Validation of a Stable Isotope Dilution Assay for the Accurate Quantitation of Mycotoxins in Maize Using UHPLC/MS/MS

Application Note

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Abstract

This application note describes a fast, easy-to-handle, and cost-effective analytical method for the quantitation of all 11 mycotoxins regulated in Europe for cereal-based food products. Based on the method published by Varga *et al*. [1], this application note provides additional useful information to quickly establish the method in other labs. The method comprises a two-step solvent extraction, a UHPLC separation using the Agilent 1290 Infinity LC System, and tandem mass spectrometric detection using the highly sensitive Agilent 6490 iFunnel Triple Quadrupole LC/MS System. Matrix effects in the electrospray ionization are compensated through the use of uniformly (^{13}C)-labeled mycotoxins as internal standards for each of the 11 target compounds. Unlike with other approaches, here the cost and time associated with extract cleanup is eliminated. Additionally, there is no need to tediously prepare matrix-matched standards.

A validation in maize has shown that the proposed two-step extraction is essentially complete (97 to 111%), allowing addition of the internal standards after extraction and, therefore, only in small quantities. Further tests on spiked maize extracts have shown that the use of internal standards allows the method to achieve good accuracies with apparent recoveries between 88 to 105%. The trueness of the method was proven by the measurement of several matrix reference materials with well-characterized concentrations.

Limits of quantitation (LOQ) for all mycotoxins were below the concentrations required by European regulation for cereal-based food and even cereal-based baby food. The method allows the accurate quantitation of all regulated mycotoxins in maize. Similar extraction recoveries and analytical accuracies may well be possible for other cereal-based foods. The key advantage besides the high accuracy is the simplicity of the method, combined with a wide scope of analytes, making it attractive for any busy routine testing laboratory.

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Introduction

Mycotoxins are secondary metabolites of fungi, which can cause acute or chronic toxicity in mammals. Adverse health effects include immunosuppressive, hepatotoxic, mutagenic, carcinogenic, or estrogenic activity caused by the toxins [2]. Out of the several hundreds of mycotoxins which have been identified, 11 are considered to be a major health risk due to their occurrence and toxicity. There are regulations in place in approximately 100 countries. In Europe, Commission Regulation (EC) 1881/2006 and its amendments specify maximum levels in food for 11 mycotoxins: aflatoxins (sum of aflatoxin B_1, B_2, G_1, and G_2, as well as for AFB_1 alone and aflatoxin M_1), the sum of fumonisin B_1 and B_2, ochratoxin A (OTA), patulin, deoxynivalenol (DON), and zearalenone (ZEN) [3]. While aflatoxin M_1 and patulin only need to be considered in milk or apple, respectively, the Fusarium toxins T-2 and HT-2 are frequently found in cereals and are considered to be a public health concern. Recently, European Commission Recommendation 2013/165/EU was published, which specifies indicative levels for cereals and cereal products, above which investigations should be performed on the factors leading to the presence of T-2 and HT-2 toxin [4]. T-2 toxin is already regulated in Russia for cereals and cereal-based food products.

Enforcement of the specified maximum levels requires the availability of reliable and accurate analytical methods. LC coupled to tandem mass spectrometry has proven to be a powerful tool for the analysis of a variety of residues and contaminants. It has been shown that LC/MS/MS allows the simultaneous determination of mycotoxins in different commodities with high sensitivity and selectivity [5]. However, in complex matrixes accurate quantitation might be hampered by matrix effects in the electrospray process causing signal suppression or enhancement of the analyte signal. Differences in the degree of matrix effects cannot only be expected between different commodities but also between individual samples of one matrix type [6].

There are different strategies to compensate for matrix effects such as the dilution of the sample, matrix-matched calibrations, standard addition, or the use of internal standards. While the dilution of the sample reduces the sensitivity of the analytical method, the use of matrix-matched calibrations is problematic due to the ubiquity of some toxins in several matrixes (for example, DON in maize) and due to the fact that variations in the matrix effects within a commodity are not fully compensated. Standard addition multiplies the amount of runs needed for each sample and is therefore not very time-efficient.

For busy routine testing laboratories, the use of internal standards which behave exactly like the target compounds but are still distinctive, is most attractive. In the past, internal standards have often been analogs of a single compound or group of compounds. However, this has limited value when the intention is to compensate for matrix effects, since such effects are retention time dependent and target compounds rarely elute concurrent with such analogs.

Stable isotopically labeled compounds are ideally suited for this purpose since they share the same physico-chemical properties (meaning they elute together with the target compound) but are still distinguishable by MS due to their different molecular mass. In addition, they are not present in naturally contaminated samples. Since the naturally abundant isotopic distribution of the analyte is diluted by the addition of stable isotopically labeled compounds, this procedure is often referred to as stable isotope dilution assay (SIDA) [7].

This application note outlines a fast, reliable, and easy-to-handle analytical method for the quantitation of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2), fumonisins (FB_1, FB_2), OTA, DON, and ZEN, as well as T-2 and HT-2 toxin in cereal-based food by UHPLC/MS/MS. To ensure accurate quantitation, a uniformly (^{13}C)-labeled homolog for each target analyte was used as the internal standard. A two-step extraction without further cleanup was combined with UHPLC separation and the highly sensitive MS/MS detection using Dynamic MRM. The method was successfully validated for maize and method performance parameters including the determination of linearity, the LOQ based on the signal-to-noise (S/N) ratio, and the repeatability have been evaluated. The trueness of the method was proven by analyzing several test materials with well-characterized concentrations. All presented results are published in more detail by Varga *et al*. [1].

