APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Multiclass-Multianalyte Method for Mycotoxin Testing in Foods Using Immunoaffinity Column Sample Clean-up with QSight LC/MS/MS

Introduction

Mycotoxins are toxic secondary metabolites produced by various fungal species growing in different agricultural products. Based on their occurrence and toxicity, the most important classes of mycotoxins are aflatoxins (AFB1, AFB2, AFG1, AFG2), 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. Mycotoxins have serious adverse effects on humans and animals and can cause economic losses on crops due to their contamination. Aflatoxins have been classified as carcinogenic to humans, while ochratoxin A and fumonisins are classified as possible carcinogens by the International Agency for Research on Cancer.[1] Therefore, mycotoxin levels in foods and feeds are regulated by regulatory agencies around the world to minimize the exposure and protect consumers.[2-8] The maximum levels (MLs) for several mycotoxins in foods and feeds have been established by the European Union and the U.S. Food and Drug Administration (FDA).[4-8]

To obtain reliable information on the presence and concentrations of mycotoxins in foods and feeds and meet the regulatory requirements, various analytical methods have been developed for analysis of mycotoxins in foods.[9-10] Recently, LC/MS/MS method has become the method of choice for quantification and confirmation of mycotoxins in various food and feed sample matrices due to its superior sensitivity, selectivity and capability of analyzing multiple target mycotoxins in a single run. [9-25]

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To cope with the increasing number of sample matrices and mycotoxins of interest, the current trend in mycotoxin analysis is to develop the "fit for all purposes" multi-mycotoxin methods that can analyze all the regulated mycotoxins in various food matrices in a single run. However, to achieve this goal, scientists have faced several challenges: (1) very different maximum levels (MLs) regulated for different mycotoxins based on their toxicity and type of food, which complicate the procedures of preparation for calibration standards and quality control samples; (2) a wide range of food and feed sample matrices, which make the extensively used matrix-matched calibration method less efficient because multiple sets of matrix-matched calibrations need be prepared for 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 obstacles and avoid sample clean up steps, simple sample preparation procedures using acetonitrile/water mixture as extraction solvents have been used in developing multi-mycotoxin LC/MS/MS methods. However, direct injection of the crude sample extracts to LC/MS/MS system can cause matrix effects, which can significantly affect the data quality and method's selectivity, sensitivity and accuracy. Thus, matrix effects should be considered seriously in this multi-mycotoxin method development and validation.[26]

Matrix-matched calibration method and stable isotope dilution assay (SIDA) can effectively compensate for matrix effects, and therefore, have been widely applied for mycotoxin analysis in food sample matrices.[14-25] However, these methods are calibration methods, which cannot remove sample matrix effects (suppression or enhancement) and matrix interfering components from samples, and thus, they cannot improve method's sensitivity and selectivity. To remove sample matrix and increase the sensitivity of a method for low level of mycotoxins, such as aflatoxin B1 (0.1µg/kg) and ochratoxin A (0.5µg/kg) in baby food samples, various sample clean-up and analyte concentration techniques are required, such as solid-phase extraction[27] and immunoaffinity column (IAC) sample cleanup methods.[23, 28-29] Traditionally, mycotoxins were analyzed by many single analyte or single-class mycotoxin methods developed based on their various physicochemical properties and different sample clean-up strategies.[27-30] Recently, PerkinElmer has introduced immunoaffinity columns that can selectively purify multiple mycotoxins from complex food sample matrices, such as the MaxSigna® IAC 4-in-1 Combo and MaxSignal® IAC 6-in-1 Combo, which can be used to extract eight and twenty mycotoxins, respectively, from different sample matrices.[31-32]

In this study, a reliable, sensitive and selective method was developed and validated for multi-mycotoxin determination in various food sample matrices using a PerkinElmer QSight 220 LC/MS/MS system. Sample clean-up was carried out using a PerkinElmer immunoaffinity column MaxSignal IAC 6-in-1 Combo. Stable isotope dilution assay was performed before sample extraction, which minimized variations during sample preparation and instrument analysis and further compensated for matrix effects on ionization. The results demonstrated that cleaner sample matrices and higher sensitivity could be achieved by coupling IAC sample preparation with QSight LC/MS/MS. This method was fully validated using eight different food sample matrices spiked at two analyte concentration levels and could be applied for multi-mycotoxin analyses to meet the most stringent regulatory limits.

