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Multi-mycotoxin Analysis of Finished Grain and Nut Products Using High-Performance Liquid Chromatography–Triple-Quadrupole Mass Spectrometry

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Supporting Information

ABSTRACT: Mycotoxins in foods have long been recognized as potential health hazards due to their toxic and carcinogenic properties. A simple and rapid method was developed to detect 26 mycotoxins (aflatoxins, ochratoxins, fumonisins, trichothecenes, and ergot alkaloids) in corn, rice, wheat, almond, peanut, and pistachio products using high-performance liquid chromatography–triple-quadrupole mass spectrometry. Test portions of homogenized grain or nut products were extracted with acetonitrile/water (85:15, v/v), followed by high-speed centrifugation and dilution with water. Mean recoveries (± standard deviations) were 84 ± 6, 89 ± 6, 97 ± 9, 87 ± 12, 104 ± 16, and 92 ± 18% from corn, rice, wheat, almond, peanut, and pistachio products, respectively, and the matrix-dependent instrument quantitation limits ranged from 0.2 to 12.8 μg/kg, depending on the mycotoxin. Matrix effects, as measured by the slope ratios of matrix-matched and solvent-only calibration curves, revealed primarily suppression and were more pronounced in nuts than in grains. The measured mycotoxin concentrations in 11 corn and wheat reference materials were not different from the certified concentrations. Nineteen mycotoxins were identified and measured in 35 of 70 commercial grain and nut products, ranging from 0.3 ± 0.1 μg/kg (aflatoxin B_1 in peanuts) to 1143 ± 87 μg/kg (fumonisin B_1 in corn flour). This rapid and efficient method was shown to be rugged and effective for the multiresidue analysis of mycotoxins in finished grain and nut products.

KEYWORDS: *mycotoxins, LC-MS/MS, multi-mycotoxin analysis, finished cereal and nut products*

INTRODUCTION

Mycotoxins are natural toxic contaminants found in foods and are produced as secondary metabolites from various molds or filamentous fungi species of the genus *Aspergillus* (aflatoxins and ochratoxin A), *Claviceps* (ergot alkaloids), *Fusarium* (trichothecenes, beauvericin, fumonisins, and zearalenone), *Penicillum* (citrinin), and *Alternaria* (alternariol), among others.[1−3] Many factors such as temperature, humidity, and insect damage in agricultural crops influence mold growth, resulting in the production and presence of these toxic compounds in foods, beverages, and animal feeds. Mycotoxins in foods and feeds are important health concerns because these chemical contaminants are stable, resistant to decomposition, and, depending on the exposure, pose human health hazards such as carcinogenicity, neurotoxicity, immunotoxicity, and reproductive and developmental toxicity.[4−6] On the basis of estimates that one-fourth of the world's agricultural commodities (cereal grains, nut crops, fresh produce, dairy products, etc.) are contaminated with mycotoxins, strategies need to be developed to monitor and limit their presence in the food supply. [6]

The frequent occurrence of aflatoxins, deoxynivalenol, fumonisins, ochratoxin A, and zearalenone in foods and feeds has resulted in the establishment of maximum residue concentrations for these mycotoxin species by government and international agencies, such as the U.S. Food and Drug Administration and the European Union.[7−9] Effective and efficient analytical methods are required to identify and quantitate mycotoxins at the low parts per billion (μg/kg, i.e., 2−10 μg/kg total aflatoxins and 3−5 μg/kg ochratoxin in grains) to parts per million (i.e, ≥1000 μg/kg total fumonisins in grains) concentrations to assess standardization and enforce regulatory limits.[9] The common laboratory testing of mycotoxins in cereal grains, finished grain products, and nuts is based on solvent extraction of the analytes, solid-phase extraction, or immunoaffinity cleanup of samples[10] followed by instrumental analyses. Such instrumental analysis includes thin layer chromatography,[11,12] derivatization/capillary gas chromatography,[13−15] high-performance liquid chromatography (HPLC) coupled with postcolumn derivatization/fluorescence or ultraviolet detection,[16−20] and enzyme-linked

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immunosorbent or immunoaffinity-based assays and techniques.[21−27] These analytical techniques are effective for measuring specific classes of mycotoxins in various food types, but they can either be labor- and/or time-intensive or lack the sensitivity and selectivity for effective and efficient screening. Multiresidue methods are preferable because several mycotoxins frequently occur in the same food product.

In the past few years, liquid chromatography coupled with mass spectrometry (LC-MS) has been an effective tool for the analysis of a wide range of chemical contaminants, such as pesticides, veterinary drugs, organic pollutants, and animal and plant toxins, including mycotoxins in various raw and finished food products.[28−37] Triple-quadrupole mass spectrometry (MS/MS) is recognized as a sensitive, selective, and specific mass spectrometric technique for targeted contaminants in complex food matrices. The potential for the simultaneous quantitation and identification of all mycotoxins of interest in a single LC-MS/MS procedure using two precursor-to-product ion transitions per mycotoxin for different agricultural commodities is achievable. Before this goal can be realized, the challenges for the development of an effective, rugged, and robust multi-mycotoxin LC-MS/MS method must be resolved, such as the accommodation of a wide range of physical−chemical properties, such as polarity and solubility, exhibited by mycotoxin species. A drawback to LC-MS-based methods is the high capital costs for equipment and hardware accessories compared to traditional mycotoxin procedures. Therefore, optimization of effective and efficient extraction, isolation, chromatographic separation, ionization, and mass spectrometric detection conditions would favor this capital-intensive approach, allowing for a cost-effective method for the analysis of several mycotoxins in a single procedure.