Experimental

Reagents and maize samples

All reagents and solvents were HPLC or LC/MS grade. Acetonitrile and methanol were purchased from Baker (Mallinckrodt Baker, Deventer, Netherlands). Ultrapure water was produced using a Milli-Q Integral system equipped with a 0.22-µm point-of-use membrane filter cartridge (EMD Millipore, Billerica, MA, USA). Formic acid was from Fluka (Fluka AG, Buchs, Switzerland) and ammonium formate solution (5 M) was from Agilent (p/n G1946-85021).

All mycotoxin analytical standards were included in the LC/MS Mycotoxin Kit (p/n KIT-001) purchased from Romer Labs Diagnostic GmbH (Tulln, Austria). The kit contains mixtures of unlabeled and uniformly (^{13}C)-labeled fumonisin B_1 and B_2, Fusarium toxins (DON, ZEN, T-2, and HT-2 toxin), aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2), and OTA in acetonitrile or acetonitrile/water (1:1, v/v).

For spiking experiments, an uncontaminated maize sample was required. For this reason, maize was collected from the field and was visually inspected for the absence of mold. The maize kernels were ground, homogenized, and analyzed for mycotoxins using this UHPLC/MS/MS method. Background contaminations were below the LODs. Various test materials for maize including official reference materials and check sample materials for several mycotoxins were purchased from EU Joint Research Centre (JRC, Geel, Belgium), Food Analysis Performance Assessment Scheme (FAPAS, York, UK), Romer Labs (Tulln, Austria), and Scientific and Technological Research Council of Turkey-Ankara Test and Analysis Laboratory (TÜBITAK-ATAL) to check the trueness of the method. Samples were prepared and analyzed using this UHPLC/MS/MS method and results were compared to certified or consensus values.

Solutions and standards

A working solution of the unlabeled mycotoxins was prepared by adding 500 µL of the aflatoxin mix, 50 µL of the fumonisin mix, 50 µL of the Fusarium toxin mix, and 50 µL of the OTA solution to 350 µL ultrapure water resulting in the following concentrations:

- AFB_1, AFB_2, AFG_1, and AFG_2, 500 ng/mL
- DON, 5,000 ng/mL
- FB_1 and FB_2, 2,500 ng/mL
- HT-2, 5,000 ng/mL
- OTA, 500 ng/mL
- T-2, 500 ng/mL
- ZEN, 1,500 ng/mL

The working solution of the U-(^{13}C)-labeled mycotoxins was prepared by adding 20 µL of the labeled aflatoxin mix, 100 µL of the labeled fumonisin mix, 50 µL of the labeled Fusarium toxin mix, and 5 µL of the labeled OTA solution to 825 µL acetonitrile/water (1:1, v/v) resulting in the following concentrations:

- ($^{13}C_{17}$)-AFB_1, ($^{13}C_{17}$)-AFB_2, ($^{13}C_{17}$)-AFG_1 and ($^{13}C_{17}$)-AFG_2, 10 ng/mL
- ($^{13}C_{15}$)-DON, 500 ng/mL
- ($^{13}C_{34}$)-FB_1 and ($^{13}C_{34}$)-FB_2, 500 ng/mL
- ($^{13}C_{22}$)-HT-2, 500 ng/mL
- ($^{13}C_{20}$)-OTA, 50 ng/mL
- ($^{13}C_{24}$)-T-2, 50 ng/m
- ($^{13}C_{18}$)-ZEN, 150 ng/mL.

The stock solutions and the two working solutions were stored at –20 °C.

Calibration samples were prepared by diluting the unlabeled working solution with a mixture of acetonitrile/water (30:70; v/v) to cover a concentration range of 0.005 to 50 ng/mL for the aflatoxins, OTA, and T-2 toxin, 0.05 to 500 ng/mL for DON and HT-2 toxin, 0.025 to 250 ng/mL for the fumonisins, and 0.015 to 150 ng/mL for ZEN. Twenty microliters of the (^{13}C)-labeled standard working solution were added to 80 µL of the neat calibration solution and thoroughly mixed. Table 1 shows the resulting concentrations of the internal standards in the injected samples.

Sample preparation

A 5-g (± 0.01 g) amount of ground and homogenized maize samples were weighed in 50-mL plastic tubes, followed by the addition of 20 mL of extraction solvent A (acetonitrile/water; 80/20, v/v, containing 0.1% formic acid), and extraction of the samples on a rotary shaker for 60 minutes (Edmund Bühler SM30, Hechingen, Germany) at room temperature. After centrifugation at 3,500 rpm for 5 minutes (Eppendorf Centrifuge 5804 R, Hamburg, Germany), the supernatant was transferred to another 50-mL plastic tube, followed by the addition of 20 mL of extraction solvent B (acetonitrile/water; 20/80, v/v, containing 0.1% formic acid) to the residue and extraction of the samples on the rotary shaker for another 30 minutes. Afterwards, the tubes were centrifuged at 3,500 rpm for 5 minutes and the supernatant was combined with the first extract. When combining the supernatants of the two extraction steps, a precipitate might occur and, therefore, the combined raw extracts were centrifuged again for 5 minutes at 3,500 rpm. An 80-µL aliquot of the final extract was transferred into an HPLC vial with a glass insert (deactivated) and 20 µL of the (^{13}C)-labeled working solution were added and thoroughly mixed. This resulted in a 10-fold dilution. A concentration of 10 ng/mL in the final solution corresponded to 100 µg/kg in the sample. Figure 1 shows a scheme of the extraction procedure.

