APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

Authors:

Jingcun Wu,

Tyrally Ordinario

Feng Qin

PerkinElmer Inc.,
Woodbridge, Canada

A Fully Validated Method for Determination of Twelve Mycotoxins in Various Food Matrices by Stable Isotope Dilution QSight LC/MS/MS

Introduction

Mycotoxins are toxic secondary metabolites produced by various fungal species growing in foods and animal feeds. Due to the widespread distribution of fungi in the agricultural environment, mycotoxins are one of the most important contaminants in foods and feeds. Although hundreds of mycotoxins have been identified in agricultural products, the most common classes of mycotoxins considered to be of major concern regarding their occurrence and toxicity are aflatoxins (aflatoxins B1, B2, G1, G2), ochratoxin A (OTA), deoxynivalenol (DON), fumonisins (B1, B2 and B3), HT-2 and T-2 toxins, and zearalenone (ZEN). The chemical structures of these mycotoxins are illustrated in Figure 1. At certain levels and combinations, these mycotoxins can be harmful to humans and animals when consumed in foods and feeds. Therefore, to minimize risk and protect consumers, mycotoxin levels in foods and/or feeds have been regulated by agencies in many countries around the world.[1-2] The European Union Commission Regulation 1881/2006 and its amendments set maximum levels (MLs) for several mycotoxins in foods and feeds.[3-6] The U.S. Food and Drug Administration (FDA) has also established action or guidance levels for aflatoxins, fumonisins, deoxynivalenol and other specific mycotoxins.[7]

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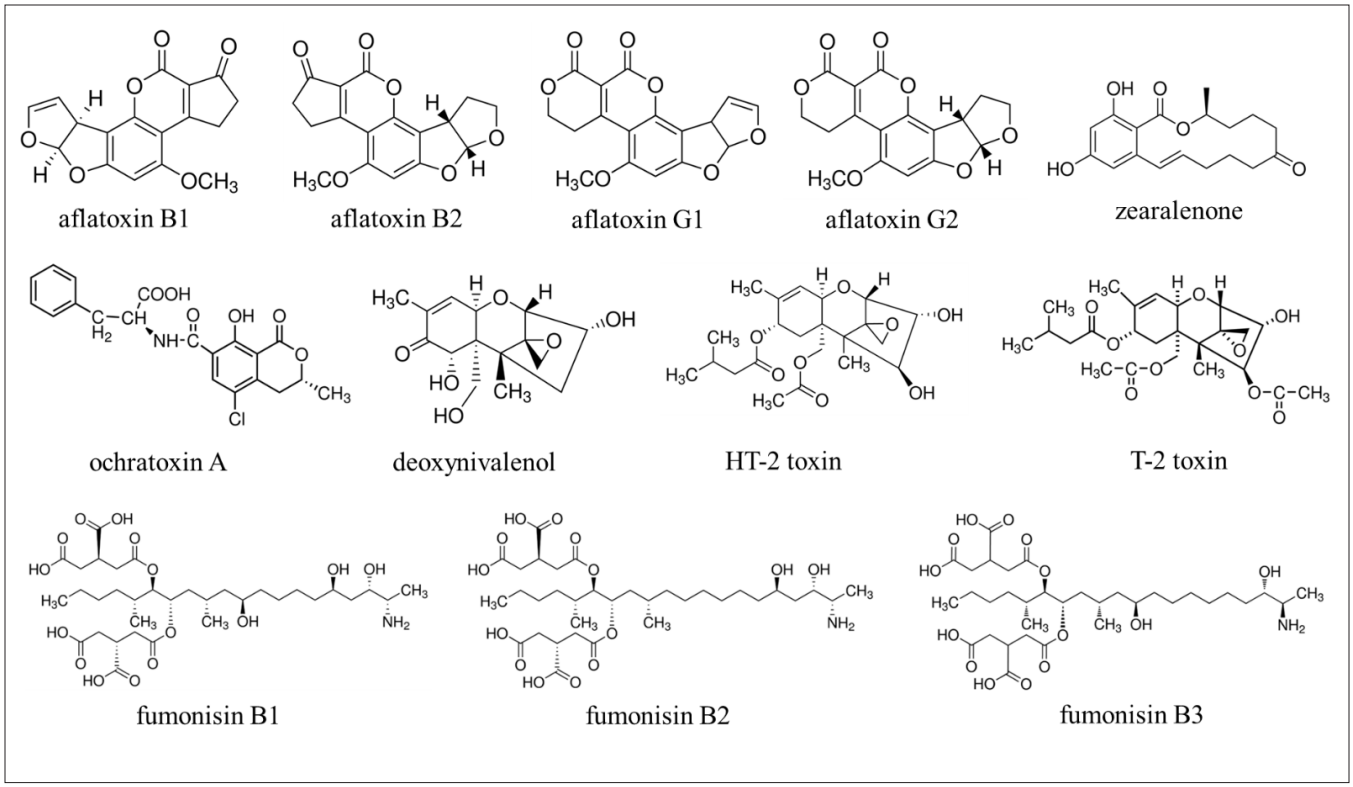

Figure 1. Chemical structures of studied mycotoxins

To obtain reliable information on the presence and concentration of mycotoxins in foods and feeds as they relate to regulatory requirements, various analytical methods, such as enzyme-linked immunosorbent assay (ELISA), thin layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC) coupled with different detection methods, have been developed for analysis of mycotoxins in foods.[8-9] Recently, an LC/MS/MS method has become the method of choice for quantification and confirmation of mycotoxins in various food sample matrices owing to its superior sensitivity, selectivity and capability of analyzing multiple target mycotoxins in a single run.[8-24]

Traditionally, mycotoxins were analyzed by many single analyte or single-class mycotoxin methods developed based on their different physicochemical properties and sample matrices. These methods often had low sample throughput owing to tedious sample preparation procedures and different clean-up steps.[8-10] To address the increasing number of sample matrices and mycotoxins of interest, the current trend in mycotoxin analysis is the development of "fit-for-all-purposes" multi-mycotoxin methods which can analyze all regulated mycotoxins in various food matrices in a single run. However, to achieve this goal, scientists have faced several challenges: (1) very different maximum level (MLs) regulations for different mycotoxins based on their toxicity and type of food (e.g. baby food has much lower MLs compared to regular food), which complicates preparation procedures for calibration standards and quality control samples; (2) a wide range of food and feed sample matrices, making widely used matrix-matched calibration method less efficient, as multiple sets of matrix-matched calibrations need be prepared for various different matrices; and (3) the diversity of mycotoxins in their physicochemical properties, which makes it difficult to effectively extract and purify all analytes by a single method.

To overcome these problems and avoid sample clean-up steps, simple sample extraction procedures using an acetonitrile/water mixture as the extraction solvent have been widely used in developing multi-mycotoxin LC/MS/MS methods. However, direct injection of the crude sample extracts into an LC/MS/MS system can cause matrix effects, which can significantly affect the data quality and the method's selectivity, sensitivity and accuracy. Thus, matrix effects should be taken into careful consideration when developing and validating a multi-mycotoxin method. To reduce or compensate for matrix effects, several approaches have been applied to LC/MS/MS method development, including sample dilutions, matrix-matched standards calibration method, standard additions method, and an isotope dilution internal standard calibration method.[13-24]

Sample dilution is the simplest approach to alleviate matrix effects, but it also reduces the sensitivity of the method owing to the dilution of the samples, and thus requires a highly sensitive instrument.

The standard addition method is feasible for samples containing a single mycotoxin, but it is quite complex and time consuming for fortification of samples containing multiple mycotoxins with significantly different concentrations. In addition, the standard addition method at a minimum doubles the analysis time, as both the sample extract and fortified extracts need to be injected for each sample.