The aim of this study is to develop a simple, reliable, and validated multi-mycotoxin LC-MS/MS method for the simultaneous determination of 26 common mycotoxins, including the major classes such as trichothecenes, aflatoxins, ochratoxins, fumonisins, and ergot alkaloids, for enforcement of tolerance levels in finished grain and nut products sold in the United States. The performance and practical applicability of the validated method were also evaluated by analyzing reference grain materials and by screening mycotoxins in 70 commercially available finished grain and nut products.

MATERIALS AND METHODS

Chemicals and Materials. Aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, beauvericin, deoxynivalenol, diacetoxyscirpenol, 15-acetyldeoxynivalenol, ergot alkaloids (ergocornine, ercocristine, ergocryptine, ergometrine, ergosine, ergotamine), fumonisin B_1, fumonisin B_2, fusarenon-X, HT-2 toxin, neosolaniol, ochratoxin A, ochratoxin B, T-2 toxin, and zearalenone standards were purchased in neat form from Romer Labs, Inc. (Union, MO, USA). Citrinin, sterigmatocystin, and verrucarin A standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Stable isotope-labeled internal standards, $^{13}C_{17}$-aflatoxin B_1 (0.5 μg/mL) $^{13}C_{20}$-ochratoxin A (10 μg/mL), $^{13}C_{18}$-zearalenone (25 μg/mL), $^{13}C_{34}$-fumonisin B_1 (25 μg/mL), and $^{13}C_{24}$-T-2 toxin (25 μg/mL), and two reference materials (aflatoxin in maize and zearalenone in maize) were also purchased from Romer Labs, Inc. Nine reference materials (aflatoxin, deoxynivalenol, and zearalenone in corn and wheat) were generously provided from the U.S. Department of Agriculture, Grain Inspection, Packers and Stockyards Administration, Technology and Science Division (USDA-GIPSA-TSD, Kansas City, MO, USA). Blank rice, wheat, corn, peanut, pistachio, and almond samples and finished grain (i.e., pasta) and nut (i.e., peanut butter) products were purchased from commercially available sources.

LC grade acetonitrile, methanol, and water and MS grade formic acid and ammonium formate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Plastic syringes (3 mL) and 13 mm × 0.2 μm PTFE syringe filters were purchased from Pall Life Sciences (Ann Arbor, MI, USA).

Standards Preparation. Stock standard solutions (200 μg/mL) of each of the 26 mycotoxin standards were prepared by dissolving 5.0 mg of the mycotoxin in 25 mL of acetonitrile. Because of the different detection limits and maximum tolerance levels of various mycotoxins in different agricultural commodities,[7−9] two working standard solutions were prepared, groups A and B. The working standard for group A, consisting of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, beauvericin, diacetoxyscirpenol, ergot alkaloids, HT-2 toxin, neosolaniol, ochratoxin A, ochratoxin B, sterigmatocystin, T-2 toxin, and verrucarin A, was prepared to a concentration of 1.0 μg/mL by preparing individual 20 μg/mL solutions and transferring 0.5 mL of the diluted individual stock standard solutions to a 10 mL volumetric flask and bringing it up to volume with acetonitrile/water (50:50, v/v). The group B working standard, consisting of citrinin, deoxynivalenol, 15-acetyldeoxynivalenol, fumonisin B_1, fumonisin B_2, fusarenon-X, and zearalenone, was prepared to a concentration of 10 μg/mL by delivering 0.5 mL of each individual stock standard (200 μg/mL) to a 10 mL volumetric flask and bringing it up to volume with acetonitrile/water (50:50, v/v). Stock solutions and working standard solutions used in the preparation of solventonly calibration standards, matrix-matched calibration standards, and method recovery studies were stored at −20 °C.

Solvent-only calibration standards were prepared from the working standard solutions by diluting the group A and B working standard solutions to 500 and 5000 ng/mL, respectively. Eight solvent-only calibration standards (0.5, 1.0, 2.5, 5.0, 10, 25, 50, and 100 ng/mL for group A; 5.0, 10, 25, 50, 100, 250, 500, and 1000 ng/mL for group B) were prepared by successive dilution of the group A and B mixed working standard solutions with acetonitrile/water (50:50, v/v). The mixed isotope-labeled internal standard solution was prepared by mixing 0.1 mL each of $^{13}C_{17}$-aflatoxin B_1 (0.5 μg/mL), $^{13}C_{20}$-ochratoxin A (2.5 μg/mL), $^{13}C_{18}$-zearalenone (5 μg/mL), $^{13}C_{34}$-fumonisin B_1 (25 μg/mL), and $^{13}C_{24}$-T-2 toxin (5 μg/mL) with 0.5 mL of acetonitrile/water (50:50, v/v).

Sample Preparation. Whole grains and nuts were homogenized with dry ice in a RobotCoupe blender (Ridgeland, MS, USA) until powdery consistencies were obtained. The shells were removed from nuts before grinding. The homogenized samples were transferred to polypropylene freezer bags stored in a −20 °C freezer; the bags were left opened to allow the carbon dioxide to sublime before sealing and then stored until further use.

A volume of 5 mL of extraction solvent (acetonitrile/water, 85:15, v/v) was added to 1.00 ± 0.02 g of ground sample in 15 mL disposable screw-capped polypropylene centrifuge tubes (Corning Inc., Corning, NY, USA). The samples were extracted for 30 min using a high-speed shaker with pulsation (Glas-Col, Terre Haute, IN, USA) using a motor speed setting of 75 (1540−1560 rpm as measured by a DPM5 digital photo tachometer, Universal Enterprises, Inc., Beaverton, OR, USA) and pulser frequency set at the middle mark of the dial (∼30−35 pulsations/min), followed by subsequent centrifugation for 5 min at 4500 rpm (4200*g*; ThermoElectro Corp., Milford, MA, USA). Five hundred microliters of the extract was transferred to a clean test tube, followed by the addition of 20 μL of the internal standard solution consisting of ($^{13}C_{17}$)-aflatoxin B_1, ($^{13}C_{34}$)-fumonisin B_1, ($^{13}C_{20}$)-ochratoxin A, ($^{13}C_{24}$)-T-2, and ($^{13}C_{18}$)-zearalenone) and 480 μL of 20 mM ammonium formate, and the tube was vortexed for 15 s. Samples were filtered through 13 mm × 0.2 μm PTFE syringe filters (Pall Life Sciences) and a 3 mL disposable syringe directly into an autosampler vial (National Scientific, Rockwood, TN, USA).