For the evaluation of matrix effects, blank maize samples were extracted according to the procedure described above. An aliquot of 975 µL of the raw extract was spiked with 25 µL of the working solution of unlabeled mycotoxins and thoroughly mixed. The spiked extract was further diluted with blank extract to obtain six different spiking levels. For the evaluation of extraction recoveries and apparent recoveries as well as working range, LOQ, and repeatability, blank maize samples were spiked before extraction with the appropriate amount of the unlabeled mycotoxin working solution. Samples were extracted following the procedure described above. The trueness of the method was assessed by the measurement of 12 matrix reference materials with known (certified or consensus) concentrations for the target analytes.

Table 1. Concentrations of the (^{13}C)-Labeled Internal Standards in the Injection Solution

Compound	Concentration (ng/mL)	Amount injected (pg)
($^{13}C_{17}$)-AFB$_1$	2	6
($^{13}C_{17}$)-AFB$_2$	2	6
($^{13}C_{17}$)-AFG$_1$	2	6
($^{13}C_{17}$)-AFG$_2$	2	6
($^{13}C_{15}$)-DON	100	300
($^{13}C_{34}$)-FB$_1$	100	300
($^{13}C_{34}$)-FB$_2$	100	300
($^{13}C_{22}$)-HT-2	100	300
($^{13}C_{24}$)-T-2	10	30
($^{13}C_{18}$)-ZEN	30	90
($^{13}C_{20}$)-OTA	10	30

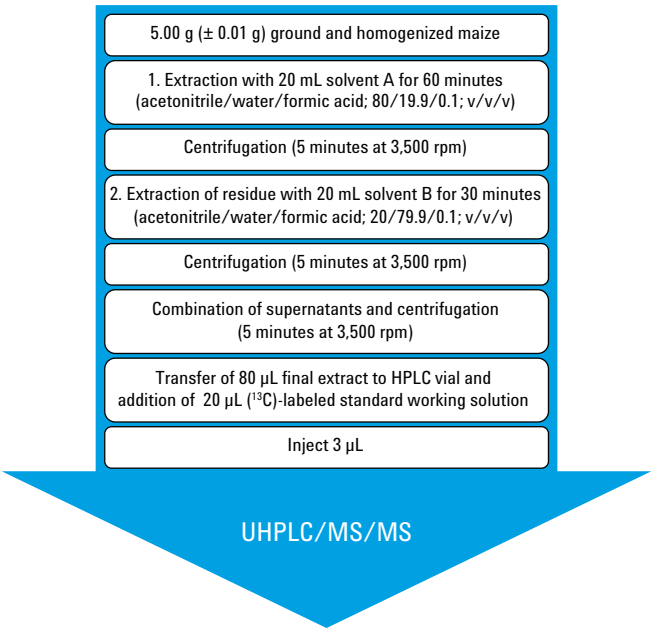

Figure 1. Schematic overview of the sample preparation and extraction procedure for maize samples.

Equipment

Separation was carried out using an Agilent 1290 Infinity UHPLC system consisting of an Agilent 1290 Infinity Binary Pump (G4220A), an Agilent 1290 Infinity High Performance Autosampler (G4226A), and an Agilent 1290 Infinity Thermostatted Column Compartment (G1316C). The UHPLC system was coupled to an Agilent G6490A Triple Quadrupole mass spectrometer equipped with an Agilent Jet Stream electrospray ionization source. Agilent MassHunter workstation B.06.00 software was used for data acquisition and analysis.

Method

The 1290 Infinity UHPLC conditions are summarized in Table 2 and the G6490A Triple Quadrupole parameters are shown in Table 3. Analysis was carried out with positive and negative electrospray ionization in Dynamic MRM mode using two major transitions per target compound and one transition for the (^{13}C)-labeled internal standards. Transitions and conditions for the native and labeled mycotoxins were optimized using the MassHunter Optimizer software with flow injection of the diluted stock solutions. For most analytes, the $[M+H]^+$ species produced the most abundant precursor ion except for T-2 and HT-2 toxin, which were analyzed as the $[M+NH_4]^+$ species. For ZEN, the highest sensitivity could be achieved in negative mode with $[M-H]^-$ as the precursor. All used transitions and conditions are summarized in Table 4. To minimize the amount of matrix going into the spray chamber, the HPLC flow was diverted into waste within the first minute of the analysis and from 7 minutes until the end of the analysis.

Table 2. UHPLC Parameters

UHPLC column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm (p/n 959758-902) at 30 °C	
Mobile phase	A: 0.1% formic acid, 5 mM ammonium formate in water B: 0.1% formic acid, 5 mM ammonium formate in methanol	
Gradient program	Min	% B
	0	30
	0.5	30
	8.0	100
	9.5	100
	9.6	30
	Post time	2 minutes
Flow rate	0.35 mL/min	
Injection volume	3 µL	
Needle wash	5 seconds with acetonitrile/water (50/50; v/v)	

Table 3. Agilent G6490A Triple Quadrupole Parameters

Ionization mode	Positive and negative ESI with Agilent Jet Stream
Scan type	Dynamic MRM
Gas temperature	140 °C
Gas Flow	16 L/min
Nebulizer pressure	25 psi
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Capillary voltage	4,000 V (pos); 3,000 V (neg)
Nozzle voltage	0 V
Cycle time	400 ms
Total number of MRMs	33
Maximum number of concurrent MRMs	12
Minimum dwell time	39.8 ms
Maximum dwell time	196.5 ms
Resolution	unit, unit

Table 4. MRM Transitions and Conditions Used for the Analysis of Native and (^{13}C)-Labeled Mycotoxins.