Although matrix-matched standards calibration methods have been extensively used in mycotoxin analysis to compensate for matrix effects,[13-16] they are time consuming and labor intensive when many different sample matrices are being analyzed, as each sample matrix needs its own matrix-matched standard calibration curve. Additionally, it is often difficult to find a blank matrix that contains no mycotoxins. Some studies showed that there were even variations within a given food matrix, and that the differences could not be fully compensated by matrix-matching, thus leading to poor analyte recoveries for some mycotoxins.[16,19] Finally, mycotoxins in unknown sample matrices, or samples that have incurred mycotoxins of interest, cannot be determined accurately by matrix-matched calibration method.

For internal standard calibration, the ideal internal standards (IS) are stable isotopically labelled standards because they share the same chemical and physical properties as the target analytes but are still distinct with 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 due to the addition of stable isotope labelled standards, this procedure is often referred to as stable isotope dilution assay (SIDA). SIDA has proven to be the most effective and preferred calibration approach for mycotoxin analysis in complex sample matrices because it can not only compensate for sample matrix effects, but also minimize variations in analytical procedures (including low extraction efficiency and/or potential analyte loss in sample preparation, and instrument performance variations).

SIDA is a technique that measures the relative response of a stable isotopically labeled IS spiked into the test samples prior to the sample extraction. Since the relative response ratio of the IS to its native analyte remains constant in both solvent-only calibration standards and the extracted sample matrix, solvent-only calibration standards can be used for quantification of mycotoxins in various different sample matrices, and therefore, significantly simplify the sample preparation and analyte quantification processes. However, previous studies have demonstrated that sample matrix effects are analyte dependent (i.e. matrix effects are different on different analytes), and therefore, individual labeled IS should be used for each analyte to effectively compensate for matrix effects on each analyte.[16, 26] An analogue IS should not be used for the closely eluting mycotoxins because significant error could result from using an inappropriate IS.[16] Recently, more and more certified stable isotope labeled mycotoxins are commercially available from various sources. The sensitivity of LC/MS/MS systems has increased dramatically, which makes it more cost-effective to use these labeled IS because only a small amount is required for sample fortification. Owing to these advancements in labeled IS and instrument sensitivity, SIDA coupled with LC/MS/MS has found increasing applications in screening and quantification of multi-mycotoxins in various food and feed matrices.[16-24]

The objective of this work was to assess the applicability of the stable isotope dilution mass spectrometry approach, using a PerkinElmer QSight® 220 LC/MS/MS system, for the determination of aflatoxin B1, B2, G1, and G2, ochratoxin A, deoxynivalenol, fumonisin B1, B2, and B3, HT toxin, T-2 toxin, and zearalenone in various food sample matrices. Method validation was carried out in all selected six food matrices using blank samples spiked at two analyte concentrations. The results of this study have demonstrated that the developed SIDA-LC/MS/MS method is simple, sensitive and reliable, and can be used for routine analysis of multiple mycotoxins in different food matrices.

Experimental

Hardware/Software

Chromatographic separations of various mycotoxins and mycotoxins from potentially interfering components were conducted utilizing a PerkinElmer QSight LX50 ultra-high-performance liquid chromatography (UHPLC) system. Subsequent detection was achieved using a PerkinElmer QSight 220 triple quadrupole mass spectrometer with a dual ionization source (ESI and APCI). All instrument control, data acquisition and data processing were performed using Simplicity™ 3Q software.

Materials and Methods

Chemicals and Materials

The below mycotoxin standard stock solutions and their ^{13}C uniformly labeled internal standard (^{13}C-IS) stock solutions were obtained from Romer Laboratories, Inc.

- Aflatoxin Mix solution consisted of aflatoxin B1, B2, G1 and G2 (1.0 µg/mL for each analyte in acetonitrile)
- Fusarium Toxins Mix consisted of deoxynivalenol (100 µg/mL), HT-2 toxin (100 µg/mL), T-2 toxin (10 µg/mL), and zearalenone (30 µg/mL) in acetonitrile
- Fumonisin Mix consisted of fumonisin B1 (50 µg/mL), fumonisin B2 (50 µg/mL), and fumonisin B3 (50 µg/mL) in acetonitrile/water (50:50, v/v)
- Ochratoxin A (10 µg/mL) in acetonitrile
- ^{13}C-aflatoxin Mix consisted of U-[$^{13}C_{17}$]- aflatoxin B1, B2, G1, and G2 (0.5 µg/mL for each analyte in acetonitrile)
- U-[$^{13}C_{20}$]-ochratoxin A (10 µg/mL)
- U-[$^{13}C_{34}$]-fumonisin B1 (25 µg/mL)
- U-[$^{13}C_{34}$]-fumonisin B2 (10 µg/mL)
- U-[$^{13}C_{34}$]-fumonisin B3 (10 µg/mL)
- U-[$^{13}C_{15}$]- deoxynivalenol (25 µg/mL)
- U-[$^{13}C_{22}$]-HT-2 toxin (25 µg/mL)
- U-[$^{13}C_{24}$]-T-2 toxin (25 µg/mL)
- U-[$^{13}C_{18}$]-zearalenone (25 µg/mL)

HPLC grade solvents (methanol, acetonitrile, water) and other chemicals such as formic acid and ammonium formate were obtained from Sigma-Aldrich. Disposable polypropylene syringe (10 mL), syringe filter (0.22 µm), polypropylene centrifuge tube (15 mL), amber autosampler vials and caps were obtained from PerkinElmer, Inc. Test samples (yellow corn, white corn, wheat, soybean, almond, oat breakfast cereal, and peanut butter) were purchased from local stores (Toronto, ON, Canada).

Standard Preparation

To prepare calibration standards and quality control samples, three working standard (WS) mix solutions (WS-Mix1, WS-Mix2 and WS-Mix3), and two internal standard spiking solutions (IS-Spike1 and IS-Spike2) were prepared from the corresponding stock solutions by appropriate dilutions with a diluent of acetonitrile/water (50:50, v/v).

WS-Mix1 contained aflatoxin B1, B2, G1, G2 (each 100 ng/mL) and ochratoxin A (200 ng/mL); WS-Mix2 consisted of the three fumonisins (B1, B2 and B3, each 2000 ng/mL); WS-Mix3 included deoxynivalenol (2000 ng/mL), HT-2 toxin (2000 ng/mL), T-2 toxin (200 ng/mL), and zearalenone (600 ng/mL).

IS-Spike1 consisted of $^{13}C_{17}$ aflatoxin B1, B2, G1, G2 (each at 50 ng/mL); $^{13}C_{20}$-ochratoxin A (200 ng/mL); $^{13}C_{34}$-fumonisin B1 (2000 ng/mL); $^{13}C_{34}$-fumonisin B2 (1000 ng/mL); $^{13}C_{34}$-fumonisin B3 (1000 ng/mL); $^{13}C_{15}$- deoxynivalenol (2000 ng/mL); $^{13}C_{22}$-HT-2 toxin (2000 ng/mL); $^{13}C_{24}$-T-2 toxin (1000 ng/mL); and $^{13}C_{18}$-zearalenone (1000 ng/mL). IS-Spike2 was prepared by 10-fold dilutions of IS-Spike1 solution using a diluent of acetonitrile/water (50:50, v/v). Seven levels of calibration standard solutions (each 1 mL) were prepared by a series of dilutions of the appropriate amount of WS-Mixes 1, 2, and 3, using the same acetonitrile/water diluent, respectively; and then IS-Spike2 solution (100 µL) was fortified into each calibration standard. The analyte concentrations in the standards are listed in Table 1. Two zero standard solutions were also prepared: standard 01 was prepared by adding the diluent directly into an auto sampler vial to check the background and potential contamination to the vials; standard 02, containing only IS, was prepared to check the isotope purity of the IS.

Standard stock solutions, working solutions and IS spiking solutions were stored in a freezer at –20 °C. The working solutions and IS spiking solutions should be brought to room temperature in the dark and mixed thoroughly before use. Calibration standard solutions were stored in a dark place in a fridge.

