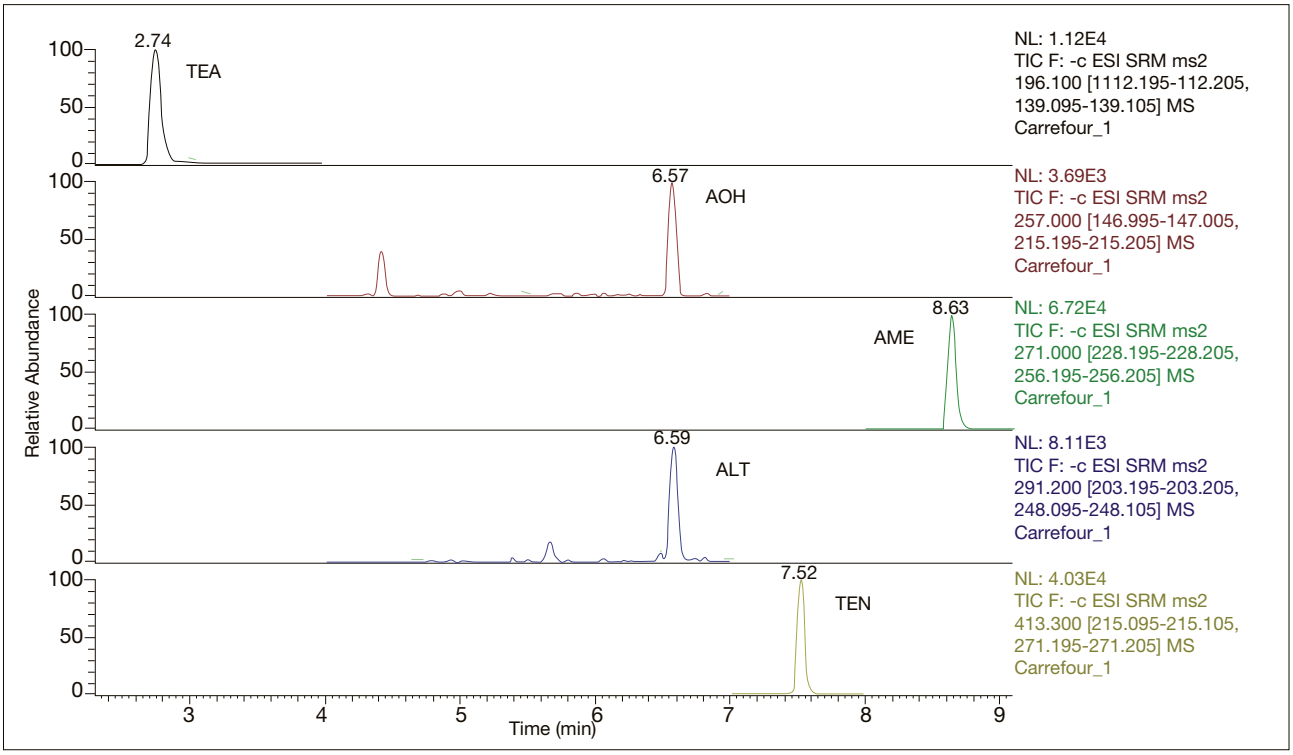

Figure 3. LC-MS/MS chromatograms of Alternaria toxins (10 µg/kg) using basic pH eluent without derivatisation

Table 3. Ion transitions of Alternatria toxins and citrinin using ESI negative ionisation without chemical derivatisation

Component	Parent ion (m/z)	Daughter ions (m/z)	Collision energy (V)
TEA	196	139 112	15 20
ALT	291	248 203	20 30
AOH	257	215 147	20 20
TEN	413	271 215	15 15
AME	271	256 228	20 20

5. Inter-laboratory validation of the method

The most important part of the standardization process is the inter-laboratory validation of the method, the main purpose of which is to check and evaluate the reproducibility of the analysis. To do this, the concentrations of the toxins must be determined in naturally contaminated (at low, medium and high levels) and spiked samples. To evaluate the concentration of a given component n a given sample, a minimum of eight independent values are required, however, only the results of two laboratories can be excluded **[9]**. It is advisable to involve at least fifteen laboratories in order to have an adequate number of results for each sample and component. This is so because, based on experience, about 2-3 laboratories do not report results, while some samples and their components do not always produce a sufficient number of reported results. This can occur mainly at low concentration levels because not all participants possess instruments with adequate sensitivity.

If, during the validation, the goal is to determine components that have long been analyzed (such as DON or aflatoxins), it is relatively easy to ask laboratories with the necessary experience for validation, based on their successful participation in previous proficiency tests. However, *Alternaria* toxins are still analyzed by very few laboratories to this day, so laboratories applying for the validation do not always have prior experience. For this reason, organization of a so-called pre-trial becomes necessary, in which the laboratories participating in the validation can master the method in advance. In this case, the pre-trial was performed with twenty-five laboratories, analyzing tomato juice samples **[8]**, and only three of the participating laboratories had prior knowledge of *Alternaria* LC-MS/MS analysis. Of the twenty-five laboratories, only sixteen eventually participated in the final validation, because either they did not return any result or their results differed significantly from the consensus average.