Compound	RT	Precursor ion	Product ion	Collision energy	Cell accelerator	Polarity
Aflatoxin B_1	4.49	313.1	285.0 241.0	21 41	3 3	Positive
Aflatoxin B_2	4.24	315.1	287.0 258.9	21 29	3 3	Positive
Aflatoxin G_1	3.93	329.1	243.0 200.1	25 41	3 5	Positive
Aflatoxin G_2	3.65	331.1	313.0 245.1	21 25	3 5	Positive
Deoxynivalenol	1.51	297.1	249.0 203.0	4 12	3 3	Positive
Fumonisin B_1	5.65	722.4	352.4 334.4	37 37	3 3	Positive
Fumonisin B_2	6.55	706.4	336.4 318.3	41 41	3 3	Positive
HT-2 toxin	5.46	442.2	263.0 215.0	9 13	3 3	Positive
Ochratoxin A	6.53	404.1	238.9 102.1	25 70	3 5	Positive
T-2 toxin	6.03	484.3	305.0 215.1	8 9	5 5	Positive
Zearalenone	6.51	317.1	272.9 130.9	17 29	7 5	Negative
(^{13}C)-Aflatoxin B_1	4.49	330.1	301.1	21	3	Positive
(^{13}C)-Aflatoxin B_2	4.24	332.2	303.0	21	3	Positive
(^{13}C)-Aflatoxin G_1	3.93	346.1	212.2	41	5	Positive
(^{13}C)-Aflatoxin G_2	3.65	348.1	259.1	25	5	Positive
(^{13}C)-Deoxynivalenol	1.51	312.2	263.1	4	3	Positive
(^{13}C)-Fumonisin B_1	5.65	756.5	374.4	37	3	Positive
(^{13}C)-Fumonisin B_2	6.55	740.5	358.3	41	3	Positive
(^{13}C)-HT-2 toxin	5.46	464.3	278.1	9	3	Positive
(^{13}C)-Ochratoxin A	6.53	424.2	250.1	25	3	Positive
(^{13}C)-T-2 toxin	6.03	508.3	322.1	8	5	Positive
(^{13}C)-Zearalenone	6.51	335.2	290.0	17	7	Negative

Quantitation of mycotoxins was based on internal standard calibration. For each mycotoxin, the corresponding (^{13}C)-labeled mycotoxin was used as internal standard. The apparent recoveries, including matrix effects and the recovery of the extraction step, were calculated by comparing the measured concentrations of the spiked maize samples versus the theoretical values. The extraction recoveries were calculated by comparing the apparent recoveries with the recoveries obtained by analyzing maize extracts which were spiked after extraction. LOQs were calculated based on a S/N ratio of 10 for the quantifier trace in the extract of the spiked maize sample and a S/N ratio of at least 3 for the qualifier trace. The LODs were calculated in a similar way, but using a S/N ratio of 3 for the less intensive qualifier transition.

Results and Discussion

Development of the UHPLC/MS/MS method

Transitions for all compounds were evaluated in positive and negative mode using the MassHunter Optimizer software. While for most of the compounds the $[M+H]^+$ ion was the most abundant precursor, for ZEN highest sensitivity could be achieved when using the $[M-H]^-$ ion as the precursor. For T-2 and HT-2 toxin, the $[M+NH_4]^+$ species was chosen as the precursor. To include all compounds in a single analytical run, fast polarity switching was applied since ZEN eluted in the same time window as OTA and FB_2.

Acidified mobile phases were used to ensure stable retention times and increased ionization efficiencies for the fumonisins.

Chromatography was optimized to achieve baseline separation for the aflatoxins as AFG_1 and AFG_2 share the same transitions as the labeled AFB_1 and AFB_2, respectively. The method also achieves baseline separation of FB_2 and FB_3. FB_3 is not regulated and so was not chosen to be included in the method but, since it is an isomer of FB_2 and can often co-occur, it needs resolving from FB_2. While the method achieves a short runtime, it also maximizes chromatographic resolution through the use of a ZORBAX UHPLC column. Improved chromatographic resolution minimizes matrix effects allowing for the injection of 3 µL of raw extract without further dilution or cleanup. Figure 2 shows the chromatogram of a calibration sample including all 11 mycotoxins and their internal standards, while Figure 3 illustrates the separation of FB_2 and FB_3 in a contaminated sample.