Recovery Studies. Method recovery samples were prepared in quadruplicates from the homogenized sample matrices at three spiking concentrations (10, 50, and 100 μg/kg for group A; 100, 500, and 1000 μg/kg for group B). Method blank samples were also prepared in each sample batch as quality control samples, as well as used in matrix effect studies. For group A recovery studies, the spiking solution

Table 1. Mycotoxin Information (Name, CAS Registry Number, Molecular Formula, Weight, and Structure), MS/MS Parameters (Precursor and Product Ion, Ion Ratio, Declustering Potential, Collision Energy, and Collision Exit Potential), and Chromatographic Retention Times Used for the Multi-mycotoxin LC-MS/MS Analysis of Finished Grain and Nut Products

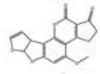
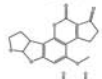
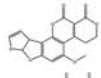
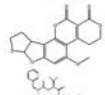
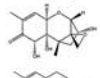
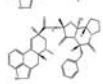
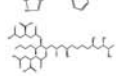
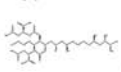
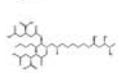
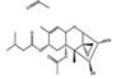
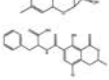
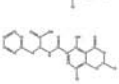
Mycotoxin	CAS Number	Molecular Formula	Molecular Weight	Group	Molecular Structure	Precursor Ion	Product Ions[1]	Ion Ratio (2°/1°)[2]	DP[3]	CE[4]	CXP[5]	Retention Time (min)
15-Acetyl Deoxynivalenol	88337-96-6	$C_{17}H_{22}O_7$	338.35	B		356 $[M+NH_4]^+$	**321**, 137	0.71	41	**19**, 21	**20**, 8	4.5
Aflatoxin B_1	1162-65-8	$C_{17}H_{12}O_6$	312.27	A		313 $[M+H]^+$	**285**, 128	0.93	106	**37**, 101	**8**, 22	5.5
($^{13}C_{17}$)-Aflatoxin B_1	1217449-45-0	$^{13}C_{17}H_{12}O_6$	329.15			330 $[M+H]^+$	**255**, 227	0.30	55	**50**, 60	**5**, 5	5.5
Aflatoxin B_2	7220-81-7	$C_{17}H_{14}O_6$	314.29	A		315 $[M+H]^+$	**287**, 259	0.60	121	**37**, 45	**16**, 46	5.4
Aflatoxin G_1	1165-39-5	$C_{17}H_{12}O_7$	328.28	A		329 $[M+H]^+$	**243**, 200	0.62	106	**41**, 57	**12**, 12	5.2
Aflatoxin G_2	7241-98-7	$C_{17}H_{14}O_7$	330.29	A		331 $[M+H]^+$	**313**, 245	0.39	111	**36**, 49	**18**, 20	5.1
Beauvreicin	26048-05-5	$C_{45}H_{57}N_3O_9$	783.95	A		801 $[M+NH_4]^+$	**244**, 262	0.68	131	**43**, 49	**12**, 14	6.9
Citrinin	518-75-2	$C_{13}H_{14}O_5$	250.25	B		251 $[M+H]^+$	**233**, 205	0.35	71	**37**, 39	**34**, 14	5.3
Deoxynivalenol	51481-10-8	$C_{15}H_{20}O_6$	296.32	B		297 $[M+H]^+$	**249**, 203	0.58	71	**17**, 23	**44**, 10	3.2
Diacetoxyscirpenol	2270-40-8	$C_{19}H_{26}O_7$	366.40	A		384 $[M+NH_4]^+$	**307**, 105	0.48	51	**17**, 57	**8**, 16	5.2
Ergocornine	564-36-3	$C_{31}H_{39}N_5O_5$	561.67	A		562 $[M+H]^+$	544, 223	0.67	71	**21**, 55	**16**, 42	5.6
Ergocristine	511-08-0	$C_{35}H_{39}N_5O_5$	609.71	A		610 $[M+H]^+$	**592**, 223	0.73	121	**21**, 57	**18**, 12	5.8
Ergocryptine	511-09-1	$C_{32}H_{41}N_5O_5$	575.70	A		576 $[M+H]^+$	**223**, 208	0.74	51	**45**, 60	**14**, 15	5.7
Ergometrine	60-79-7	$C_{19}H_{23}N_3O_2$	325.41	A		326 $[M+H]^+$	**223**, 208	0.78	66	**41**, 36	**15**, 14	3.8
Ergosine	561-94-4	$C_{30}H_{37}N_5O_5$	547.65	A		548 $[M+H]^+$	**223**, 208	0.48	86	**55**, 63	**12**, 16	5.5
Ergotamine	113-15-5	$C_{33}H_{35}N_5O_5$	581.66	A		582 $[M+H]^+$	**223**, 208	0.72	116	**49**, 65	**18**, 10	5.6
Fumonisin B_1	116355-83-0	$C_{34}H_{59}NO_{15}$	721.83	B		722 $[M+H]^+$	**352**, 334	0.91	101	**53**, 61	**18**, 26	5.7
($^{13}C_{34}$)-Fumonisin B_1		$^{13}C_{34}H_{59}NO_{15}$	755.58			756 $[M+H]^+$	**356**, 374	0.74	121	**59**, 53	**20**, 10	5.7
Fumonisin B_2	116355-84-1	$C_{34}H_{59}NO_{14}$	705.83	B		706 $[M+H]^+$	**336**, 318	0.39	98	**59**, 50	**9**, 3	6.1
Fusarenon-X	88337-96-6	$C_{17}H_{22}O_7$	338.40	B		355 $[M+H]^+$	**247**, 229	0.62	36	**13**, 15	**18**, 44	3.8
HT-2	26934-87-2	$C_{22}H_{32}O_8$	424.48	A		442 $[M+NH_4]^+$	**263**, 215	0.70	41	**19**, 19	**14**, 18	5.5
Neosolaniol	36519-25-2	$C_{19}H_{26}O_8$	382.40	A		400 $[M+NH_4]^+$	**185**, 215	0.84	81	**25**, 25	**10**, 20	4.1
Ochratoxin A	303-47-9	$C_{20}H_{18}ClNO_6$	403.81	A		404 $[M+H]^+$	**239**, 102	0.61	66	**41**, 101	**16**, 16	6.0
($^{13}C_{20}$)-Ochratoxin A		$^{13}C_{20}H_{18}ClNO_6$	423.67			424 $[M+H]^+$	**250**, 232	0.26	30	**30**, 45	**5**, 5	6.0