Sample Preparation

Before sample extraction, each solid food sample (250 g) was ground to a fine powder using a food grade grinder, passed through a food grade sieve (particle ≤1 mm), and then mixed thoroughly to ensure that the matrix was homogeneous.

Sample preparation was carried out based on a published work from U.S. FDA[23] with minor modifications. The method included simple sample extraction, centrifugation, and filtration. Briefly, 1.0 g of the ground and homogenized sample was weighed into a 15 mL screw capped polypropylene centrifuge tube, fortified with 50 µL of the IS-Spike1 solution (and appropriate amount of WS-Mixes 1, 2 and 3 for recovery studies), and vortexed for one minute. The sample was then extracted with 5 mL of a 50% acetonitrile solution (in water, 1:1 in v/v) for 30 minutes on a shaker. The sample solution was centrifuged at 4 °C for 15 minutes at 4000 rpm, and 2 mL of the supernatant was then filtered through a 0.22 µm syringe filter directly into an amber autosampler vial for LC/MS/MS analysis.

Since ^{13}C-IS was added prior to sample extraction, the fortified IS would go through the entire sample preparation and instrumental analysis as the target mycotoxins, and thus any signal variation of target mycotoxins caused by sample preparation or matrix effects could be offset by monitoring the relative response between the mycotoxins and the corresponding ^{13}C-IS.

Table 1. Analyte Concentrations (ng/mL) in Calibration Standards.

Analyte	STD 7	STD 6	STD 5	STD 4	STD 3	STD 2	STD 1	STD 02
Aflatoxin B1	25	10	5	1	0.5	0.1	0.05	IS only (0.5) *
Aflatoxin B2	25	10	5	1	0.5	0.1	0.05	IS only (0.5)
Aflatoxin G1	25	10	5	1	0.5	0.1	0.05	IS only (0.5)
Aflatoxin G2	25	10	5	1	0.5	0.1	0.05	IS only (0.5)
Ochratoxin A	50	20	10	2	1	0.2	0.1	IS only (2.0)
Fumonisin B1	500	200	100	20	10	2	1	IS only (20)
Fumonisin B2	500	200	100	20	10	2	1	IS only (10)
Fumonisin B3	500	200	100	20	10	2	1	IS only (10)
Deoxynivalenol	500	200	100	20	10	2	1	IS only (20)
Zearalenone	150	60	30	6	3	0.6	0.3	IS only (10)
HT-2 Toxin	500	200	100	20	10	2	1	IS only (20)
T-2 Toxin	50	20	10	2	1	0.2	0.1	IS only (10)

* IS concentrations in each standard are the same and shown in the column of STD 02.

For method validation, a laboratory reagent blank (LRB) was prepared and tested first to ensure that there is no interference or contamination from reagents or materials used, or from the sample preparation processes. Next, all food blank samples were examined for any mycotoxin peaks and any interfering components. Finally, to evaluate sample matrix effects and analyte recovery from sample matrix, laboratory fortified matrix samples (LFM) were prepared by following the same sample preparation procedures described above, using each of the blank samples (corn, wheat, soybean, almond, oat cereal and peanut butter) as a sample matrix, spiked with analyte at two concentration levels. At each spiking level, LFM samples were prepared in triplicates. Since some mycotoxins were detected in blank samples used for recovery studies, the recovered mycotoxin results were corrected by subtracting those values from the blank samples.

LC Method and MS Source Conditions

The optimized LC method and MS source parameters are shown in Table 2. The multiple reaction monitoring mode (MRM) transitions of mycotoxins and their internal standards (^{13}C-IS), as well as their optimized parameters, are shown in Table 3. Multiple MRM transitions were monitored to evaluate potential interfering components for certain transitions in real samples, which will help confidently identify analytes from complex sample matrices, reduce false positive and false negatives in the results, and increase the accuracy of analyte quantification.

Optimization of MS/MS parameters, such as collision energies (CE), entrance voltages (EV), and lens voltages (CCL2), were performed by infusion of standards and use of autotune feature in the Simplicity 3Q software. MS source parameters, including gas flows, temperature and probe position settings, were optimized for maximum sensitivity by infusion of standard solution with a T-unit connected to the mobile phase flow. Based on the optimized conditions, the MS acquisition method was generated using Simplicity software in the time-managed-MRM module, with the retention times and corresponding retention time windows for all analytes.

Table 2. LC Method and MS Source Conditions.

LC Conditions	
LC Column	PerkinElmer Quasar SPP C18 (100 x 2.1 mm, 2.6µm, P/N9308917)
Mobile Phase A	0.1% formic acid and 5 mM ammonium formate in water
Mobile Phase B	0.1% formic acid and 5 mM ammonium formate in methanol
Mobile Phase Gradient (Flow Rate: 0.3mL/min)	Start at 10% mobile phase B and hold at 10% B for 0.5 min, then increase B to 100% at 7 min and keep at 100% B for 1 min to clean the column, finally return to initial condition at 8.1 min and keep running at initial conditions for 3 min.
Column Oven Temperature	35 °C
Auto Sampler Temperature	8 °C
Injection Volume	5.0 µL
MS Source Conditions	
ESI Voltage (Positive)	4500 V
ESI Voltage (Negative)	-4800 V
Drying Gas	120
Nebulizer Gas	300
Source Temperature	350 °C
HSID Temperature	220 °C
Detection mode	Time-managed MRM™

Table 3. Optimized MRM Transitions and Parameters.

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Aflatoxin B1_1	Positive	313.1	285.1	-29	51	-76
Aflatoxin B1_2	Positive	313.1	241.1	-49	43	-108
Aflatoxin B1_3	Positive	313.1	128.2	-99	47	-140
$^{13}C_{17}$-Aflatoxin B1_1	Positive	330.2	301.2	-31	49	-72
$^{13}C_{17}$-Aflatoxin B1_2	Positive	330.2	255.2	-50	49	-140
Aflatoxin B2_1	Positive	315.1	287.3	-32	55	-84
Aflatoxin B2_2	Positive	315.1	259.1	-40	46	-84
Aflatoxin B2_3	Positive	315.1	115.2	-100	53	-148
$^{13}C_{17}$-Aflatoxin B2_1	Positive	332.0	303.2	-32	55	-84
$^{13}C_{17}$-Aflatoxin B2_2	Positive	332.0	273.1	-37	46	-84

Table 3. Optimized MRM Transitions and Parameters - Continued.