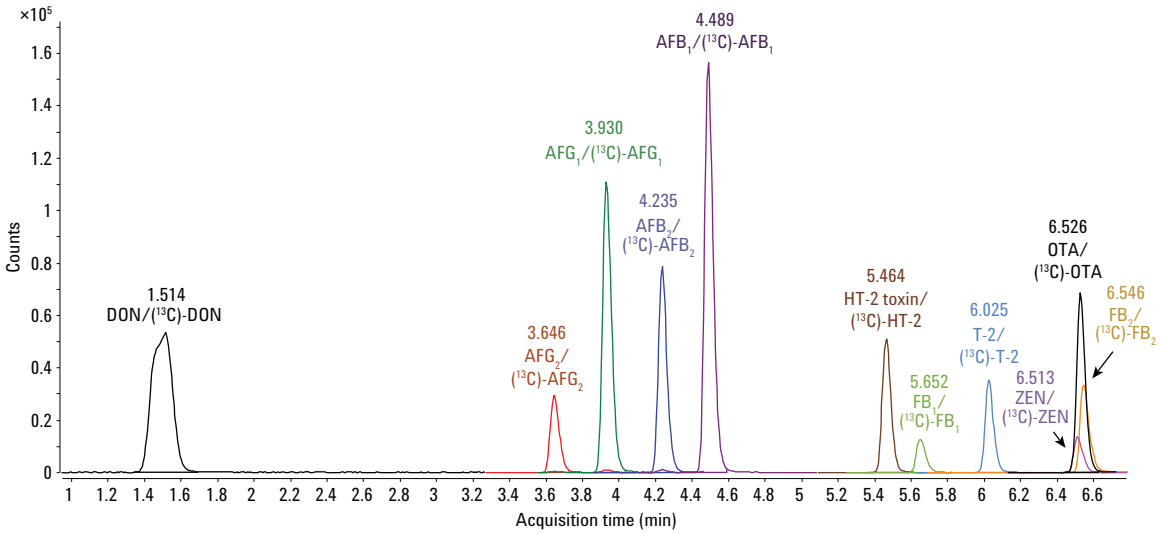

Figure 2. Chromatograms of the internal standards in a calibration sample containing all 11 mycotoxins and their internal standards, illustrating the separation efficiency of the UHPLC method.

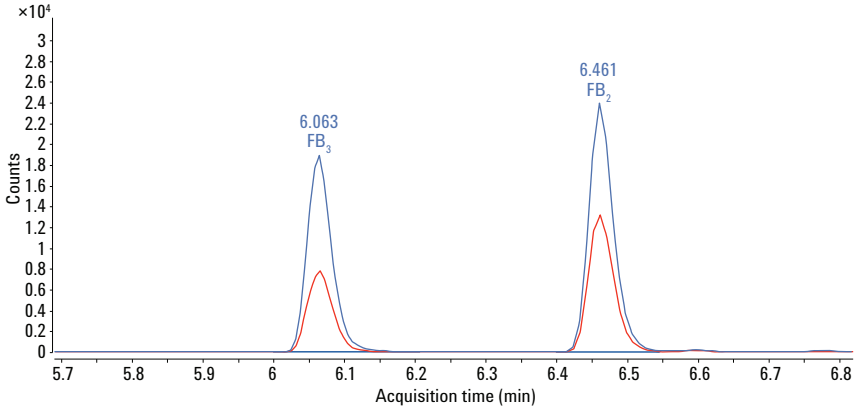

Figure 3. Chromatographic separation of fumonisin B_3 and fumonisin B_2 in a contaminated maize sample.

Development of a generic extraction method

Extraction of mycotoxins from food samples is often accomplished by acidified acetonitrile or acetonitrile/water mixtures with high acetonitrile contents, or even with QuEChERS-like extractions using a salt partitioning step and cleanup. This typically results in high extraction recoveries for most of the target compounds except for fumonisins which, due to their polar character, have a higher affinity to polar solvents and polar surfaces. A sample preparation for fumonisins requires more polar extraction solvents and carefully optimized or no cleanup. However, as the extraction recovery for fumonisins is increased by using more polar solvents or a higher water content in the acetonitrile/water mixture, the extraction recoveries for other target compounds, as for example aflatoxins, are reduced. Therefore, a two-step extraction, first with acetonitrile/water/formic acid (80/19.9/0.1; v/v/v) over 60 minutes, followed by a 30-minute extraction with acetonitrile/water/formic acid (20/79.9/0.1; v/v/v) was applied. This extraction strategy resulted in high extraction recoveries (between 97 and 111% for maize, see Table 6) even for fumonisins with a minimal dilution of the sample so that the final extract can be injected in the UHPLC/MS/MS system without further treatment. For fumonisins, a loss of signal intensity over time was observed in neat standard solutions, which was most likely caused by adsorption to the glass vials, so special care in the choice of HPLC vials and inserts is required. This effect was not observed in the presence of matrix.

Method performance characterization

The method was validated for the model matrix maize since most of the regulated mycotoxins are regulated in maize and maize is considered to be a complex matrix that can cause severe matrix effects. Method performance was characterized by the linear working range, LODs, and LOQs. Figure 4 shows the exemplary calibration curves for the internal standard calibration of DON, AFB_1, OTA, and ZEN.