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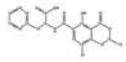
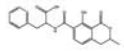
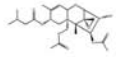
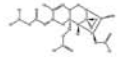
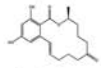
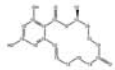
Table 1. continued

Mycotoxin	CAS Number	Molecular Formula	Molecular Weight	Group	Molecular Structure	Precursor Ion	Product Ions[1]	Ion Ratio (2°/1°)[2]	DP[3]	CE[4]	CXP[5]	Retention Time (min)
($^{13}C_{20}$)-Ochratoxin A		$^{13}C_{20}H_{18}ClNO_6$	423.67			424 $[M+H]^+$	**250**, 232	0.26	30	**30**, 45	**5**, 5	6.0
Ochratoxin B	4825-86-9	$C_{20}H_{19}NO_6$	369.37	A		370 $[M+H]^+$	**205**, 103	0.46	51	**31**, 77	**10**, 18	5.8
Sterigmatocystin	10048-13-2	$C_{18}H_{12}O_6$	324.28	A		325 $[M+H]^+$	**281**, 253	0.18	63	**20**, 39	**16**, 25	6.5
T-2	21259-20-1	$C_{24}H_{34}O_9$	466.52	A		484 $[M+NH_4]^+$	**215**, 185	0.90	57	**28**, 30	**17**, 11	5.8
($^{13}C_{24}$)-T-2		$^{13}C_{24}H_{34}O_9$	490.35			508 $[M+NH_4]^+$	**198**, 229	0.93	41	**26**, 30	**3**, 3	5.8
Verrucarin A	3148-09-2	$C_{24}H_{34}O_9$	502.55	B		520 $[M+NH_4]^+$	**249**, 457	0.74	51	**25**, 19	**16**, 12	5.9
Zearalenone	17924-92-4	$C_{18}H_{22}O_5$	318.36	B		319 $[M+H]^+$	**283**, 187	0.61	81	**19**, 27	18, 18	6.2
($^{13}C_{18}$)-Zearalenone		$^{13}C_{18}H_{22}O_5$	336.23			337 $[M+H]^+$	**301**, 199	0.41	106	**19**, 29	**6**, 12	6.2

[1]Primary product ion transition used for quantitation is indicated in **bold**. [2]Ion ratio (2°/1°) determined by area ratios of the primary product (1°) transition by the secondary (2°) product transition. [3]DP, declustering potential. [4]CE, collision energy. [5]CXP, collision cell exit potenti.

volumes of 100 μL (0.1 μg/mL), 50 μL (1 μg/mL), or 100 μL (1 μg/mL) were added to the sample tubes containing 1.00 ± 0.02 g of ground samples to achieve fortification levels of 10, 50, or 100 μg/kg, respectively. For group B recovery studies, spiking solution volumes of 100 μL (1 μg/mL), 50 μL (10 μg/mL), or 100 μL (10 μg/mL) were added into sample tubes containing 1.00 ± 0.02 g of ground samples to achieve fortification levels of 100, 500, or 1000 μg/kg, respectively. After the addition of 5 mL of extraction solvent (acetonitrile/water, 85:15, v/v), the sample tubes were placed on the high-speed shaker and extracted as described in the previous section. The availability of stable ^{13}C-isotope-labeled internal standards allowed for the quantitation of aflatoxin B_1, fumonisin B_1, HT-2, ochratoxin A, and zearalenone to be determined by using the peak area ratio of responses of the native analytes to that of their corresponding labeled internal standards. For those native compounds without labeled internal standards, quantitation was performed by the external standard method by determining the concentration of the analyte using a calibration curve based on the peak areas of matrix-matched calibration standards. Matrix-matched standards were prepared by extracting rice, wheat, corn, peanut, pistachio, and almond blanks (as described above) and fortifying the extracts with the calibration standards to the appropriate concentrations. Calibration curves consisted of the eight calibration standards from the two mycotoxin groups (A and B) and were constructed using least-squares regression.

Analysis of Reference Materials and Commercial Products. Reference materials obtained from Romer Labs and USDA-GIPSA-TSD and commercial finished grain and nut products were prepared in triplicates using procedures described under Sample Preparation. Whole samples were homogenized, whereas finished commercial products that were already in homogeneous form were used as-is. The samples were quantitated using matrix-matched standards using the internal or external methods from matrices that were screened and determined to be free of the mycotoxin of interest.