During the final validation, the following samples were sent to the sixteen laboratories **[8]**:

- Cereals naturally contaminated with *Alternaria* toxins: wheat, triticale and sorghum;
- Tomato juice naturally contaminated with *Alternaria* toxins: 3 batches;
- Sunflower seeds naturally contaminated with *Alternaria* toxins: 2 batches of unpeeled seeds and 1 seed mixture, which was a mixture of peeled and unpeeled seeds;
- Participants received each sample under two codes (blind replicates) so that we could evaluate repeatability within the laboratory and to have more date available to analyze reproducibility;
- For the preparation of spiked samples, separate test samples were sent for each matrix, for which a standard solution mixture containing *Alternaria* toxins in unknown concentrations was also provided to the participating laboratories. Spiked samples were prepared by the laboratories according to the „spiking guide";
- Blank samples for each matrix for matrix-matched calibration;
- In the case of sunflower seeds, the blank was peeled sunflower, because the unpeeled samples are high in TEA and therefore not suitable for calibration;
- The analytical standards of the target components and their standard solution mixture were also provided, so that all laboratories would use the same calibration solution, and no deviation would result from this;
- The homogeneity of the samples was checked according to the harmonized protocol before dispatch **[10]**;
- Simultaneously with the sending of the samples, stability testing of the samples was initiated at different temperatures and for different durations.

Concentration levels required by CEN for validation: 1-10 µg/kg for ALT, AOH and AME, and 10-1,000 µg/kg for TEA and TEN. Recovery was assessed from the concentrations measured in the spiked samples, with spiking levels of 2 and 8 µg/kg for ALT, AOH and AME, and 50 and 200 µg/kg for TEA and TEN. These levels were unknown to participants.

Statistical evaluation of the results obtained (concentrations not corrected for recovery) focused mainly on reproducibility **[9]**. The reproducibility of the method is well characterized by the so-called HorRat value. The HorRat value is the quotient of the reproducibility of a given target component calculated for the given sample and the target reproducibility expected by the organizers. The latter reproducibility value (the „target reproducibility") can be calculated from the Horwitz-Thompson equation: below 120 µg/kg it is uniformly 22%, while above this value the classical Horwitz relationship can be applied **[11]**. Based on the validation criteria, the HorRat value must be less than two; this condition was indeed met, except for TEA, in the case of the unpeeled sunflower samples. ***Table 4*** shows the HorRat values calculated for TEA in the case of different sunflower samples. While in unpeeled sunflowers the calculated HorRat values were uniformly 2.4 regardless of the concentration **[8]**, in the case of peeled samples, which contained much lower concentrations of TEA, the values were below two. The lower reproducibility observed during the analysis of unpeeled samples can be explained by the calibration and the matrix effect, which is a typical feature of LC-MS/MS-based measurements, and mainly effects the precision and accuracy of the method **[11]**. During the validation, a peeled sunflower sample was provided for calibration, because it contained a small amount of TEA contamination of natural origin, as opposed to unpeeled sunflower that was contaminated with high concentrations of TEA. The extracts of the unpeeled and peeled sunflower samples contain significantly different matrices, which can even be noticed by their color. Consequently, the calibration from the peeled sample could not compensate for the matrix effect in the unpeeled sunflower samples, so the detected concentrations were significantly affected by the matrix effect. The reason for this is that the endogenous constituents of unpeeled sunflower differ from those of peeled sunflower.

It is important to note that laboratories reported only the detected concentrations; the measured values were not corrected for recovery, in contrast to the usual procedure for conventional proficiency tests. Different laboratories used different instruments in which the matrix effect during the analysis of unpeeled sunflowers may have been different. Since the calibration recorded from the peeled sample did not adequately compensate for the matrix effect, there were large differences between the values measured by the participants. The same problem did not occur in the analysis of peeled sunflowers, because a similar degree of matrix effect may have occurred in the calibration and the test sample, due to the similarity of the samples. It is worth noting that the repeatability was also acceptable in the case of unpeeled samples (<20%). The reason for this is that repeated analysis of the same sample has the same matrix effect in the same instrument, so laboratories detected similar concentrations within the laboratory for duplicate samples, while inter-laboratory results were different due to the different matrix effects in the different instruments.

Table 4. HorRat values calculated for TEA for sunflower samples with matrix-matched calibration.