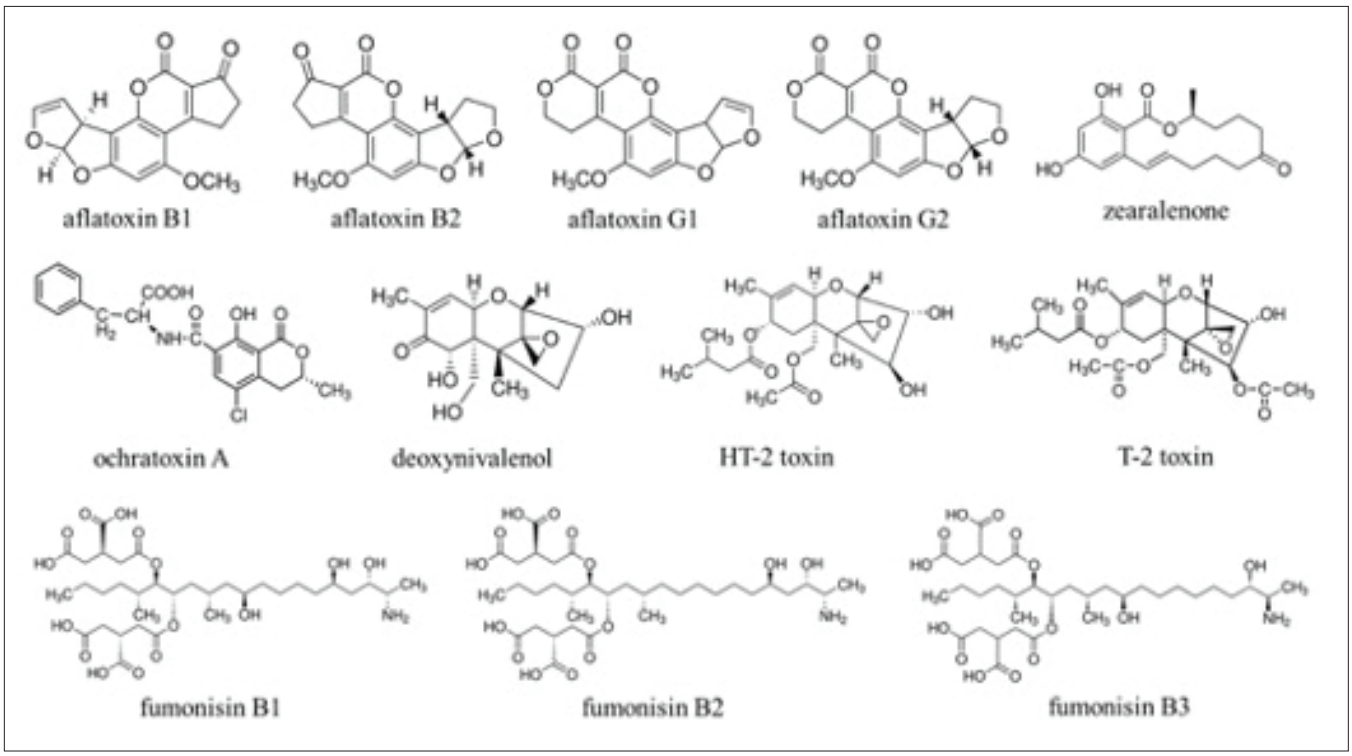

Figure 1. The chemical structures of the studied mycotoxins.

Experimental

Hardware/Software

Chromatographic separations of different mycotoxins and mycotoxins from potential interfering components were conducted by a PerkinElmer QSight LX 50™ ultra-high-performance liquid chromatography (UHPLC) system, and 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 the Simplicity™ 3Q Software.

Materials and Methods

Chemicals and Materials

The following 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); and U-[$^{13}C_{18}$]-zearalenone (25 µg/mL). HPLC grade solvents (methanol, acetonitrile, water) and other chemicals such as formic acid, ammonium formate and phosphate buffered saline (PBS, pH 7.4) were obtained from Sigma-Aldrich. Immunoaffinity columns (MaxSignal IAC 6-in-1 Combo, PerkinElmer P/N FOOD-1505-01), disposable polypropylene syringe (10 mL, 20 mL), syringe filter (0.45 and 0.22 µm), polypropylene centrifuge tube (50 mL), amber autosampler vials and caps were obtained from PerkinElmer Inc.

Test samples (yellow corn, white corn, wheat, soybean, almond, oat breakfast cereal, peanut butter, chili powder, black pepper powder) 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 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 1mL) 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.

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.

Sample Preparation

1. Principle of immunoaffinity column sample clean-up

This sample preparation method applied mycotoxin immunoaffinity column (MaxSignal IAC 6-in-1 Combo, PerkinElmer P/N FOOD-1505-01) for sample clean up based on antibody-antigen specific interactions as illustrated in Figure 2: Twenty antibodies of the six main classes of mycotoxins were immobilized to the stationary phase in column concurrently. After the sample was extracted and filtered, the sample solution was slowly passed through the immunoaffinity column (IAC). The mycotoxins were bound to the corresponding antibodies in the column. The IAC column was then washed to remove other unrelated substances (including matrix interfering components) that were not bound to the column. Finally, the mycotoxins (analytes) were eluted from the column using an appropriate eluent (methanol containing 2% acetic acid in this study).

Since twenty antibodies of the six main classes of mycotoxins were immobilized onto the stationary phase, this IAC can simultaneously adsorb twenty mycotoxins of six major toxin types: Aflatoxin (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2), Zearalenone (ZEN) and its derivatives (ZAN, α-ZOL, β-ZOL, α-ZAL, β-ZAL), Deoxynivalenol (DON) and 15-Acetyldeoxynivalenol (15-ADON), Ochratoxin (OTA), T-2 toxin and HT-2 toxin, fumonisins (FB1, FB2, FB3) from various sample matrices, and has a highly targeted purification effect on these twenty analytes. In this study, twelve regulated mycotoxins from the six major toxin classes were selected to evaluate the sample clean up performance of the IAC from eight different food sample matrices. The purified sample solutions were analyzed by LC/MS/MS after concentration and filtration.

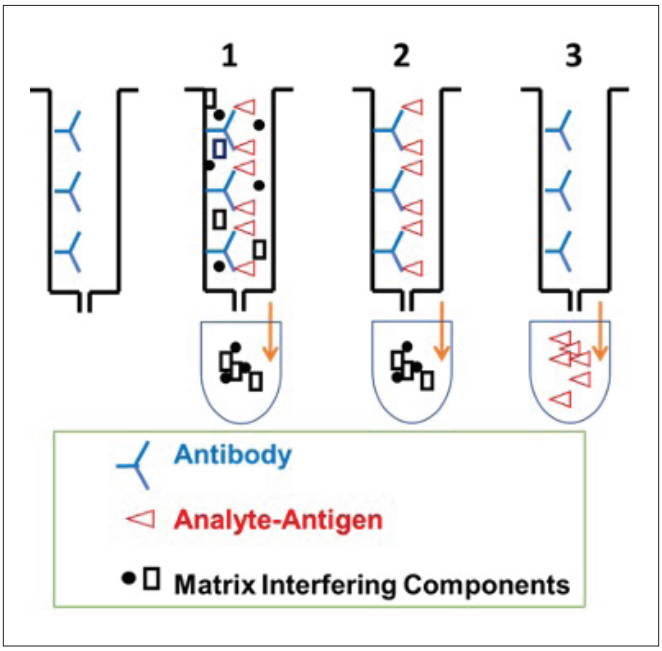

Figure 2. Schematic illustration of the procedures of immunoaffinity column sample clean-up: 1. Load sample; 2. Wash matrix components; and 3. Elute analytes from column.