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Aflatoxin G1_1	Positive	329.3	200.2	-54	45	-108
Aflatoxin G1_2	Positive	329.1	243.2	-35	44	-72
Aflatoxin G1_3	Positive	329.1	115.2	-100	45	-136
$^{13}C_{17}$-Aflatoxin G1_1	Positive	346.1	124.2	-100	45	-136
$^{13}C_{17}$-Aflatoxin G1_2	Positive	346.1	257.1	-29	44	-72
Aflatoxin G2-1	Positive	331.1	313.1	-33	50	-76
Aflatoxin G2-2	Positive	331.1	189.2	-55	49	-120
Aflatoxin G2-3	Positive	331.1	245.1	-38	51	-100
$^{13}C_{17}$-Aflatoxin G2_1	Positive	348.0	330.0	-33	50	-76
$^{13}C_{17}$-Aflatoxin G2_2	Positive	348.0	259.0	-38	51	-100
Ochratoxin A_1	Positive	404.1	239.1	-32	15	-68
Ochratoxin A_2	Positive	404.1	358.0	-19	19	-64
Ochratoxin A_3	Positive	404.1	102.1	-99	10	-124
$^{13}C_{20}$-Ochratoxin A_1	Positive	424.1	250.2	-30	2	-88
$^{13}C_{20}$-Ochratoxin A_2	Positive	424.1	377.1	-18	4	-72
Deoxynivalenol_1	Positive	297.1	249.2	-15	0	-52
Deoxynivalenol_2	Positive	297.0	231.2	-17	9	-56
Deoxynivalenol_3	Positive	297.1	77.0	-86	5	-92
$^{13}C_{15}$-Deoxynivalenol_1	Positive	312.0	263.0	-13	21	-60
$^{13}C_{15}$-Deoxynivalenol_2	Positive	312.0	216.2	-23	22	-60
Fumonisin B1_1	Positive	722.4	334.5	-52	12	-152
Fumonisin B1_2	Positive	722.5	352.4	-47	19	-176
Fumonisin B1_3	Positive	722.5	81.2	-97	40	-164
$^{13}C_{34}$-Fumonisin B1_1	Positive	756.4	374.5	-48	59	-196
$^{13}C_{34}$-Fumonisin B1_2	Positive	756.4	356.4	-51	62	-200
Fumonisin B2_1	Positive	706.3	336.4	-50	51	-152
Fumonisin B2_2	Positive	706.4	318.4	-50	53	-160
Fumonisin B2_3	Positive	706.3	354.5	-44	64	-132
$^{13}C_{34}$-Fumonisin B2_1	Positive	740.3	358.4	-50	51	-152
$^{13}C_{34}$-Fumonisin B2_2	Positive	740.3	340.5	-52	53	-160
Fumonisin B3_1	Positive	706.3	336.4	-50	51	-152
Fumonisin B3_2	Positive	706.3	354.5	-44	64	-132
Fumonisin B3_3	Positive	706.4	318.4	-50	53	-160
$^{13}C_{34}$-Fumonisin B3_1	Positive	740.3	358.4	-50	51	-152
$^{13}C_{34}$-Fumonisin B3_2	Positive	740.3	340.5	-52	53	-160
HT-2 Toxin_1	Positive	442.2	263.2	-20	16	-72
HT-2 Toxin_2	Positive	442.2	215.1	-18	6	-64
HT-2 Toxin_3	Positive	442.2	105.1	-85	12	-100
$^{13}C_{22}$-HT-2 Toxin_1	Positive	464.2	229.2	-17	10	-72
$^{13}C_{22}$-HT-2 Toxin_2	Positive	464.2	278.4	-18	10	-84
T-2 Toxin_1	Positive	484.2	215.2	-28	26	-84
T-2 Toxin_2	Positive	484.2	185.1	-35	25	-84
T-2 Toxin_3	Positive	484.2	305.2	-20	2	-84
$^{13}C_{24}$-T-2 Toxin_1	Positive	508.3	322.3	-19	11	-88
$^{13}C_{24}$-T-2 Toxin_2	Positive	508.3	229.2	-26	5	-88

Table 3. Optimized MRM Transitions and Parameters - Continued.

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
Zearalenone_1	Positive	319.2	301.2	-14	16	-52
Zearalenone_2	Positive	319.3	283.1	-16	3	-52
Zearalenone_3	Positive	319.2	187.2	-27	13	-64
$^{13}C_{18}$-Zearalenone_1	Positive	337.2	301.4	-20	8	-56
$^{13}C_{18}$-Zearalenone_2	Positive	337.2	199.3	-27	16	-80
Zearalenone_1	Negative	317.1	131.1	39	-43	64
Zearalenone_2	Negative	317.1	175.1	30	-41	88
Zearalenone_3	Negative	317.1	160.0	42	-27	88
$^{13}C_{18}$-Zearalenone_1	Negative	335.1	140.3	40	-22	80
$^{13}C_{18}$-Zearalenone_2	Negative	335.1	169.1	42	-35	120

Results and Discussion

LC/MS/MS Method Optimization

To optimize mass detection conditions, both positive and negative electrospray ionization (ESI) modes were evaluated initially for all analytes. The results showed that higher signal intensity and better signal-to-noise ratio were observed for all mycotoxins under positive mode, with the exception of zearalenone (ZEN), which showed slightly higher signal intensity in negative mode. Therefore, both positive and negative MRM transitions of ZEN were included in the method in this study, as shown in Table 3. A previous study on animal feed showed that better signal-to-noise (S/N) ratios could be achieved using negative ionization for ZEN,[22] but no significant differences in ZEN results were obtained between positive and negative modes in this study, and thus, it is possible to use positive mode for all analytes in this study to simplify the method.

Although deoxynivalenol (DON) and ochratoxin A (OAT) were determined in negative ionization mode in a previous work,[27] the best results were obtained using positive ionization in this study. For most mycotoxins in the positive mode, the highest abundant precursor ions were protonated $[M+H]^+$ species. But for HT-2 and T-2 toxins, their ammonium adducts $[M+NH_4]^+$ showed higher abundance than their $[M+H]^+$ ions, and therefore, their ammonium adducts $[M+NH_4]^+$ were used as precursors in the method. Three MS/MS transitions for each mycotoxin analyte, and two MS/MS transitions for each ^{13}C-IS, were employed in this study to improve analyte identification and method accuracy. The optimized MS/MS (or MRM) parameters are listed in Table 3.

Chromatographic separation of mycotoxins was conducted by reversed phase UHPLC using a PerkinElmer Quasar superficially porous particle (SPP) C18 column (100 x 2.1 mm, 2.6 µm). Owing to the diverse physicochemical properties of the twelve studied mycotoxins (with different polarity and acidity characteristics), a compromise needed to be made between mobile phase composition (keeping suitable chromatography retention) and MS response for the target mycotoxins. It was shown that signal intensities were increased for aflatoxins and DON when a small amount of ammonium formate was added to the mobile phase. In addition, small amounts of ammonium ions in the mobile phases could help inhibit the formation of sodium adducts, especially in the case of HT-2 and T-2 toxin, and favored the formation of $[M+NH4]^+$ precursor ions. However, higher concentrations of ammonium formate could lead to ion suppression.

In this study, the optimized concentration of ammonium formate was 5 mM in both mobile phases, although previous studies had used it in concentrations ranging from 0.5 mM to 10 mM.[13-24] It was found by previous researchers,[10,15] that the addition of 0.1% formic acid in mobile phases not only enhanced the signal intensities of fumonisins (FB1 and FB2) significantly (five-fold increase in peak areas), but also improved their peak shapes. The peak areas of aflatoxins were also increased. However, the signal intensities for OTA, DON, ZEN, HT-2 and T-2 toxins were decreased slightly with the addition of acid in the mobile phases. Thus, as a compromise for the determination of all mycotoxins, 0.1% of formic acid was added to the mobile phases in this study. Since fumonisin B2 (FB2) and fumonisin B3 (FB3) have the same MS/MS transitions, baseline separation of the two peaks is required to avoid interference from each other. As shown in Figure 2, these two peaks were well separated in this study. As illustrated in Figure 3, all analytes show good peak shapes except for DON, which shows a broad peak because the injected sample solvent (50% acetonitrile) is stronger than the initial mobile phase composition (10% methanol). However, this broadened DON peak does not affect its quantification significantly.

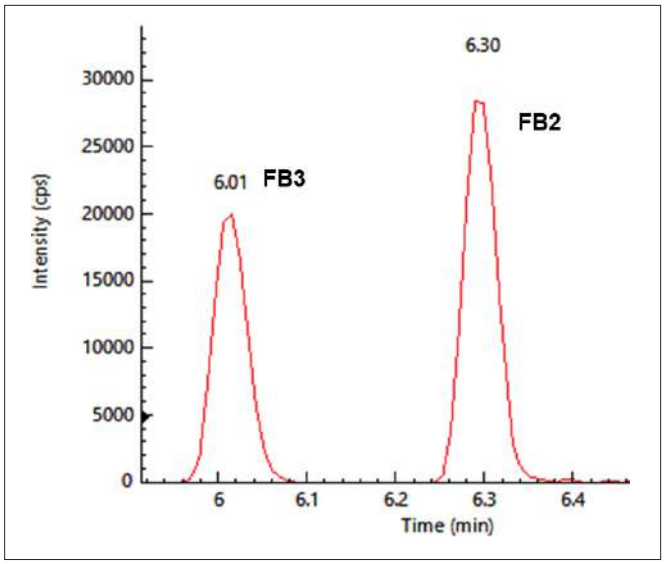

Figure 2. Baseline separation of fumonisin B2 (FB2) and fumonisin B3 in a spiked corn sample extract.