Limits of Detection and Quantitation Studies. The matrix-dependent instrument detection (MD-IDL) and quantitation (MD-IQL) limits for each analyte were obtained by using procedures from the U.S. Environmental Protection Agency's (EPA) protocol.[38] This was achieved by applying the sample preparation method, which involved the extraction, centrifugation, and dilution steps, to analyte-free matrices free of the mycotoxins (i.e., corn, wheat, rice, almond, peanut, and pistachio samples free of the mycotoxins) and fortifying at concentrations near the LOD levels (levels that generate a 3:1 signal-to-noise response).

The MD-IDL of each mycotoxin was determined on the basis of replicate (n = 8) analysis at the lowest concentration of a matrix-matched calibration standard that is statistically different from the matrix-matched blank and multiplying the standard deviation by 2.998 (critical $t_{0.010}$ = 2.998 for degree of freedom (d_f) of 7). The MD-IQL of each mycotoxin was calculated by multiplying the MD-IDL value by 3.3.

LC-MS/MS Analysis. A Shimadzu Prominence/20 series (Columbia, MD, USA) liquid chromatograph coupled with an Applied Biosystems (Foster City, CA, USA) 4000 Qtrap mass spectrometer equipped with an electrospray ionization (ESI) interface source were employed for all sample analyses using LC-ESI-MS/MS. Scheduled multiple reaction monitoring (sMRM) data were acquired and processed for all compounds in positive ion mode. Nitrogen gas of 99% purity generated from a nitrogen generator (Parker Balston, Haverhill, MA, USA) was used in the ESI source and the collision cell. Identification of target mycotoxins was performed using two specific MRM transitions for each mycotoxin according to the European Commission (EC) criteria.[39] Quantification was carried out using matrix-matched calibration curves, and isotope-labeled internal standards were used for aflatoxin B_1, ochratoxin A, zearalenone, fumonisin B_1, and T-2 toxin, whereas the other mycotoxins were quantitated using the external standard method. A Restek LC column (Bellefonte, PA, USA; Ultra Aqueous, C-18, 100 mm × 2.1 mm i.d., 3 μm) and a 10 mm × 2.1 mm guard cartridge were used for analysis. The solvent systems used were (A) HPLC grade water containing 0.1% formic acid and 10 mM ammonium formate and (B) HPLC grade methanol containing 0.1% formic acid and 10 mM ammonium formate. After an initial time of 1 min at 90% A, the proportion of B was increased linearly to 100% in the following 6 min, followed by a hold time of 3 min at 100% B. The mobile phase was returned to the initial conditions in 0.01 min, and the column was equilibrated for 5 min. The total chromatographic time was 15 min. The column temperature was set at 40 °C, the flow rate was 0.5 mL/min, and the injection volume was set at 10 μL.

Data Analysis. Mycotoxin concentrations from LC-MS/MS analysis were determined using Analyst software version 1.5 (Applied Biosystems). The data were exported to Microsoft Excel 2007 (Microsoft Co., Redmond, WA, USA) to determine average, standard deviation (SD), and relative standard deviation (RSD) values and to perform statistical (ANOVA) analysis.

Table 2. Average Recoveries (n = 4) and Relative Standard Deviations (RSDs) of 26 Mycotoxins Extracted from 6 Matrices (3 Grain Products (Corn, Rice, and Wheat) and 3 Nut Products (Almond, Peanut, and Pistachio))[a]