2. Solution Preparation

- Preparation of extraction solution 1: acetonitrile/water/acetic acid mixture (80:19:1 in v/v)

  Combine 800 mL of acetonitrile and 10 mL of acetic acid in a graduated cylinder, then bring to 1 L volume with distilled/deionized water and mix well.

- Preparation of extraction solution 2: methanol/water mixture

  Combine 800 mL of methanol and 200 mL of distilled/deionized water. Bring to 1 L final volume with distilled/deionized water and mix well.

- Preparation of PBS solution: phosphate buffered saline (PBS, pH 7.4)

  Dissolve one pouch of the premix PBS salt powder with 800 mL of distilled/deionized water, then bring to a final volume of 1 L and mix well.

- Preparation of wash solution: 0.1% v/v Tween-20 aqueous solution

  Combine 1 mL of Tween-20 and 999 mL of distilled/deionized water. Mix well.

- Preparation of eluent solution: 2% v/v acetic acid in methanol

  Combine 2 mL of acetic acid and 98 mL of methanol. Mix well.

3. Sample Extraction

Before sample extraction, each solid food sample (250 g) was ground to fine powders using a food grade grinder and passed through a food grade sieve (particle ≤1mm), then mixed thoroughly to make sure they were homogeneous.

- Weigh 5 g ± 0.01 g of the ground and homogenized sample into a 50mL centrifuge tube. Add 100 µL of ^{13}C-labled IS solution (and suitable amounts of working standard solutions when performing recovery study) and vortexed for 2 minutes.
- Add 20 mL of extraction solution one and shake vigorously on a shaker for 20 minutes.
- Centrifuge at 4 °C and 4000rpm for 5 minutes. Transfer the supernatant to a new bottle.
- Add 20 mL of extraction solution two to the centrifuged residue and shake vigorously on a shaker for 20 minutes.
- Centrifuge at 4 °C and 4000rpm for 5 minutes. Transfer the supernatant to the same bottle as step (3) and mix the solution well.
- Dilute 5 mL of the combined supernatant with 35 mL of PBS solution. Mix well.
- Filter the solution with disposable syringe filter (0.45µm) or microfiber filter paper and collect the filtrate to a clean sample bottle.
- Use 32 mL of the filtrate (equivalent to 0.5 g of the sample, dilution factor = 2) as the final sample for IAC cleaning up.

4. IAC Sample Clean-Up Procedure

- Remove the IAC column from a fridge and place into a column holder. Remove the plunger of a syringe, then attach the syringe through the connector plug above the column to complete the connection. Secure to an air-pressure controller, if available.
- Transfer the 32 mL of the sample solution to fill the syringe.
- Remove the cap under the affinity column (do not discard it as this will be used in the next step). Adjust the air-pressure to have a flow rate of 1–2 drops/second.
- After all the sample solution has flowed through the column, add 10 mL of water to wash the column at a flow rate of 2–3 drops per second. Repeat this wash step one more time.

  Note: for complex sample matrices such as red chili, black pepper and peanut butter samples in this study, pre-wash one or two times with 10 mL of Wash Solution before washing with water.
- After wash solution has flowed through the column, load 2 mL of Eluent Solution. Cap the opening under the column using the plug and allow the column to incubate for 3 minutes. Place a clean collection tube under the column. After 3 minutes, remove the plug and allow the liquid to flow through at a rate of one drop per second. Collect this liquid known as the eluate.
- Add another 1 mL of the Eluent Solution. Cap the opening under the column using the plug and allow the column to incubate for 3 minutes. Place the same collection tube under the column. After 3 minutes, remove the plug and allow the liquid to flow through at a rate of one drop per second.
- Place the eluate under a slow stream of nitrogen gas at 50°C (in a water bath) to evaporate any residual solvents. Dissolve the dried residue with 1 mL of the acetonitrile/water (50:50) diluent and the solution was vortex-mixed and filtered through a 0.22 µm syringe filter directly into an amber autosampler vial for LC/MS/MS analysis.

Since ^{13}C-labled 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.

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 and from the sample preparation processes. Then, 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 as described above, using each of the eight blank samples (corn, wheat, soybean, almond, oat cereal, red chili, black pepper, and peanut butter) as a sample matrix, respectively, 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), and 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 analyte from complex sample matrices, reduce false positive and false negative 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), was performed by infusion of standards and use of autotune feature in software. MS Source parameters including gas flows, temperature and probe position settings, were optimized for maximum sensitivity by infusion of standards with a T-unit connected to LC mobile phases flow and ion source. 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: 0.3 mL/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	5.0µL
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
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

Table 3. Optimized MRM Transitions and Parameters. - Continued.

Compound Name	Polarity	Precursor Ion	Product Ion	CE	EV	CCL2
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
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 except for zearalenone (ZEN), which showed slightly higher signal intensity in negative mode, and 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,[23] similar results were also obtained in this study (refer to the sample matrix effects section for further discussion). Although deoxynivalenol (DON) and ochratoxin A (OAT) were determined in negative ionization mode in a previous work,[33] 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's accuracy. The optimized MS/MS (or MRM) parameters were 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, 2.6µm). Due to the diverse physicochemical properties of the twelve studied mycotoxins (with different polarity and acidity characteristics), a compromise need 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 small amount of ammonium formate was added to the mobile phase. In addition, small amounts of ammonium ions in mobile phases could help inhibit the formation of sodium adducts, especially in the case of DON, HT-2 and T-2 toxin, and favored the formation of $[M+NH_4]^+$ 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 its concentrations ranging from 0.5 mM to 10 mM.[14-25] It was found by previous researchers,[11, 16] 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 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 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% acetonitrile). However, this broadened DON peak does not affect its quantification significantly. The two peaks of FB3 (at 6.05 min) and FB2 (at 6.36 min) were well separated in this study.