	Shelled	**Shelled**	**Shelled**	**Husked**	**Husked**
Concentration (µg/kg)	804	1102	452	53.0	153
Repeatability (RSD%)	18.8	14.9	15.3	10.4	11.6
Reproducibility (RSD%)	39.5	38.3	43.7	35.7	25.0
Target reproducibility (RSD%)	16.5	15.8	18.0	22.0	21.2
HorRat	2.4	2.4	2.4	1.2	1.6

6. Final method with isotope dilution and its inter-laboratory validation

As the HorRat values were not below two for all components and samples during the validation, further development of the method became necessary. The reproducibility of LC-MS/MS methods can be greatly enhanced by isotope dilution (Isotope Dilution Mass Spectroscopy – IDMS), which compensates well for the matrix effect varying from sample to sample. In this case, a stable isotope-labeled analogue of the target compound is added to the sample as an internal standard (ISTD). the physicochemical properties of the internal standard are the same as those of the target component (a small difference in polarity may occur with deuterated standards), so the target compound and its isotopically labeled analogue ideally elute at the same retention time. As a result of the co-elution, the target component and its internal standard are subjected to the same direction and extent of matrix effect in the ion source, so the ratio of the responses (areas) of the target compound and the ISTD, the isotope ratio (IR), will be independent of the matrix effect.

The ISTD does not interfere with the signal of the target component, because it is detected at other m/z values that are sufficiently distant (preferably at least +3 Da) from the m/z value of the target component due to the isotope label.

This requires isotope-labeled ISTDs, which were not yet available in 2015, so we first used matrix-matched calibration. However, stable isotope-labeled ISTDs (labeled with ^{13}C or deuterium) of *Alternaria* became commercially available in 2018 (TEA-$^{13}C2$, ALT-d6, AOH-d3, TEN-d3 and AME-d3), making revalidation of the method possible using the IDMS technique.

After 2018, the JRC repeated the in-laboratory and inter-laboratory validation using the method supplemented with isotope-labeled ISTDs (***Table 5***). The concept was the same during the first and second validation, with the difference being that cereal-based samples only included wheat samples and tomato-based samples were tomato purees during the second procedure. In the case of TEA, HorRat values ranged from 0.40 to 0.66 with IDMS detection in unpeeled samples, while the value was 0.53 in peeled samples, which is significantly better than the values without ISTD (***Table 4***). As previously expected, isotope dilution greatly improved inter-laboratory reproducibility. During the validation, the expected precision could only be achieved with ISTDs, which is common in LC-MS quantitative studies. This is always due to matrix effect compensation.

Table 5. Ion transition values of Alternaria toxins and the isotope labelled ISTDs using negative ESI

Component	Parent ion (m/z)	Daughter ions (m/z)	Collision energy (V)
TEA	196	139 112	-27 -30
TEA-$^{13}C2$	198	141	-27
ALT	291	214 186	-29 -35
ALT-d6	296	189	-35
AOH	257	215 212	-35 -35
AOH-d3	260	218	-35
TEN	413	141 271	-25 -22
TEN-d3	416	274	-22
AME	271	256 228	-27 -36
AME-d3	274	259	-36

7. Documentation

The full validation dossier was completed by 2020 **[12]**, together with the draft standard. Review and revision of the draft standard will be completed soon and the proposed standard is expected to be adopted by CEN in the end of 2021 (the standard has been issued since the article was submitted: CEN EN 17521:2021 Foodstuffs - Determination of Alternaria toxins in tomato, wheat and sunflower seeds by SPE clean-up and HPLC-MS/MS. The Editor).

8. Deviation from the standard method

LC-MS/MS instruments from different vendors may vary significantly in terms of sensitivity. One of the main reasons for this is the ion source **[11]**. While the standard describes the use of ESI (Electrospray Ion Source), there is hardly any application in the literature where the ESI ion source of the instrument showed sufficient efficiency to achieve the desired detection limit, so the use of atmospheric pressure chemical ionization (APCI) became necessary **[13]**. Another possibility is when the instrument used is so sensitive that no solid phase purification or enrichment (Solid Phase Extraction – SPE) is required, but the extract of the sample can be injected directly into the device („dilute-and-shoot") **[11]**, **[14]**. The important feature of a standard is that all laboratories should be able to use the method described in it, so the application of SPE enrichment was unavoidable due to the low concentration levels and the complexity of the unpeeled sunflower seed samples.

If the first validation is successful, matrix-matched calibration would probably be recommended by the standard. However, with the advent of ISTDs, a group of laboratories would prefer to use IDMS later on.

From this point of view, it is fortunate that IDMS has been introduced in the standard, which is simpler and more accurate, but the acquisition of ISTDs is more expensive. In the absence of ISTDs, standard addition (as a quantitative evaluation) can also be used to adequately compensate for the matrix effect, but this is time-consuming, because each sample must be prepared at least four or five times. Yet there are laboratories that use this type of evaluation.

9. Acknowledgment

I would like to give thanks Carlos Gonçalves for the successful completion of the standardization project.

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