	average recovery, % (RSDs, %)																	
	rice fortification levels, μg/kg			wheat fortification levels, μg/kg			corn fortification levels, μg/kg			peanut fortification levels, μg/kg			pistachio fortification evels, μg/kg			almond fortification levels, μg/kg		
group A	10	50	100	10	50	100	10	50	100	10	50	100	10	50	100	10	50	100
aflatoxin B_1	94 (4)	96 (7)	99 (3)	89 (7)	114 (4)	104 (4)	83 (7)	88 (10)	94 (7)	92 (15)	110 (11)	121 (5)	111 (9)	91 (16)	106 (22)	104 (8)	111 (10)	114 (11)
aflatoxin B_2	107 (5)	98 (4)	93 (3)	98 (7)	86 (5)	90 (10)	81 (9)	95 (12)	86 (9)	77 (18)	117 (8)	111 (6)	78 (8)	105 (14)	104 (11)	84 (10)	91 (10)	79 (15)
aflatoxin G_1	79 (7)	94 (9)	92 (8)	91 (7)	111 (9)	108 (4)	73 (10)	97 (12)	95 (5)	105 (12)	126 (9)	113 (9)	93 (11)	112 (12)	111 (7)	91 (6)	80 (11)	87 (15)
aflatoxin G_2	110 (7)	88 (7)	95 (7)	101 (8)	109 (4)	112 (9)	83 (8)	93 (9)	92 (11)	78 (12)	106 (7)	122 (7)	74 (8)	112 (10)	102 (11)	91 (14)	88 (10)	89 (11)
beauvericin	113 (5)	87 (9)	95 (7)	105 (9)	102 (8)	95 (5)	103 (4)	93 (8)	91 (8)	102 (13)	104 (15)	114 (8)	71 (9)	82 (10)	94 (8)	89 (12)	89 (13)	90 (7)
diacetoxyscirpenol	84 (10)	85 (5)	88 (6)	83 (9)	112 (4)	103 (6)	77 (10)	78 (5)	88 (7)	91 (21)	114 (15)	115 (10)	86 (17)	118 (12)	116 (7)	88 (18)	93 (13)	100 (10)
ergocornine	83 (6)	89 (7)	81 (8)	82 (11)	95 (6)	80 (7)	88 (11)	94 (10)	82 (9)	112 (7)	122 (14)	108 (7)	72 (11)	119 (14)	114 (11)	72 (17)	91 (17)	98 (13)
ergocristine	81 (11)	83 (4)	97 (5)	95 (6)	112 (8)	105 (6)	86 (11)	87 (11)	88 (8)	111 (9)	111 (9)	126 (15)	72 (12)	100 (18)	93 (12)	90 (9)	83 (15)	84 (16)
ergocryptine	85 (13)	88 (8)	95 (6)	92 (12)	82 (6)	80 (6)	84 (9)	87 (14)	75 (9)	108 (3)	109 (19)	125 (13)	76 (12)	106 (8)	96 (12)	78 (18)	95 (7)	89 (15)
ergometrine	95 (6)	86 (5)	90 (7)	92 (8)	94 (6)	100 (6)	77 (7)	80 (7)	81 (6)	95 (12)	95 (7)	128 (9)	67 (11)	81 (10)	85 (5)	77 (7)	77 (19)	80 (12)
ergosine	83 (10)	81 (9)	84 (8)	85 (7)	90 (9)	86 (9)	81 (10)	81 (9)	79 (9)	112 (17)	116 (13)	129 (18)	85 (4)	113 (6)	114 (9)	77 (11)	108 (15)	108 (4)
ergotamine	84 (12)	78 (11)	78 (4)	90 (8)	106 (6)	117 (6)	78 (11)	91 (9)	80 (11)	103 (14)	117 (12)	101 (12)	68 (8)	109 (15)	98 (14)	77 (11)	98 (11)	92 (14)
HT-2	98 (13)	81 (6)	99 (5)	96 (10)	95 (9)	108 (6)	87 (12)	91 (13)	88 (7)	99 (16)	61 (7)	76 (15)	79 (15)	76 (19)	80 (12)	111 (11)	68 (10)	71 (14)
neosolaniol	83 (6)	76 (4)	79 (8)	84 (7)	115 (10)	108 (5)	76 (4)	82 (6)	75 (7)	109 (11)	128 (10)	132 (12)	68 (14)	78 (16)	79 (15)	94 (16)	86 (5)	84 (11)
ochratoxin A	110 (5)	86 (2)	89 (7)	101 (7)	107 (5)	109 (7)	79 (9)	93 (6)	89 (8)	81 (14)	79 (8)	117 (10)	123 (10)	102 (12)	81 (5)	95 (15)	98 (6)	99 (11)
ochratoxin B	86 (12)	80 (10)	81 (9)	93 (9)	117 (5)	90 (8)	87 (4)	85 (8)	85 (11)	125 (12)	118 (11)	132 (11)	78 (10)	87 (22)	84 (23)	94 (18)	87 (11)	81 (16)
sterigmatocystin	108 (5)	91 (6)	85 (5)	105 (6)	114 (5)	111 (5)	92 (5)	89 (6)	77 (9)	103 (6)	97 (6)	131 (10)	77 (7)	78 (16)	77 (19)	88 (6)	87 (7)	82 (9)
T-2	92 (5)	81 (8)	92 (6)	89 (6)	111 (7)	98 (5)	73 (7)	88 (9)	84 (12)	128 (16)	105 (13)	132 (13)	77 (16)	81 (8)	103 (10)	85 (5)	81 (13)	88 (12)

	average recovery, % (RSDs, %)																	
	rice fortification levels, μg/kg			wheat fortification levels, μg/kg			corn fortification levels, μg/kg			peanut fortification levels, μg/kg			pistachio fortification levels, μg/kg			almond fortification levels, μg/kg		
group B	100	500	1000	100	500	1000	100	500	1000	100	500	1000	100	500	1000	100	500	1000
15-acetyldeoxynivalenol	116 (3)	84 (4)	81 (4)	108 (1)	103 (9)	94 (6)	107 (2)	81 (6)	77 (8)	75 (14)	132 (8)	116 (9)	129 (19)	121 (13)	100 (11)	72 (16)	71 (9)	80 (10)
citrinin	95 (9)	86 (9)	89 (5)	87 (6)	106 (4)	90 (6)	79 (11)	79 (6)	84 (8)	82 (20)	122 (12)	111 (14)	113 (10)	81 (18)	95 (11)	121 (12)	113 (9)	109 (7)
deoxynivalenol	76 (9)	80 (9)	84 (4)	77 (5)	97 (7)	89 (6)	77 (11)	80 (10)	77 (9)	106 (11)	124 (7)	101 (8)	114 (17)	95 (14)	102 (16)	103 (16)	72 (17)	82 (9)
fumonisin B_2	78 (3)	87 (4)	85 (6)	80 (4)	77 (7)	71 (13)	73 (6)	79 (11)	70 (14)	70 (11)	63 (7)	73 (15)	45 (16)	52 (11)	60 (11)	62 (8)	69 (6)	67 (8)
fumonisin B_1	79 (5)	86 (10)	82 (8)	79 (7)	73 (12)	79 (17)	66 (3)	68 (11)	72 (16)	69 (6)	54 (5)	52 (13)	42 (15)	46 (14)	49 (10)	59 (20)	57 (10)	53 (15)
fusarenon-X	93 (8)	78 (7)	103 (6)	89 (13)	93 (10)	103 (7)	89 (12)	81 (11)	92 (10)	123 (16)	117 (16)	74 (16)	125 (20)	143 (10)	110 (18)	125 (7)	90 (16)	114 (9)
verrucarin A	74 (7)	98 (5)	78 (8)	82 (9)	96 (6)	114 (7)	76 (9)	87 (10)	76 (12)	96 (16)	83 (11)	88 (10)	83 (17)	85 (6)	94 (12)	76 (10)	82 (17)	78 (15)
zearalenone	111 (4)	87 (6)	91 (3)	93 (8)	108 (8)	111 (5)	81 (11)	82 (6)	82 (9)	84 (11)	103 (9)	117 (6)	109 (13)	123 (14)	95 (6)	87 (6)	83 (9)	82 (14)

[a]The recoveries were the average of three spiking levels (10, 50, and 100 μg/kg for group A; 100, 500, and 1000 μg/kg for group B). Quantification was performed using matrix-matched calibration.