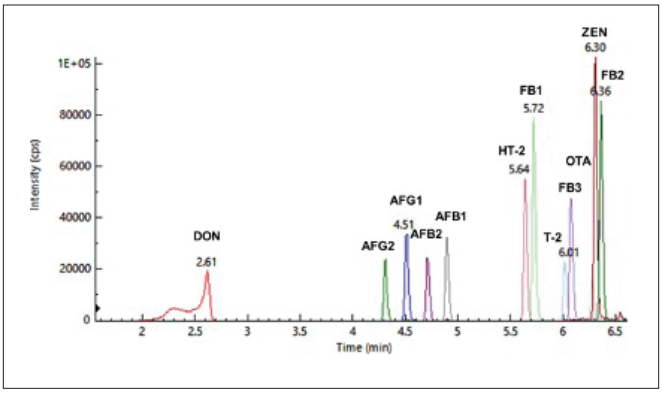

Figure 3. Extracted ion chromatograms of the 12 mycotoxins in a spiked corn sample.

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 the calibration curves show good linearity with correlation coefficients (R_2) greater than 0.99. Example calibration curves of the six different classes of mycotoxins are shown in Figure 4.

Stable isotope dilution assay (SIDA) 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 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 simplify sample preparation and analyte quantification, and thus save time and money when testing many samples with different sample matrices.

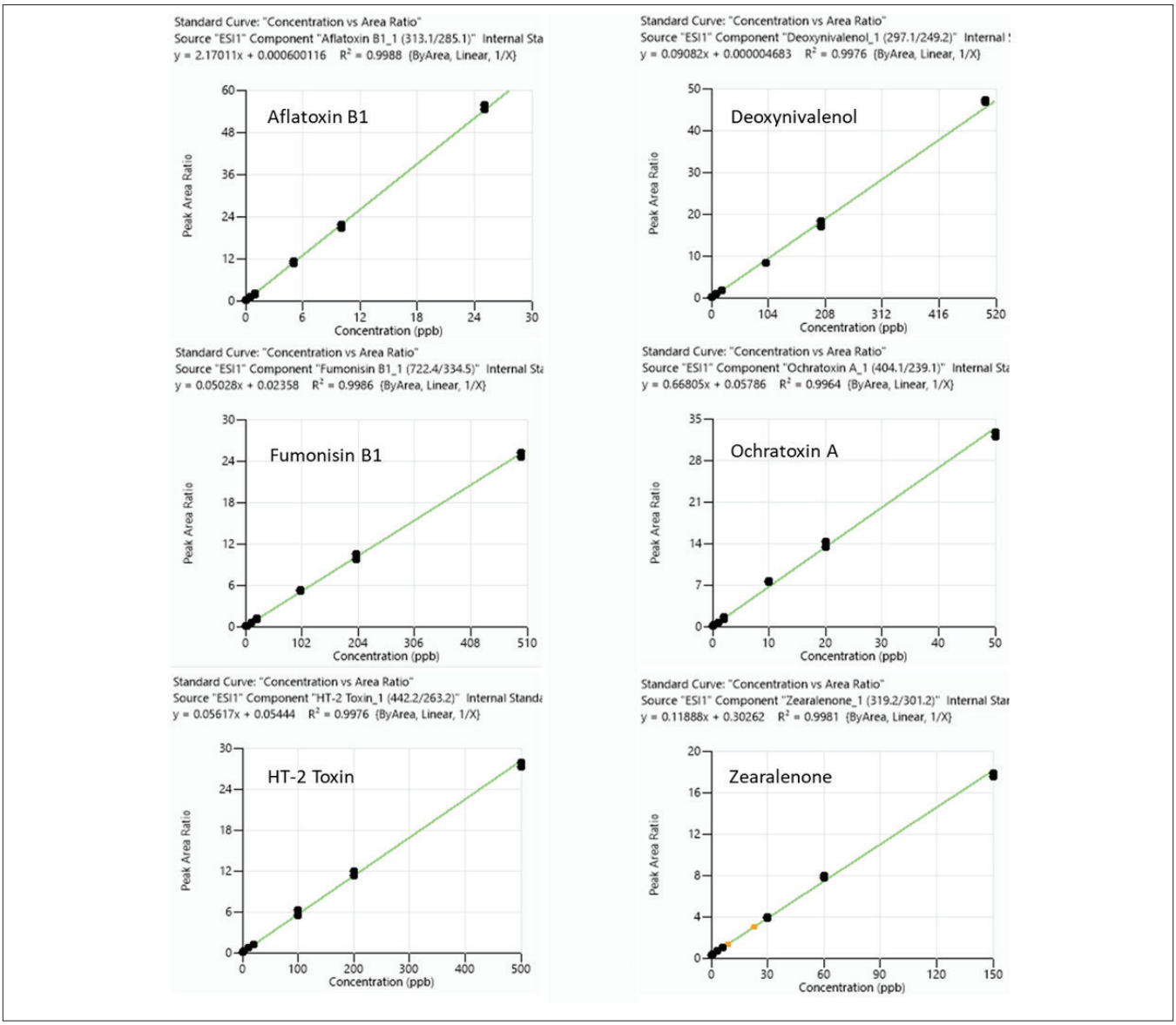

Figure 4. Example calibration curves for the six major types of mycotoxins.

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's selectivity, sensitivity and accuracy, especially for complex sample matrices such as various food commodities. Isotope dilution with 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 cannot remove matrix interfering components from samples and thus cannot improve method's sensitivity and selectivity. Sample clean-up techniques have been demonstrated to be the most effective approach for matrix effects reduction and analyte concentration, which lead to better selectivity and sensitivity of a method. One of the widely used sample clean-up techniques is immunoaffinity chromatographic (IAC) separation based on specific antibody-antigen interactions.