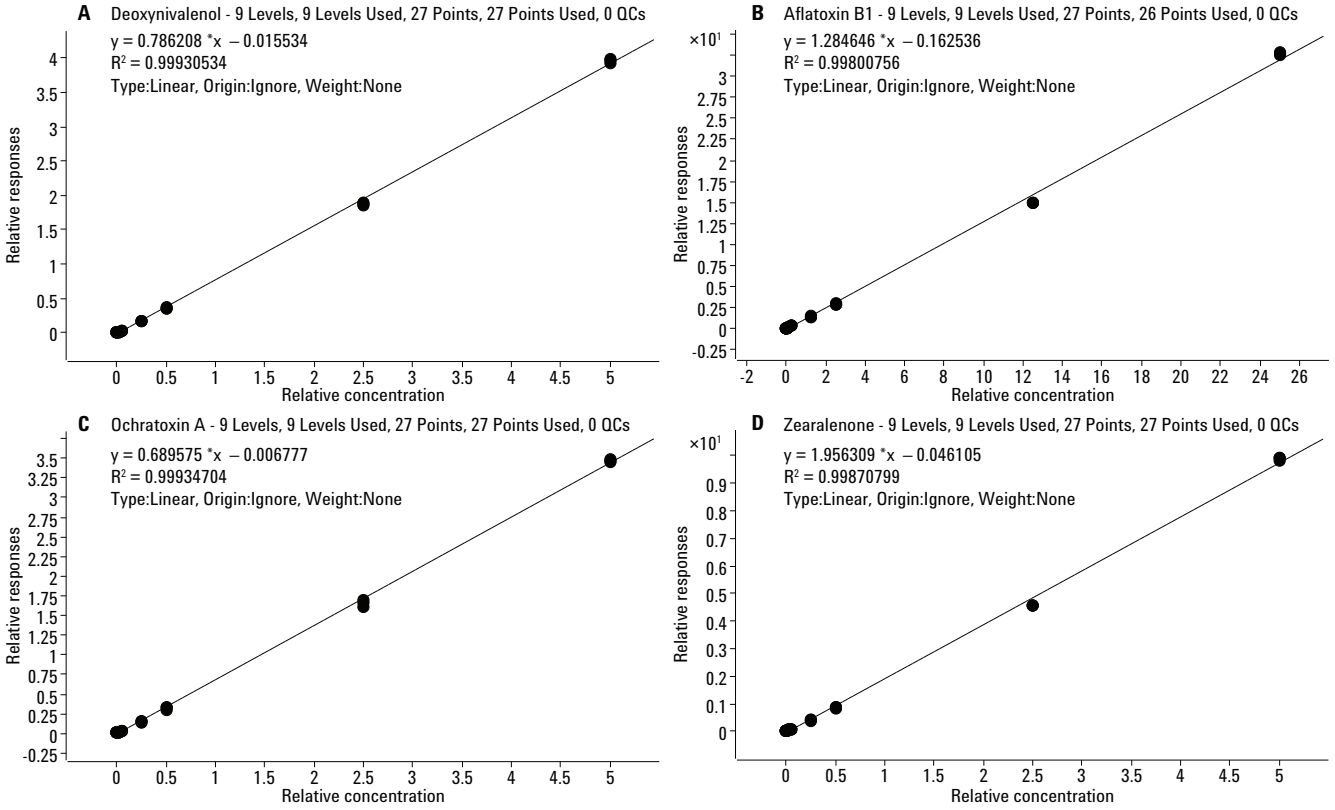

Figure 4. Calibration curves for the internal standard calibration of DON (A), AFB_1 (B), OTA (C), and ZEN (D) using neat solvent standards.

Linear calibration curves were obtained for all targeted mycotoxins over at least 3 orders of magnitude, covering the relevant concentration ranges. The relevant concentration ranges are different for the individual mycotoxins depending on the maximum limits specified in current legislation. The method allows the reliable quantitation of AFB_1 down to 0.1 µg/kg (maximum limit for cereal-based baby food), while for DON, fumonisins, and ZEN, for which higher maximum limits are specified, the upper limit of quantitation is in a range of up to several hundreds of µg/kg, which allows direct analysis even beyond the regulatory limit without further dilution. Table 5 gives the LOQs as well as the linear ranges of the method. Figure 5 shows the chromatograms of AFB_1 and OTA, the compounds with the lowest regulatory limits, spiked into blank maize before extraction.

The lowest spiking concentration for both compounds was 0.5 µg/kg. This is well below the specified maximum limits for maize and cereal-based foodstuff, and corresponds to the maximum limit of OTA and five times the maximum limit for AFB_1 in cereal-based baby food. Extrapolation to an S/N ratio of 10 results in LOQs of approximately 50% of the maximum limit for cereal-based baby food for both compounds. For all other compounds, the observed LOQs are far below the specified maximum limits or indicative levels.

Table 5. Method Performance Parameters for Mycotoxins in Maize Matrix

	Linear working range (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Aflatoxin B_1	0.05–500	0.02	0.03
Aflatoxin B_2	0.25–500	0.04	0.06
Aflatoxin G_1	0.05–500	0.03	0.05
Aflatoxin G_2	0.25–500	0.06	0.24
Deoxynivalenol	5.0–5,000	1.7	3.2
Fumonisin B_1	12.5–2,500	1.4	3.3
Fumonisin B_2	2.5–2,500	0.8	2.2
HT-2 toxin	2.5–5,000	0.6	2.0
Ochratoxin A	0.25–500	0.13	0.24
T-2 toxin	0.25–500	0.06	0.18
Zearalenone	7.5–1,500	1.2	2.9