Sample Extraction

Efforts were made by several research groups to extract the target mycotoxins from various food matrices.[13-15] However, it was very difficult to achieve high extraction efficiency for all target analytes using a single extraction step owing to their different physicochemical properties. Different solvents and solvent mixtures at different ratios were examined; acetonitrile was proved to be a better organic solvent than methanol, and the best results were obtained by a mixture of acetonitrile/water (80/20), with good recoveries (>70%) for all mycotoxins except for fumonisins. Several experiments were carried out to improve extraction efficiency for fumonisins, and it was found that increasing the water content in the extraction solvent in a second extraction step, or adding formic acid in the solvent mixture, could achieve this goal. Therefore, to obtain the best extraction efficiency for all analytes, two consecutive extraction steps had to be employed. The first extraction was carried out using acetonitrile/water/formic acid (80/19.9/0.1, in v/v/v), followed by extraction with acetonitrile/water/formic acid (20/79.9/0.1, in v/v/v).[13,19]

Since the use of ^{13}C-IS before extraction can compensate for variations in extraction efficiency and matrix effects, the single extraction step is commonly used in stable isotope dilution assay (SIDA) to simplify the extraction procedures, using either acetonitrile/water (50/50) or acetonitrile/water/formic acid (80/19.9/0.1) as the extraction solution.[16, 20-24] In this study, the mixture of acetonitrile/water (50/50) was used as the extraction solution after fortification of ^{13}C-IS to the samples.

Calibration Curves and Linearity

Several sets of calibration curves with seven concentration levels (as listed in Table 1) were generated on separate days for each of the twelve mycotoxins, based on internal standard calibration method. All calibration curves show good linearity, with correlation coefficients (R^2) greater than 0.99. Example calibration curves representative of the six different classes of mycotoxins are shown in Figure 4. The accuracies for most of the calibration points evaluated by the RSD% of the residuals are less than 15% (it is less than 20% for the lowest standard).

Stable isotope dilution assay and LC/MS/MS are based on the fortification of samples with isotope labeled internal standards (IS) (e.g., ^{13}C-IS in this study) prior to sample preparation and instrumental analysis. The target analytes and fortified IS have the same physicochemical properties, which results in identical recoveries and MS responses. By measuring the relative response ratio between a target analyte and its corresponding labeled IS, signal variations caused by potential analyte loss during sample preparation or ionization suppression during LC/MS/MS detection are corrected, as the relative response ratio remains constant. Therefore, the application of SIDA should be instrument- and sample matrix-independent, unlike the matrix-matched calibration method, in which multiple sets of matrix-matched calibration standards need be prepared to match for different sample matrices. For SIDA LC/MS/MS, one set of calibration standards prepared in solvent (the "so-called" solvent-only calibration) can be used directly for analyte quantification for different sample matrices, which significantly simplifies sample preparation and analyte quantification, and thus save time and money when testing many samples with different sample matrices.

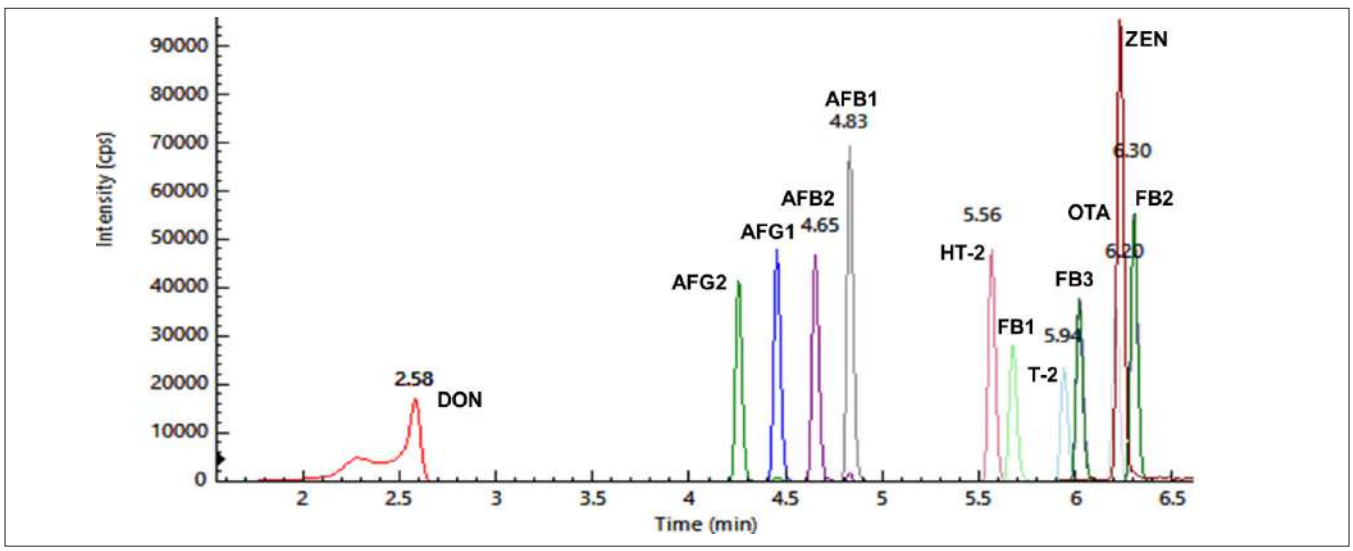

Figure 3. Extracted ion chromatograms of the 12 mycotoxins in a calibration standard.