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Table 3. Matrix-Dependent Instrument Limits of Detection and Limits of Quantitation (in Parentheses) of Mycotoxins Fortified in Six Matrices (Corn, Rice, Wheat, Almond, Peanut, and Pistachio)

	matrix limit of detection (and quantitation), μg/kg					
mycotoxin	corn	rice	wheat	almond	peanut	pistachio
15-acetyldeoxynivalenol	3.1 (10.2)	3.4 (11.3)	3.2 (10.5)	8.1 (10.3)	3.1 (10.1)	3.9 (12.8)
aflatoxin B_1	0.1 (0.3)	0.1 (0.3)	0.1 (0.4)	0.1 (0.3)	0.1 (0.3)	0.1 (0.4)
aflatoxin B_2	0.2 (0.7)	0.2 (0.5)	0.2 (0.7)	0.1 (0.2)	0.1 (0.4)	0.1 (0.3)
aflatoxin G_1	0.2 (0.8)	0.2 (0.8)	0.3 (1.0)	0.1 (0.2)	0.1 (0.3)	0.1 (0.3)
aflatoxin G_2	0.3 (0.9)	0.2 (0.8)	0.3 (0.9)	0.1 (0.2)	0.2 (0.5)	0.2 (0.7)
beauvreicin	0.1 (0.2)	0.1 (0.2)	0.1 (0.2)	0.2 (0.7)	0.2 (0.6)	0.2 (0.7)
citrinin	2.2 (7.1)	2.5 (8.2)	2.3 (7.7)	2.6 (8.6)	2.7 (8.9)	2.5 (8.3)
deoxynivalenol	3.1 (10.1)	3.1 (10.1)	3.2 (10.4)	3.1 (10.2)	3.2 (10.6)	3.4 (11.3)
diacetoxyscirpenol	0.2 (0.7)	0.2 (0.7)	0.2 (0.7)	0.3 (0.9)	0.3 (0.8)	0.3 (0.8)
ergocornine	0.2 (0.7)	0.2 (0.6)	0.1 (0.5)	0.2 (0.7)	0.3 (0.8)	0.2 (0.7)
ergocristine	0.3 (0.9)	0.2 (0.8)	0.2 (0.8)	0.2 (0.7)	0.3 (0.9	0.2 (0.7)
ergocryptine	0.2 (0.7)	0.3 (0.9)	0.2 (0.7)	0.2 (0.7)	0.3 (0.9)	0.3 (0.8)
ergometrine	0.1 (0.5)	0.1 (0.4)	0.1 (0.4)	0.2 (0.7)	0.3 (0.9)	0.2 (0.7)
ergosine	0.2 (0.5)	0.1 (0.4)	0.1 (0.3)	0.2 (0.6)	0.2 (0.5)	0.1 (0.4)
ergotamine	0.2 (0.8)	0.2 (0.8)	0.2 (0.7)	0.2 (0.8)	0.3 (0.9)	0.3 (0.8)
fumonisin B_1	2.4 (7.8)	2.5 (8.2)	2.8 (9.1)	2.4 (7.9)	2.6 (8.5)	2.2 (7.3)
fumonisin B_2	2.4 (7.9)	2.2 (7.4)	2.3 (7.6)	2.8 (9.4)	2.9 (9.6)	2.5 (8.3)
fusarenon-X	3.1 (10.3)	3.1 (10.2)	3.2 (10.4)	3.8 (12.4)	3.7 (12.1)	3.8 (12.5)
HT-2	1.2 (3.8)	1.0 (3.2)	0.9 (3.1)	1.4 (4.6)	1.1 (3.5)	1.3 (4.2)
neosolaniol	0.8 (2.6)	0.7 (2.4)	0.8 (2.6)	0.3 (1.2)	0.8 (2.6)	0.5 (1.7)
ochratoxin A	0.1 (0.4)	0.1 (0.3)	0.1 (0.3)	0.1 (0.4)	0.2 (0.6)	0.2 (0.7)
ochratoxin B	0.2 (0.8)	0.2 (0.7)	0.3 (0.9)	0.1 (0.3)	0.2 (0.7)	0.2 (0.5)
sterigmatocystin	0.1 (0.5)	0.1 (0.3)	0.1 (0.3)	0.2 (0.6)	0.2 (0.6)	0.2 (0.5)
T-2	0.3 (0.9)	0.2 (0.7)	0.2 (0.7)	0.2 (0.8)	0.2 (0.6)	0.2 (0.5)
verrucarin A	3.1 (10.1)	3.1 (10.3)	3.2 (10.5)	3.2 (10.4)	3.1 (10.3)	3.1 (10.2)
zearalenone	2.6 (8.6)	2.5 (8.3)	2.6 (8.7)	2.2 (7.3)	3.0 (9.8)	2.5 (8.2)
geometric mean	0.5 (1.6)	0.4 (1.4)	0.4 (1.5)	0.4 (1.4)	0.5 (1.6)	0.5 (1.5)