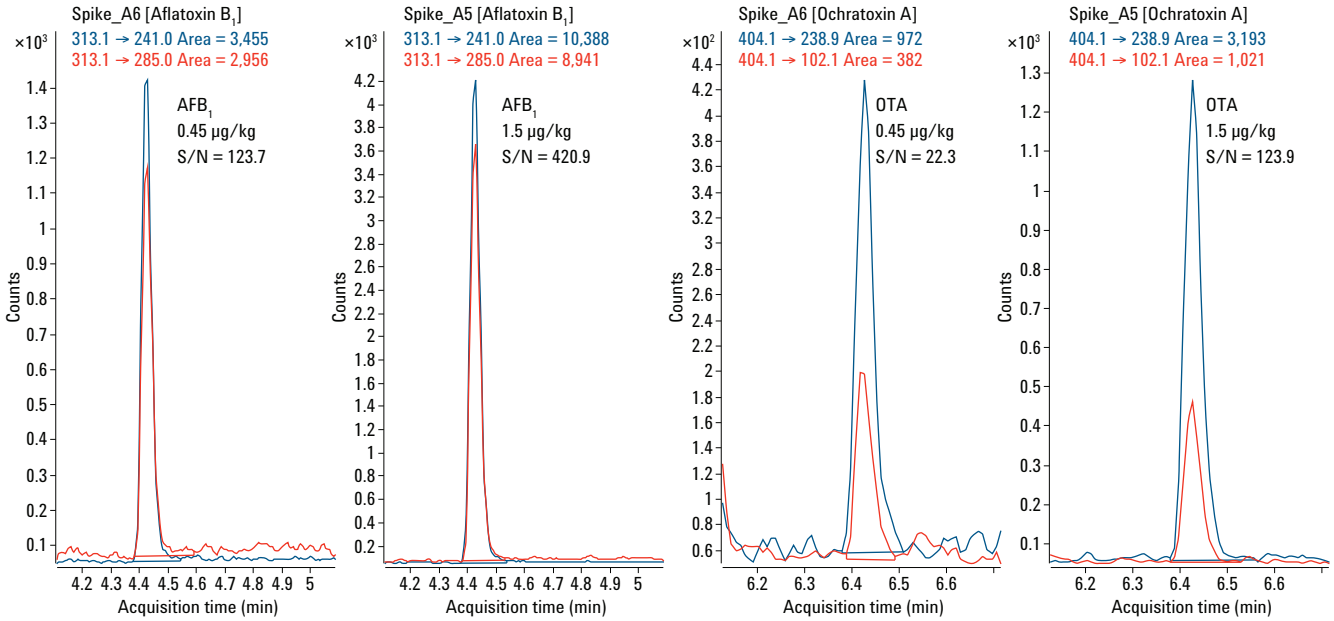

Figure 5. Chromatograms of AFB_1 and OTA spiked into blank maize before extraction in concentrations close to the regulatory limits for cereal-based baby food (S/N ratios calculated for quantifier transitions with peak-to-peak noise algorithm based on signal height).

The generic extraction with two acetonitrile/water mixtures with different polarities results in a considerable matrix load in the sample extracts. When calculating the apparent recoveries for the samples spiked before extraction based on an external calibration, matrix effects were observed leading to reduced recoveries for the aflatoxins and DON, while signal enhancement was observed for fumonisins, OTA, T-2, and HT-2 toxin. However, when calculating the apparent recoveries based on internal calibrations as shown in Table 6, excellent recoveries with good RSD values were obtained. On average over all spiking levels apparent recoveries were in a range between 88 and 105% for all targeted mycotoxins. The extraction recoveries were calculated by comparing apparent recoveries for the maize samples spiked before extraction with the results obtained for the samples spiked after extraction (both with internal calibration). With extraction recoveries between 97 and 111% for the average of all spiking levels, it can be concluded that the two step extraction procedure allows for a complete extraction of mycotoxins from maize, even for fumonisins. Complete extraction in combination with apparent recoveries close to 100% also means, that matrix effects in the electrospray ionization are effectively compensated by the addition of the (^{13}C)-labeled internal standards. Signal suppression thus only decreases the sensitivity of the method but does not negatively affect the accurate quantitation of the targeted mycotoxins.

Table 6. Apparent Recoveries (R_A) and Extraction Recoveries (R_E) for All Mycotoxins in Maize Matrix for up to Six Spiking Levels (n = 3)

	Level 1		**Level 2**		**Level 3**		**Level 4**		**Level 5**		**Level 6**	
	0.45 µg/kg		**1.5 µg/kg**		**4.5 µg/kg**		**15 µg/kg**		**45 µg/kg**		**150 µg/kg**	
	R_A	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**
AFB_1	104 ± 2	108 ± 4	98 ± 1	108 ± 5	100 ± 7	104 ± 2	109 ± 4	115 ± 4	104 ± 6	106 ± 8	111 ± 7	111 ± 10
AFB_2	102 ± 1	111 ± 4	96 ± 1	108 ± 2	95 ± 4	108 ± 4	101 ± 3	108 ± 6	100 ± 6	105 ± 10	102 ± 5	106 ± 10
AFG_1	101 ± 4	103 ± 4	97 ± 2	106 ± 8	99 ± 6	111 ± 4	104 ± 5	114 ± 4	105 ± 1	112 ± 8	106 ± 7	110 ± 6
AFG_2	107 ± 11	116 ± 16	96 ± 3	109 ± 3	95 ± 5	107 ± 6	101 ± 6	112 ± 8	100 ± 6	109 ± 8	108 ± 9	111 ± 9
T-2	97 ± 3	101 ± 9	100 ± 5	111 ± 10	92 ± 4	108 ± 3	99 ± 2	116 ± 0	100 ± 10	111 ± 14	107 ± 3	109 ± 5
OTA	99 ± 2	95 ± 5	95 ± 11	111 ± 3	85 ± 4	99 ± 6	93 ± 5	106 ± 2	92 ± 2	103 ± 5	97 ± 5	102 ± 6
	Level 1		**Level 2**		**Level 3**		**Level 4**		**Level 5**		**Level 6**	
	1.5 µg/kg		**5 µg/kg**		**15 µg/kg**		**50 µg/kg**		**150 µg/kg**		**500 µg/kg**	
	R_A	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**
FB_1	< LOQ	< LOQ	99 ± 10	105 ± 13	92 ± 6	102 ± 9	96 ± 4	104 ± 2	93 ± 5	103 ± 9	102 ± 4	105 ± 4
FB_2	< LOQ	< LOQ	89 ± 12	98 ± 18	82 ± 2	94 ± 5	85 ± 4	100 ± 8	88 ± 5	101 ± 12	93 ± 5	98 ± 6
ZEN	< LOQ	< LOQ	102 ± 10	111 ± 17	95 ± 13	117 ± 12	98 ± 7	110 ± 11	98 ± 8	97 ± 13	104 ± 7	107 ± 8
	Level 1		**Level 2**		**Level 3**		**Level 4**		**Level 5**		**Level 6**	
	4.5 µg/kg		**15 µg/kg**		**45 µg/kg**		**150 µg/kg**		**450 µg/kg**		**1,500 µg/kg**	
	R_A	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**	**R_A**	**R_E**
DON	114 ± 6	95 ± 8	98 ± 5	101 ± 4	92 ± 6	102 ± 6	98 ± 3	109 ± 3	94 ± 5	104 ± 7	102 ± 5	107 ± 6
HT-2	97 ± 8	108 ± 12	92 ± 6	107 ± 8	89 ± 1	106 ± 7	97 ± 4	114 ± 5	95 ± 7	107 ± 12	101 ± 6	112 ± 7