In this study, a PerkinElmer IAC column was employed for isolating the twelve mycotoxins from various sample matrices. As demonstrated in Figure 5, this IAC technique significantly reduced matrix components from a peanut butter sample matrix (figure 5B) as compared to the results obtained by the method without using IAC sample treatment (figure 5A). In addition, as shown clearly in Figure 5, the sensitivity of the method, in terms of signal intensity and signal to noise ratio, could be improved dramatically due to less matrix suppressions on analyte signal and the analyte concentration steps during IAC sample preparation procedures. Similar results were also observed for other mycotoxins in this sample and in all other sample matrices, especially for the early eluting analytes such as aflatoxins (AFB1, AFB2, AFG1 and AFG2, data not shown, but available upon request).

Another approach to reduce matrix effects is to evaluate the analyte's signal to noise ratio and responses from sample matrix interfering components under both positive and negative ionization modes and select a better ionization mode for quantification. A previous study on mycotoxins in animal feed showed that better signal to noise (S/N) ratios were achieved using negative ionization for ZEN,[23] similar results were also obtained in this study as illustrated in Figure 6.

To minimize any variations in analytical procedures (including variations in sample preparation and instrument analysis) and further compensate for matrix effects (in case that some matrix components may not be eliminated completely by IAC), isotope dilution with stable ^{13}C-labeled IS for each analyte was applied to all sample matrices before sample extraction to improve method's reproducibility, accuracy and robustness.

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 standard and samples. According to regulatory guidance on analytical method validation, at least two structurally specific MS/MS transitions should be used in a LC/MS/MS method.[34-39] In this study, three MS/MS ion pairs were employed for each analyte in the method to assist confidently identify the peaks of interest in the studied samples. For examples, 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.[40] 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.

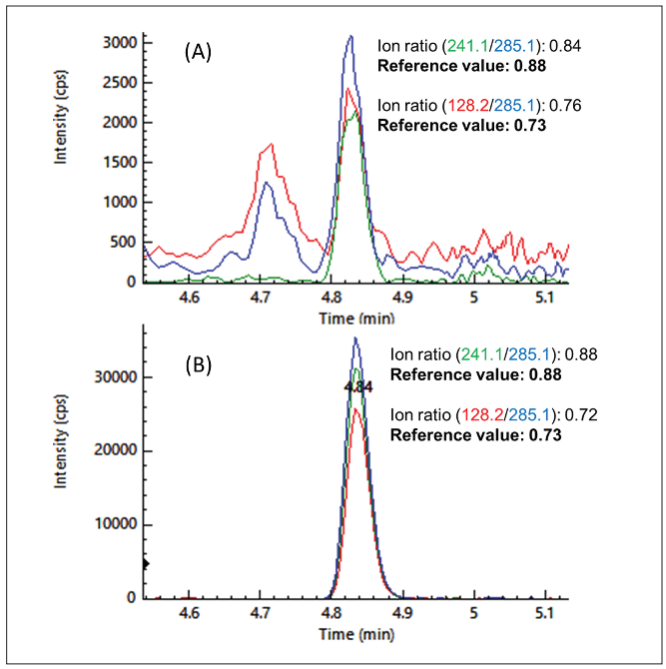

Figure 5. Overlapped three MS/MS chromatograms and their ion ratios of qualifier/quantifier ions for aflatoxin B1 in a peanut butter blank sample prepared by (A) extraction without IAC sample clean-up, and (B) with IAC sample clean-up.

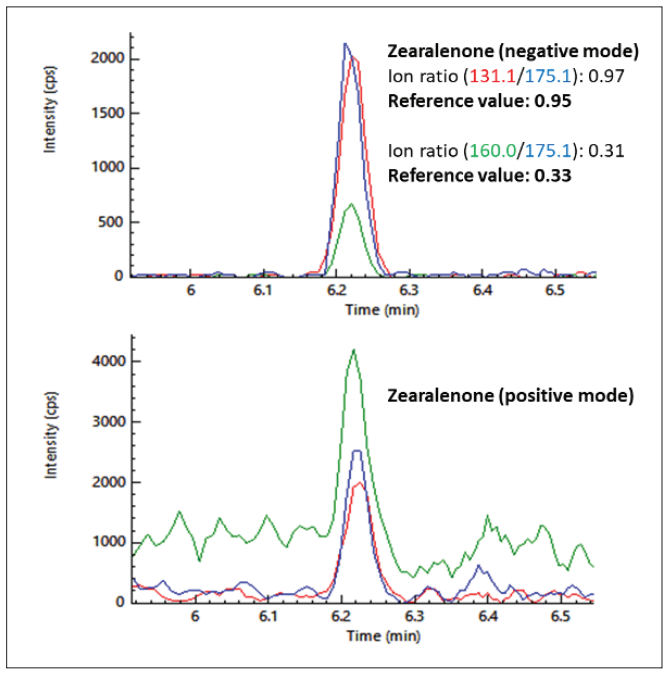

Figure 6. Overlapped three MS/MS chromatograms and their ion ratios of qualifier/quantifier ions of zearalenone at negative mode and at positive mode in a black pepper sample blank.

Eight blank sample matrices were evaluated during method validation. As shown in Figure 7, ochratoxin A (3.92 µg/kg) in a chili powder sample blank was determined and confirmed by comparing its retention time and ion ratios of qualifier ions to quantifier ion with the reference standard. The retention time of the analyte in the sample matches with that of the standard perfectly and the deviations for ion ratios are less than 10%. Similar results were also obtained for aflatoxin B1 (6.26 µg/kg) in a peanut butter sample blank and zearalenone (125 µg/kg) in a black pepper sample blank as illustrated in Figures 5-6, demonstrating good selectivity of the method for mycotoxin analysis. Again, as shown in Figure 8 in a yellow corn blank sample, fumonisins B1 (88.6 µg/kg), B2 (16.7 µg/kg) and B3 (11.1 µg/kg) were confirmed by their retention times and ion ratios of qualifier/quantifier ions, which are consistent with those of reference standard. HT-2 toxin (15.9 µg/kg) and T-2 toxin (7.1 µg/kg) were determined only in an oat cereal sample blank as seen in Figure 9.