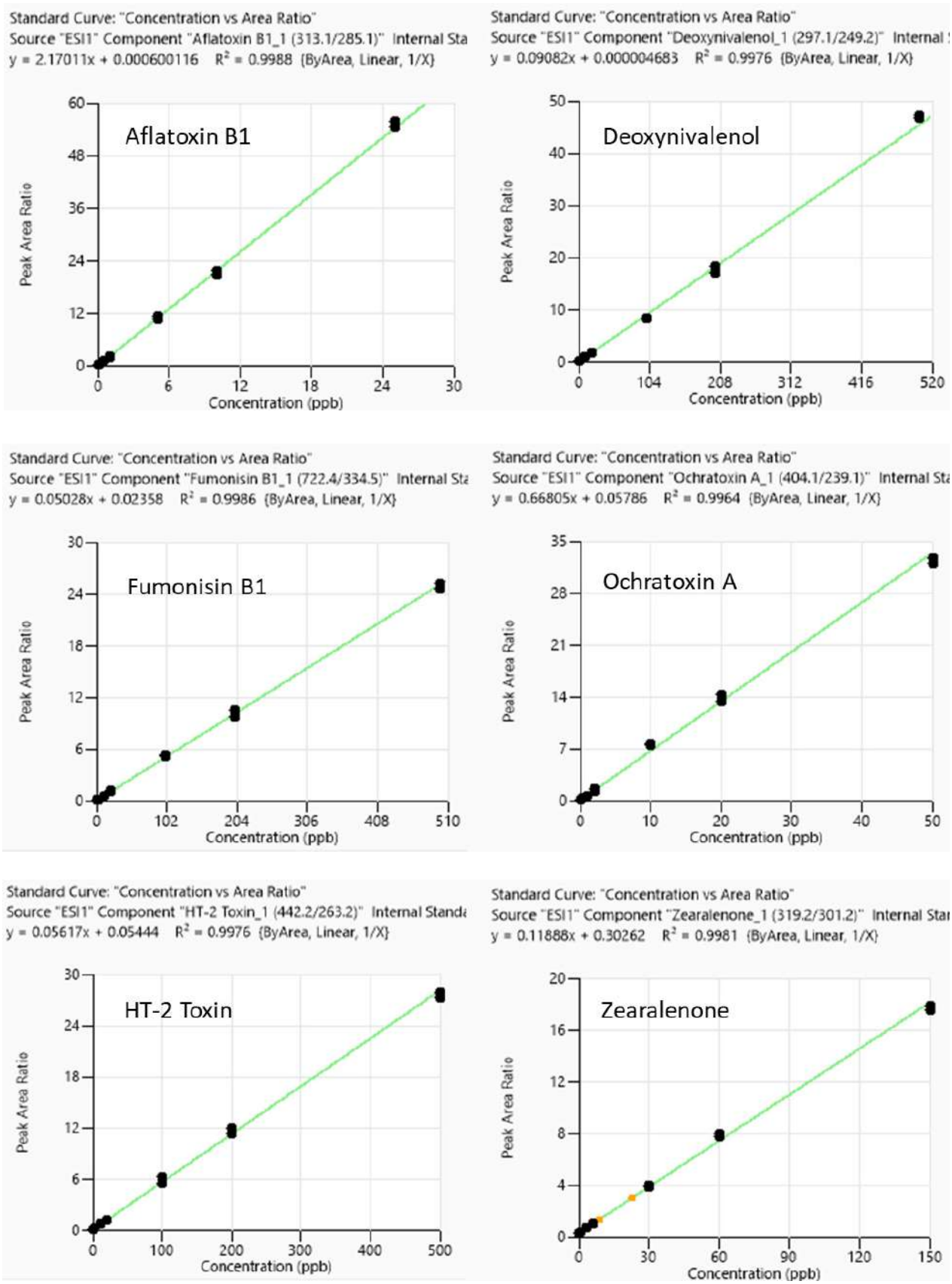

Figure 4. Example calibration curves for the six major types of mycotoxins.

Method Performance and Validation

Sample Matrix Effects, Method's Selectivity and Sensitivity

Sample matrix effects have attracted great attention in LC/MS/MS method development and validations because they can affect data quality and method selectivity, sensitivity and accuracy, especially for complex sample matrices such as various food commodities. Isotope dilution with a stable isotopically labeled internal standard calibration method (SIDA) has proven to be the most effective and simple method to correct matrix effects. However, this method had limited applications in the past for mycotoxin analysis owing to the cost and availability of the labeled IS. Some researchers have attempted to use a few selected labeled IS to cover all analytes in order to cut the cost of the analysis.[16, 26] However, poor recoveries and inaccurate results for those analytes that do not have the corresponding labeled IS were observed, as matrix effects are analyte dependent, and each analyte must have its corresponding labeled IS.

In this study, isotope dilution with stable ^{13}C-labeled IS for each analyte was applied to all sample matrices before sample extraction to compensate for matrix effects and any variations in analytical procedures (including variations in sample preparation and instrument analysis). Additionally, sample matrix effects were also reduced to a certain extent by sample dilutions in this study with a dilution factor of five.

The method's selectivity and analyte confirmation from food samples can be evaluated by comparing the analyte retention time and mass spectrum information (such as the peak area ratios of qualifier to quantifier ions of the analyte) between reference standards and samples. According to regulatory guidance on analytical method validation, at least two structurally specific MS/MS transition ion pairs should be used in an LC/MS/MS method.[28-31] In this study, three MS/MS ion pairs were employed for each analyte in the method to identify the peaks of interest in the studied samples. For example, ideally the blank samples used for recovery study should not contain any analyte. However, the blank samples obtained may contain some analytes since mycotoxins are prevalent in many grain products, even when they are still in the field.[32] Therefore, to achieve accurate results and obtain correct analyte recoveries, it is important to unambiguously identify or confirm the peaks of interest in blank sample matrices.

Six blank sample matrices were evaluated during method validation. Although no mycotoxin was found in almond and soybean blank samples, at least one mycotoxin was identified from each of the other four sample blanks (yellow corn, wheat, oat cereal, and peanut butter) by this method. As shown in Figure 5 for a yellow corn blank sample, fumonisins B1 and B2 can be confirmed by their retention times and ion ratios of qualifier/quantifier ions, which are consistent with those of reference standard (deviations of retention time <2%, and deviation of ion ratio < 10%), demonstrating good selectivity of the method for mycotoxin analysis. For the wheat blank sample, only deoxynivalenol (DON) was identified and determined as illustrated by Figure 6. For the oat cereal blank, only HT-2 and T-2 toxins were identified (see Figure 7). For the peanut butter blank, as demonstrated in Figure 8, a relatively high amount of aflatoxin B1 was determined and confirmed by retention time and ion peak area ratio comparisons with its reference standard.

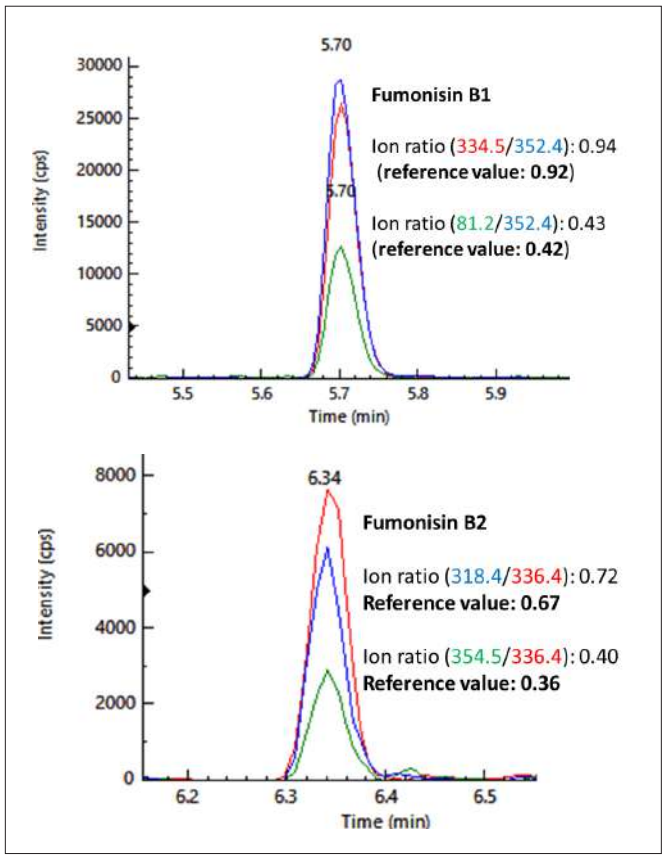

Figure 5. Overlapped three MS/MS chromatograms and their ion ratios of qualifier/quantifier ions of fumonisin B1 (top) and fumonisin B2 (bottom) in a yellow corn sample blank.

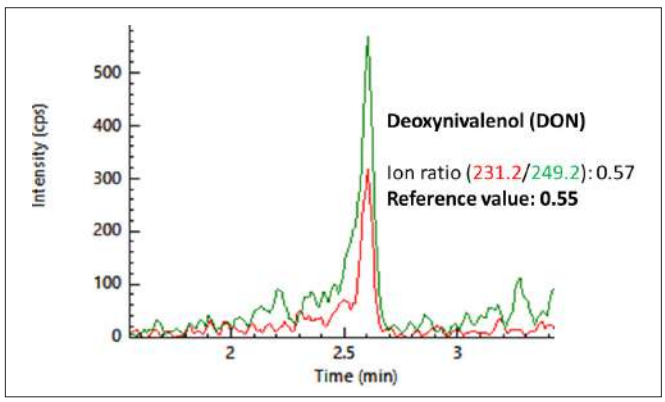

Figure 6. Overlapped two MS/MS chromatograms and their ion ratios of qualifier/quantifier ions for DON in a wheat blank sample.