The trueness of the method was proven by analyzing matrix reference materials with well characterized analyte concentrations for 8 of the 11 tested compounds. As no reference material is available with specified concentrations for all tested mycotoxins, Table 7 summarizes the determined concentrations for five different test materials. Further matrix reference materials have been analyzed, and results are included in [1]. In general, the measured concentrations were in good agreement with the values specified for the matrix reference materials, showing that the 2-step extraction without any cleanup and the UHPLC/MS/MS measurement with stable isotope dilution allows for the accurate quantitation of mycotoxins in cereals.

Table 7. Assigned and Measured Values from the Analysis of Matrix Reference Materials

Matrix reference material	Analyte	Assigned value (µg/kg)	Measured value (µg/kg)
TM_01	ZEN	83 ± 4.5	86 ± 10
TM_02	Sum of aflatoxins	3.79 ± 1.67	4.6 ± 0.2
	AFB_1	1.87 ± 0.83	2.3 ± 0.1
	AFB_2	0.51 ± 0.23	0.6 ± 0.03
	AFG_1	0.96 ± 0.43	1.0 ± 0.1
	AFG_2	0.52 ± 0.23	0.7 ± 0.1
TM_03	FB_1	1,650 ± 53	1,960 ± 198
	FB_2	461 ± 16	496 ± 32
TM_04	DON	1,714 ± 64	1,660 ± 145
TM_05	DON	901 ± 55	908 ± 79
	ZEN	79 ± 13	84 ± 10

As a proof of concept, the presented method was applied to a red wine sample that was spiked with OTA to a concentration of 2 µg/L. After filtration (0.2 µm, regenerated cellulose), 3 µL of the sample were directly injected into the UHPLC/MS/MS system. The measured concentration for OTA was 2.01 ± 0.08 µg/L (n = 7), which shows that the added internal standard also compensated for the red wine matrix. Figure 6 shows the chromatogram for OTA in the red wine sample.

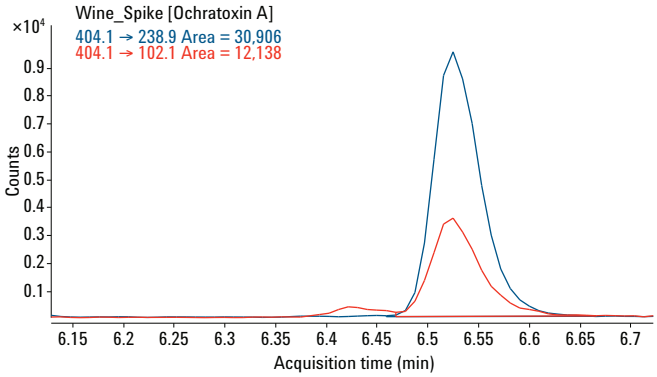

Figure 6. Chromatograms of the quantifier and qualifier transition of OTA spiked into red wine at a concentration of 2 µg/L.

Conclusions

A fast and simple UHPLC-based multitarget method for the accurate quantitation of all mycotoxins regulated in Europe in cereal-based food was created. It takes full advantage of the low delay volumes of the 1290 Infinity LC System and its ability to reliably handle high backpressures in UHPLC separations to increase the chromatographic resolution and reduce the runtime. The method also benefits from the innate sensitivity of the Agilent 6490 Triple Quadrupole LC/MS System, which is of particular importance in achieving LOQs for AFB_1 and OTA in line with the regulatory limits for cereal-based baby food. This achievement is even more significant considering the 10 fold dilution that occurs in the process of sample preparation. Another factor is the ability to make rapid positive/negative switching on the fly to allow the use of the most abundant ionization mode for each compound. Since the method enables the simultaneous analysis of analytes with very different chemical and physical properties, it also offers the potential for increased scope to further mycotoxins.

The method comprises a fast, easy, and cheap two-step solvent extraction, leading to good recoveries so that addition of the (^{13}C)-labeled internal standards could be reserved for the final extracts where they compensated for matrix effects in the electrospray ionization. It was successfully validated for maize, where an efficient compensation of all matrix effects was observed and quantitation accuracy was increased. Maize is known to produce complex extracts that can cause severe matrix effects. A method that performs well for maize most likely can be applied to other cereals and cereal-based food products.

Red wine spiked with OTA was also analyzed using this approach. Matrix effects were equally well compensated and OTA was quantified with high accuracy in the red wine even though it showed a completely different matrix profile.

Additional costs for the application of (^{13}C)-labeled internal standards range between 0.02 € for HT-2 and T-2 toxin and 0.48 € for FB_2 and result in total additional costs of less than 3.80 € per sample for all 11 internal standards. The native and (^{13}C)-labeled mycotoxins can be obtained as the LC/MS Mycotoxin Kit (p/n KIT-001) from Romer Labs Diagnostic GmbH (Tulln, Austria). Purchasing this kit with a 1290/6490 allows a laboratory to easily duplicate this approach, simply by applying the same conditions outlined.

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