The method's sensitivity depends on the instrument sensitivity, sample matrix effects (signal suppression or enhancement) and sample preparation methods (sample dilution factors). In this study, signal suppression or enhancement effects were reduced significantly by IAC sample clean-up steps. However, there were still some matrix effects, which 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 (S/N) 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 difference 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 superior sensitivity of the method for the twelve mycotoxins in all food matrices including baby foods for low level of aflatoxin B1 (0.1 µg/kg) and ochratoxin A (0.5 µg/kg). Figure 10 demonstrates an example of highly sensitive determination of aflatoxin B1 (0.056 µg/kg) in a complex chili powder sample matrix using this IAC-LC/MS/MS method.

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, respectively. For example, good precision was obtained for the six spiked corn samples as demonstrated in Table 5.

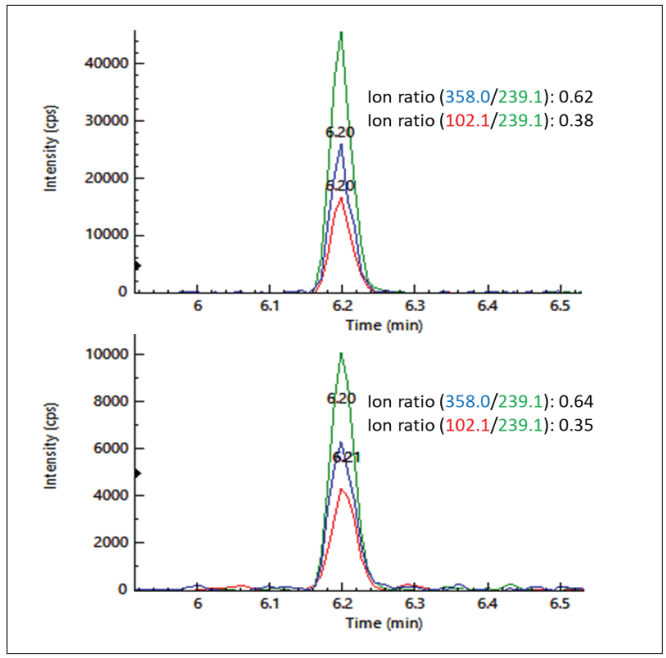

Figure 7. Overlapped three MS/MS chromatograms and their ion ratios of qualifier/quantifier ions of ochratoxin A in a reference standard (top), and in a chili powder sample blank (bottom).

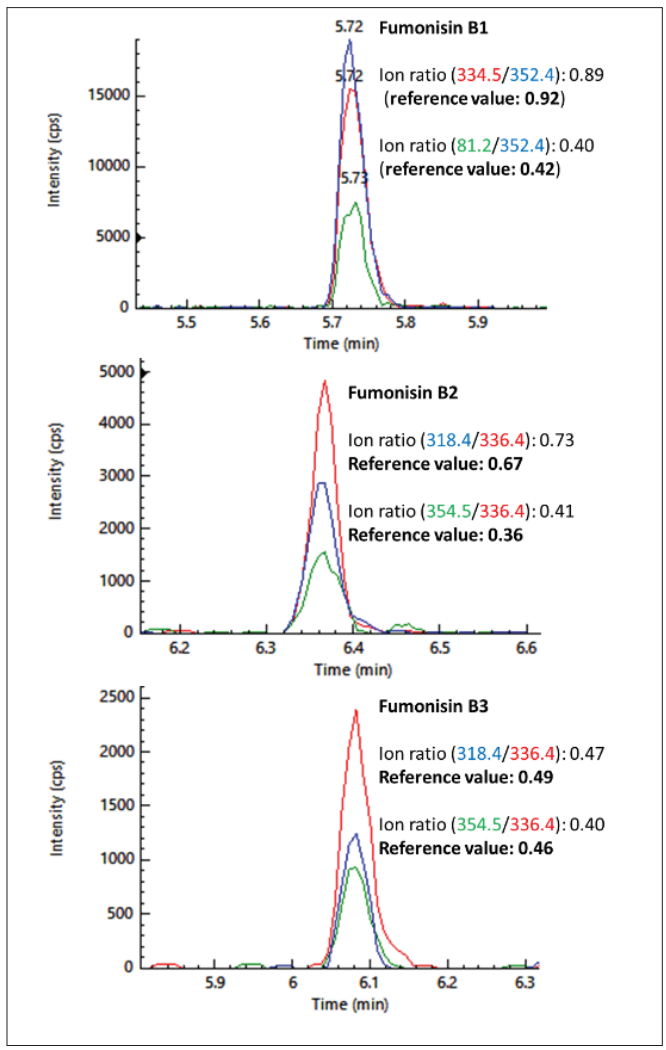

Figure 8. Overlapped three MS/MS chromatograms and their ion ratios of qualifier/quantifier ions of fumonisin B1, fumonisin B2, and fumonisin B3 in a yellow corn sample blank.