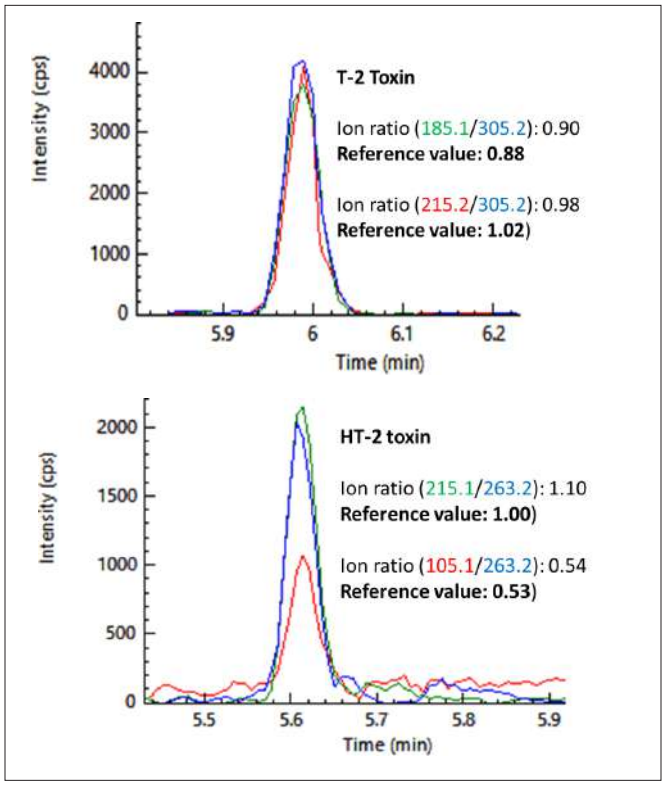

Figure 7. Overlapped three MS/MS chromatograms and their ion ratios of qualifier/quantifier ions for T2-toxin (top) and HT2-toxin (bottom) in an oat cereal blank sample.

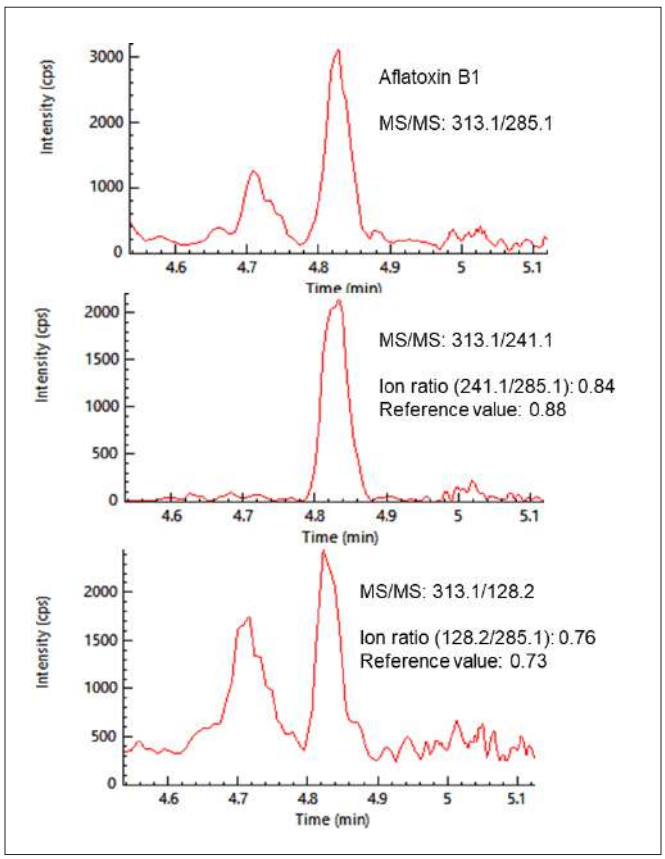

Figure 8. Three MS/MS chromatograms and their ion ratios of qualifier/quantifier ions for aflatoxin B1 in a peanut butter blank sample.

Monitoring multiple MS/MS transitions for each analyte in complex sample matrices not only facilitates compound identification with ion ratio comparison, but also provides the opportunity to select a better MS/MS transition for accurate analyte quantification. For an example, although the top MS/MS transition in Figure 8 has higher signal intensity, the second MS/MS transition shows fewer interfering peaks, and is thus more suitable for analyte quantification. Another example is illustrated in Figure 9 for aflatoxin G1 analysis in a peanut butter sample. Although the first MS transition (329.3/200.2) shows a stronger signal in a solvent-only standard solution, it suffers from matrix suppression, and has a strong matrix peak in front of the analyte peak, which will interfere with the analyte if the peaks are not separated chromatographically. In contrast, the second MS/MS transition (329.3/243.2) has almost no matrix interferences, and therefore is better suited for analyte quantification in this sample matrix.

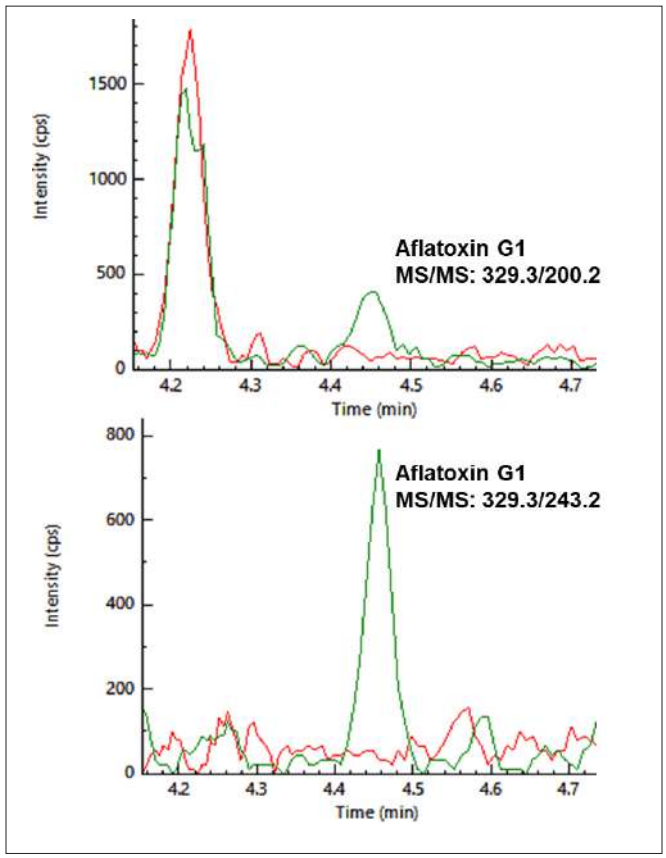

Figure 9. Two MS/MS chromatograms of aflatoxin G1 in a peanut butter sample. Red – peanut butter sample blank; green – peanut butter spiked with 1µg/kg of analyte.

The method's sensitivity depends on a number of factors, including the instrument sensitivity, sample matrix effects (signal suppression or enhancement) and sample preparation methods (sample dilution factors). In this study, signal suppression and enhancement effects were evaluated by comparing the responses of the same amount of IS spiked in solvent (solvent-only calibration standards) and in different food sample matrices. The results showed that matrix effects were both analyte dependent and sample matrix dependent. Therefore, the limit of detection (LOD) and the limit of quantification (LOQ) of the method were estimated by the signal-to-noise ratio of each analyte in each sample matrix (S/N = 3 for LOD and S/N = 10 for LOQ). Since the matrix effects on each analyte are slightly different in different sample matrices, the LOD and LOQ values are slightly different for the different matrices (thus, a range of values were reported for some analytes in this study as shown in Table 4). Overall, the LOQ values of the method for all analytes are below the regulated maximum limits (MLs) for the studied food matrices, as shown in Table 4, demonstrating good sensitivity of the method for the selected food matrices. However, for aflatoxin B1 and ochratoxin A in baby food samples, sample clean up and analyte concentration steps are needed to achieve the low limits of quantification.[24] Our work of using a immunoaffinity column for sample clean up coupled with QSight LC/MS/MS for highly sensitive mycotoxin analysis is currently under preparation and will be published in a separate application note.

Precision, Recovery and Accuracy

Method precision was assessed based on replicate analyses of a middle level standard and spiked samples (3 replicates) in each sample matrix. The precision was then calculated based on the coefficient of variation (RSD %) of the collected data. The RSDs were <10% for all the analytes in the standard, and < 20% for analytes in the spiked samples.