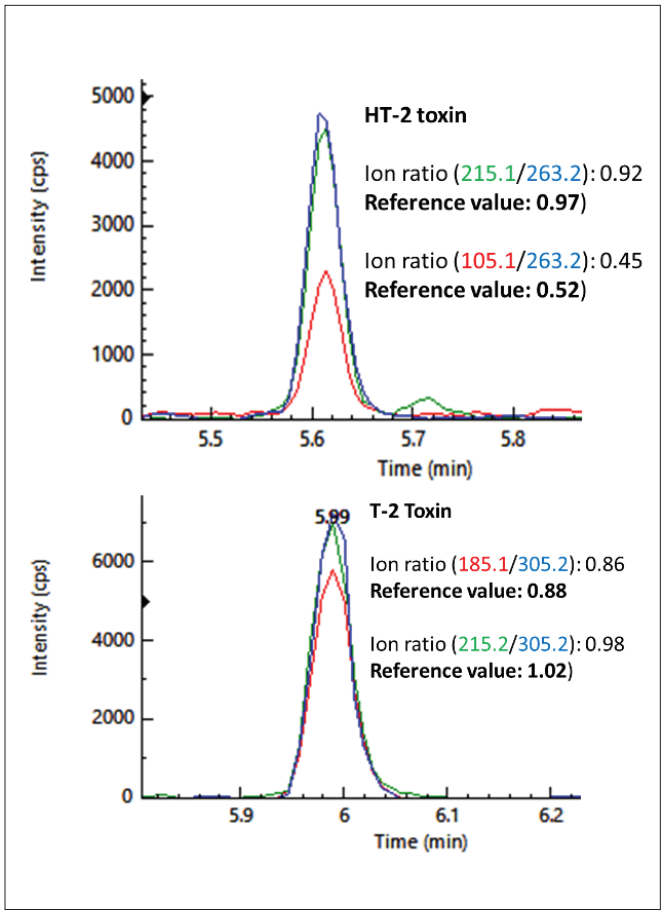

Figure 9. Overlapped three MS/MS chromatograms and their ion ratios of qualifier/quantifier ions for T2-toxin and HT2-toxin in an oat cereal blank sample.

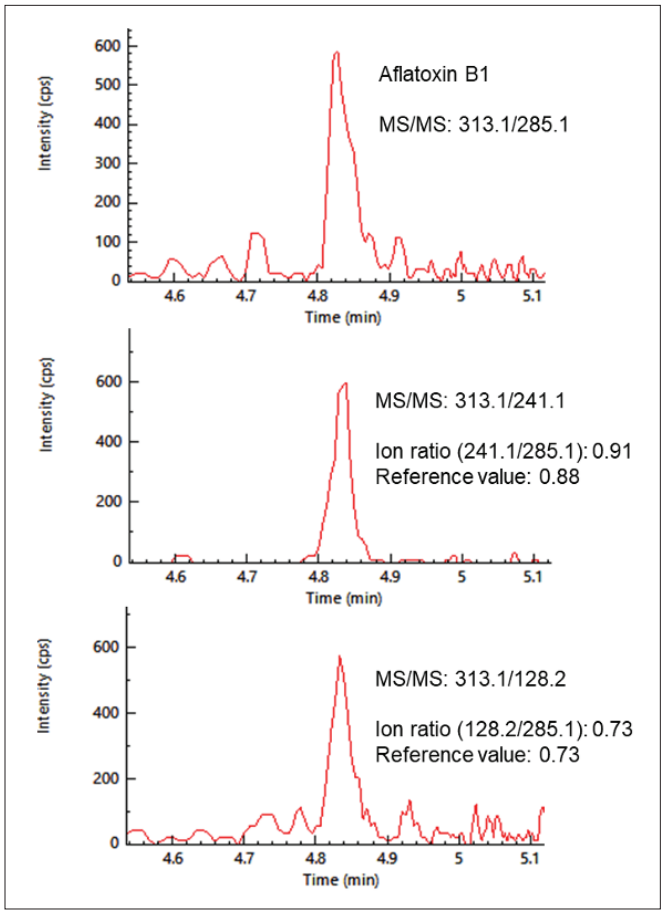

Figure 10. Three MS/MS chromatograms of aflatoxin B1 (0.056 µg/kg) in a chili powder sample.

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	0.02	0.05		2 (0.1 baby food)
Aflatoxin B2	0.02	0.07	20 (total Aflatoxins)	(Aflatoxins B1+ B2+G1 +G2): 4 - 15
Aflatoxin G1	0.02	0.06		
Aflatoxin G2	0.03	0.1		
Ochratoxin A	0.08	0.2 - 0.5	20	3 - 80 (0.5 baby food)
Fumonisin B1	1.0	2	(B1+B2+B3): 2000 - 4000	(B1+B2): 400 - 4000
Fumonisin B2	1.0	2		(B1+B2): 200 baby food
Fumonisin B3	1.0	3		
Deoxynivalenol	2 - 3	5 - 6	1000	500 - 1750 (200 baby food)
Zearalenone	0.5-2	1.5 - 6		50 - 400 (20 baby food)
HT-2 Toxin	1	3		(HT-2 + T-2): 25 - 1000
T-2 Toxin	0.3	0.6		(HT-2 + T-2): 20 baby food

* see reference 8; ** see references 4-7.

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. 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 6 and 7, the recoveries for most analytes from the spiked LFM samples were between 80% and 120%, demonstrating good accuracy of the methods.

Table 5. Recovery and Precision Obtained for Spiked Corn Samples by IAC-LC/MS/MS.

Analyte	Spiked	Analyte Recovery from Sample (%), Standard Deviation (SD) and RSD (%)								
	(µg/kg)	Corn S1	Corn S2	Corn S3	Corn S4	Corn S5	Corn S6	Average	SD	RSD
Aflatoxin B1	1	90.8	92.4	86.9	97.0	92.8	107	92.0	3.6	3.9
Aflatoxin B2	1	98.5	77.7	83.6	94.4	77.0	81.8	86.2	9.8	11.3
Aflatoxin G1	1	82.3	90.8	108	99.2	94.3	103	94.9	9.5	10.1
Aflatoxin G2	1	95.7	95.3	88.1	92.7	82.8	84.7	90.9	5.5	6.0
Ochratoxin A	2	81.9	112	98.0	85.6	82.2	111	91.9	12.9	14.0
Fumonisin B1	100	96.9	107	101	109	105	98.3	104	4.9	4.7
Fumonisin B2	100	101	94.9	110	112	103	99.6	104	6.8	6.6
Fumonisin B3	100	98.1	90.3	112	111	100	96.3	102	9.2	9.0
Deoxynivalenol	100	106	97.1	96.7	97.4	97.1	114	98.8	3.8	3.9
Zearalenone	30	116	109	107	103	105	118	108	5.0	4.6
HT-2 Toxin	100	86.5	88.5	84.6	91.0	86.5	84.4	87.4	2.4	2.8
T-2 Toxin	10	103	88.8	92.9	96.2	84.3	89.3	92.9	7.0	7.5