No interference or contamination from reagents, glassware, and sample tubes was observed in this study (no analyte was detected in all LRB samples). Method accuracy assesses how close the experimental value is to the expected value. The method's accuracy was evaluated by the recovery of a known amount of analyte spiked to a sample matrix (LFM samples). As shown in Tables 5 and 6, the recoveries for most analytes from the spiked LFM samples were between 70% and 114%, demonstrating good accuracy of the methods.

Stability of Standards and Samples

According to the recommendation from the supplier, all mycotoxin standards and uniformly ^{13}C-labeled internal standards (IS) stock solutions can be stored in a freezer for at least one year. Working standard and IS solutions are stable for a month if kept in a dark freezer after preparation. Calibration standards and sample extracts are stable for at least a week if kept in a dark refrigerator after preparation.

Determination of Mycotoxins in Selected Food Samples

The validated method was successfully applied to the determination of twelve targeted mycotoxins in seven different commercial food products including soybean, wheat, almond, oat breakfast cereal, peanut butter, white corn and yellow corn samples. All mycotoxins determined in this study are below the regulated maximum levels (MLs), except aflatoxin B1 in a peanut butter sample. No mycotoxins were detected in almond and soybean samples, while different amounts of deoxynivalenol (DON) were determined in the remaining three samples, as listed in Table 7. The result for DON is in-line with previous findings, in that DON is the most prevalent mycotoxin, and is commonly found in various grain products.[32] A high amount of aflatoxin B1 (4.62 µg/kg), and a small amount of aflatoxin B2 (0.653 µg/kg) were determined in the peanut butter sample. Similar results of aflatoxin B1 (4.38 µg/kg) and aflatoxin B2 (1.62 µg/kg) in a peanut powder sample were also obtained recently by another research group.[16] As shown in Table 7, fumonisins (B1, B2, and B3) were found only in corn (maize) samples because they are mainly produced by Fusarium verticillioides, a fungus predominant in maize and maize-based products.[32] HT-2 and T-2 toxins were determined only in the oat cereal samples. The confirmation and identification of these mycotoxins in various food samples was carried out by comparing the retention time and peak area ratio of the qualifier ion to the quantifier ion peaks of the analyte between the samples and the reference standard. All deviations from reference standards (deviations of retention time <2%, and deviation of ion ratio < 15%) were within the limits established by the European Union guidance.[29-31]

Table 4 The Estimated LOD and LOQ and Regulatory Maximum Limits (MLs).

Analyte	LOD	LOQ	US. FDA MLs*	EU MLs (Food)**
	(µg/kg)	(µg/kg)	(µg/kg)	(µg/kg)
Aflatoxin B1	0.2	0.5		2 (0.1 baby food)
Aflatoxin B2	0.25	0.56	20 (total Aflatoxins)	(Aflatoxins B1+ B2+G1 +G2): 4 -15
Aflatoxin G1	0.25	0.54		
Aflatoxin G2	0.3	0.6		
Ochratoxin A	0.4	1	20	3 - 80 (0.5 baby food)
Fumonisin B1	0.5 - 0.7	2 - 3	(B1+B2+B3): 2000 - 4000	(B1+B2): 400 - 4000
Fumonisin B2	0.5 - 0.7	2 - 3		(B1+B2): 200 baby food
Fumonisin B3	1	3 - 4		
Deoxynivalenol	5 - 6.5	15 - 20	1000	500 - 1750 (200 baby food)
Zearalenone	1	3		50 - 400 (20 baby food)
HT-2 Toxin	2	6		(HT-2 + T-2): 25 - 1000
T-2 Toxin	1	3		(HT-2 + T-2): 20 baby food

* see reference 7; ** see references 3-6.

Table 5. Mycotoxin Recovery from Food Samples at Spiking Level One.

Analyte	Spiked	Method Accuracy or Analyte Recovery from Sample Matrix (%)					
	(µg/kg)	Corn	Wheat	Soybean	Oat Cereal	Almond	Peanut Butter
Aflatoxin B1	1	100	106	95.3	89.4	93.4	101
Aflatoxin B2	1	81.4	96.1	85.3	73.3	83.6	111
Aflatoxin G1	1	93.5	95.3	98.4	85.7	90.7	98.1
Aflatoxin G2	1	91.1	107	104	85.8	92.7	92.2
Ochratoxin A	2	83.8	89.3	68.2	81.1	62.6	97.4
Fumonisin B1	100	97.4	80.1	86.5	77.1	83.8	114
Fumonisin B2	100	97.7	95.6	93.9	84.6	97.9	103
Fumonisin B3	100	91.1	95.3	95.3	89.1	92.9	107
Deoxynivalenol	100	98.7	113	102	96.6	96.8	93.2
Zearalenone	30	92.9	101	99.4	104	97.4	90.7
HT-2 Toxin	100	90.9	94.1	80.3	102	94.2	108
T-2 Toxin	10	97.3	110	98.9	106	111	98.1

Table 6. Mycotoxin Recovery from Food Samples at Spiking Level Two.

Analyte	Spiked	Method Accuracy or Analyte Recovery from Sample Matrix (%)					
	(µg/kg)	Corn	Wheat	Soybean	Oat Cereal	Almond	Peanut Butter
Aflatoxin B1	5	104	109	97.3	97.2	104	111
Aflatoxin B2	5	91.8	86.9	105	86.9	89.4	113
Aflatoxin G1	5	91.0	106	100	99.4	98.5	84.2
Aflatoxin G2	5	95.8	103	91.6	86.5	87.2	105
Ochratoxin A	10	98.7	82.0	88.8	86.3	90.0	99.2
Fumonisin B1	250	111	81.7	85.9	87.6	88.8	104
Fumonisin B2	250	99.7	101	95.5	104	98.8	100
Fumonisin B3	250	96.6	106	99.9	99.6	96.7	102
Deoxynivalenol	250	101	103	102	93.2	95.1	92.5
Zearalenone	75	99.3	100	103	101	105	81.7
HT-2 Toxin	250	95.9	93.7	83.1	94.8	94.2	112
T-2 Toxin	25	101	102	105	113	94.5	96.9

Table 7. Mycotoxin Results Obtained from Various Food Samples by SIDA-LC/MS/MS.

Analyte	Mycotoxin Contents (µg/kg) in the Samples*				
	Yellow Corn	White Corn	Wheat	Oat Cereal	Peanut Butter
Aflatoxin B1					4.62
Aflatoxin B2					0.653
Fumonisin B1	157	15.8			
Fumonisin B2	36.5	2.12			
Fumonisin B3	24.0				
Deoxynivalenol	26.8	20.5	20.6		
HT-2 Toxin				15.2	
T-2 Toxin				17.2	

* Blank space means: no mycotoxin was detected.

Conclusions

In this study, a multi-analyte UHPLC/MS/MS method has been developed and validated for the fast and reliable screening, confirmation, and quantification of twelve mycotoxins in various food matrices. All the mycotoxins, with very different physicochemical properties, can be determined simultaneously within eleven minutes, in a single chromatographic run. The high selectivity of the QSight triple quadrupole mass analyzer and the high efficiency UHPLC separation, in combination with the use of stable isotope dilution assay, have made it possible to simplify the sample preparation procedures, without suffering from matrix interferences and matrix effects.

Applying isotope dilution prior to sample extraction can not only compensate for sample matrix effects, but also minimize variations in the entire analytical process, including sample preparation and instrument analysis. Therefore, a more robust method with more accurate results can be obtained. For each analyte, three MRM transitions have been acquired, which allows confident identification and confirmation of the compounds detected in the samples.

The method presented herein has been validated in six different food matrices (maize, wheat, soybean, oat cereal, almond, and peanut better) with good sensitivity, selectivity, accuracy and precision for most of analyte/ matrix combinations, and can therefore be used in routine testing laboratories to meet regulatory requirements.

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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
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