Table 6. 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	Black Pepper	Chili Powder
Aflatoxin B1	1	94.5	112	91.1	102	101	91.0	120	102
Aflatoxin B2	1	85.5	105	86.9	86.8	89.1	89.6	102	107
Aflatoxin G1	1	96.2	103	90.4	97.9	101	104	101	99.3
Aflatoxin G2	1	89.9	105	88.5	86.0	88.5	96.7	95.6	98.2
Ochratoxin A	2	95.0	95.4	91.4	88.9	100	105	97.5	96.1
Fumonisin B1	100	103	95.5	109	95.2	95.2	98.6	101	104
Fumonisin B2	100	104	112	103	100	96.5	96.8	94.2	105
Fumonisin B3	100	101	114	110	95.0	95.1	104	97.9	105
Deoxynivalenol	100	101	76.6	103	94.0	115	97.7	97.4	95.8
Zearalenone	30	110	92.2	94.0	102	112	105	85.1	105
HT-2 Toxin	100	86.9	86.1	82.7	103	92.4	104	99.9	102
T-2 Toxin	10	92.3	100	99.9	94.1	105	103	104	111

Table 7. 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	Black Pepper	Chili Powder
Aflatoxin B1	5	98.0	88.2	97.3	97.7	114	93.0	103	103
Aflatoxin B2	5	93.3	86.8	95.6	87.3	112	105	107	103
Aflatoxin G1	5	104	88.3	104	98.3	117	103	98.7	102
Aflatoxin G2	5	91.2	87.7	97.2	93.4	110	109	103	105
Ochratoxin A	10	97.6	90.9	95.2	90.4	116	101	83.2	96.3
Fumonisin B1	250	90.6	108	109	95.0	129	97.6	97.4	94.7
Fumonisin B2	250	107	115	113	100	123	104	104	106
Fumonisin B3	250	98.0	115	118	99.6	126	102	103	100
Deoxynivalenol	250	98.5	95.9	101	95.7	113	97.7	99.9	96.0
Zearalenone	75	101	99.0	99.8	112	117	108	95.2	111
HT-2 Toxin	250	85.4	92.3	91.5	96.7	102	102	101	99.1
T-2 Toxin	25	90.4	110	107	104	115	105	104	106

Table 8. Deoxynivalenol Contents and Ion Ratios of Qualifier Ions to Quantifier Ion for the Studied Food Samples.

Sample ID	Content	Ion Ratio of Qualifier/Quantifier	
	(µg/kg)	231.2/249.2	77.0/249.2
Yellow Corn	23.6	0.53	0.38
White Corn	40.4	0.51	0.43
Wheat	42.3	0.56	0.43
Soybean	21.6	0.48	0.41
Oat Cereal	22.0	0.50	0.39
Almond	11.6	0.50	0.40
Peanut Butter	10.2	0.54	0.39
Black Pepper	10.8	0.53	0.38
Chili Powder	8.51	0.47	0.35
Reference Standard		0.50	0.36

Determination of mycotoxins in selected food samples

The validated method was successfully applied to the quantification and confirmation of the targeted mycotoxins in nine different commercial food products including soybean, wheat, almond, oat breakfast cereal, peanut butter, white corn, yellow corn, black pepper, red chili powder samples. All the mycotoxins determined in this study are below the regulated maximum levels (MLs) except for aflatoxin B1 in a peanut butter sample. A high amount of aflatoxin B1 (6.26 µg/kg) and a small amount of aflatoxin B2 (0.834 µg/kg) were determined in this peanut butter sample. Trace amount of aflatoxin B1 were also determined in a black pepper sample (0.186 µg/kg) and in a chili powder sample (0.056 µg/kg). Various amounts of deoxynivalenol (DON) were determined in the nine studied samples as listed in Table 8. Zearalenone (ZEN) was also found in all the samples at low concentrations (ranging from 2 to 6 µg/kg) except for a black pepper sample, which contained a high amount of ZEN at about 125 µg/kg. Fumonisins (FB1, FB2, and FB3) were found only in corn (maize) samples because they are mainly produced by Fusarium verticillioides, a fungus predominant on maize and maize-based products.[41] The yellow corn sample contained FB1 (88.6 µg/kg), FB2 (16.7 µg/kg), and FB3 (11.1 µg/kg), respectively, and the white corn sample contained FB1(15.2 µg/kg), and FB2 (3.94 µg/kg). The HT-2 toxin (15.9 µg/kg) and T-2 toxin (7.1 µg/kg) were determined only in an oat cereal sample. Ochratoxin A (3.92 µg/kg) was found only in a chili powder sample. The confirmation and identification of these mycotoxins in these food samples were carried out by comparing the retention time and peak area ratio of qualifier ion to quantifier ion peaks of the analyte between samples and reference standard (refer to Table 8 and Figures 5-10) and all the deviations from reference standards (deviations of retention time <2%, and deviation of ion ratio < 15%) are within limits established by the European Union guidance[37-39]

Conclusions

In this study, a multianalyte UHPLC/MS/MS method has been developed and validated for the reliable confirmation and quantification of twelve mycotoxins in various food matrices. All the mycotoxins with very different physicochemical properties can be determined simultaneously in a single chromatographic run in eleven minutes. Immunoaffinity column (IAC) sample clean-up can significantly improve data quality by effectively removing matrix interfering components from samples and enhancing the sensitivity with analyte concentrations. By coupling IAC sample treatment with the highly sensitive and selective QSight LC/MS/MS, it is possible to determine low levels of mycotoxins accurately from complex food matrices.

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

The method has been validated in eight different food matrices (maize, wheat, soybean, oat cereal, almond, peanut better, red chili and black pepper) with good sensitivity, selectivity, accuracy and precision for all the analyte/ matrix combinations, and therefore can be used in routine testing laboratories to meet all regulatory requirements.

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