RESULTS AND DISCUSSION

Optimization of LC-MS/MS. A LC-MS/MS method was developed for the analysis of various mycotoxin classes including trichothecenes, aflatoxins, ochratoxins, fumonisins, and ergot alkaloids. Two specific MRM transitions for each native and isotope-labeled mycotoxin were selected to achieve identification according to the European Commission (EC) and U.S. Food and Drug Administration criteria.[39−41] In MRM mode, the transition of the most abundant product transition (target or quantitative) ion was selected for quantitation, and the ratio of the target ion to the second least abundant (confirmatory) ion was used for identification. sMRM was used for optimal data acquisition based on the retention time and peak width of the precursor-to-product ion transition rather than populate MRM time segments with the ion transitions typically used in previous MS/MS procedures.[42] Table 1 provides the optimum MS/MS parameters along with the chemical information for 26 native mycotoxins and 5 stable isotope-labeled mycotoxins used for the sMRM monitoring. The ammoniated ion adduct $[M + NH_4]^+$ was used for 15-acetyldeoxynivalenol, beauvericin, diacetoxyscirpenol, HT-2, neosolaniol, T-2, and verrucarin A, whereas the proton adduct $[M + H]^+$ was used for the remaining mycotoxins.

In previous studies, analysis times of 30−40 min were required to detect 10 or fewer mycotoxins in cereal-based foods.[27,34−36] The advances by Sulyok et al.[28,31] expanded the LC-MS/MS analysis from 39 to 87 mycotoxins and other fungal metabolites in 21 and 15 min, respectively, for wheat, maize, and moldy food products. Twenty-six native mycotoxins, including aflatoxins (B_1, B_2, G_1, and G_2), trichothecenes (DON, 15-acetyl-DON, diacetoxyscirpenol, T-2, HT-2, and neosolaniol), ochratoxins (A and B), fumonisins (B_1 and B_2), ergot alkaloids, sterigmatocystin, verrucarin A, and zearalenone, known to be present or have maximum tolerance concentrations in grain and nut products,[16−19,21,22,30,34−37] were selected and analyzed using an efficient 15 min LC-MS/MS procedure.

Optimized Extraction Procedure. On the basis of studies[28,31] of the extraction solvent (0:100, 20:80, 40:60, 80:20, 85:15, 90:10, 100:0 acetonitrile/water and 85:14:1 acetonitrile/water/formic acid), solvent-to-sample ratio (3:1, 5:1, and 10:1), sample analysis size (0.5, 1.0, 2.0, and 5.0 g), extraction time (5, 10, 30, and 60 min), and selection of the membrane filter (nylon versus polytetrafluoroethylene), the optimal extraction procedure was finalized for method validation using aflatoxin B_1, ochratoxin A, and beauvericin as model mycotoxins in a rice matrix or a reference maize material containing aflatoxin B_1. These preliminary conditions were then expanded to the validation studies for all of the mycotoxins and matrices. Although the 85:14:1 acetonitrile/water/formic acid extraction solvent showed no differences in the recoveries of the three model mycotoxins compared to the extraction solvent without acid, studies have shown that the addition of acid improves the extraction efficiencies of the fumonisins[28] but can potentially degrade the basic ergot alkaloids.[43] To account for these differences in the physical and chemical properties of the mycotoxins, the 85:15 acetonitrile/water composition was selected as the extraction solvent.[33,44] The finalized procedure involves the extraction of 1 g of homogenized sample with 5.0 mL of acetonitrile/water (85:15

Table 4. Matrix Suppression/Enhancement (MSE) Effect of Mycotoxins Extracted from Six Matrices (Corn, Rice, Wheat, Almond, Peanut, and Pistachio)[a]

	matrix suppression/enhancement effect, %					
mycotoxin	corn	rice	wheat	almond	peanut	pistachio
15-acetyldeoxynivalenol	−14	−13	−23	−32	−54	−14
aflatoxin B_1	−5	−11	−7	6	−21	−14
aflatoxin B_2	−8	−18	−9	9	−22	−7
aflatoxin G_1	−16	−15	−10	11	−14	−5
aflatoxin G_2	−12	−3	−14	−25	−39	−39
beauvericin	−12	−17	−14	57	−29	−10
citrinin	−13	−8	−16	13	−26	−38
deoxynivalenol	−7	−17	−16	45	−54	−35
diacetoxyscripenol	−24	−16	−10	4	−51	−26
ergocornine	−22	−11	−21	−13	−26	−21
ergocristine	−7	−15	−14	4	−37	−29
ergocryptine	−11	−2	−22	−10	−40	−21
ergometrine	−15	−15	−23	−18	−56	−31
ergosine	−16	−15	−17	−13	−28	−23
ergotamine	−16	−10	−19	25	−12	−13
fumonisin B_1	−16	−19	−7	12	−38	−11
fumonisin B_2	−17	−3	−4	55	−13	−8
fusarenon-X	−24	−16	−23	−10	−36	−10
HT-2	−6	−14	−7	−39	−35	−30
neosolaniol	−13	−2	−21	6	−38	−11
ochratoxin A	−7	−11	−15	−32	−33	−30
ochratoxin B	−8	−5	−25	−25	−39	−22
sterigmatocystin	−11	−5	−22	−28	−31	−42
T-2	−14	−19	−18	6	−20	−12
verrucarin A	−7	−14	−24	43	−34	8
zearalenone	−15	−3	−15	−35	−30	26

[a]The effect was determined from the expression MSE effect = 100 × ($slope_M/slope_S$ − 1)%, where $slope_M$ and $slope_S$ are the slopes obtained from the matrix-matched and solvent-only calibration curves, respectively.

Table 5. Analysis of Reference Materials and Comparison between Assigned and Measured Concentrations[a]

reference material	analyte	matrix	certified concn, μg/kg	measured concn, μg/kg) (n = 3)	% difference
GIPSA-MRM2010-004A	aflatoxins[b]	corn	10.2 ± 1.1	9.3 ± 1.2	−8.8
GIPSA-MRM-2010-006	aflatoxins[b]	corn	78.2 ± 5.7	73.8 ± 10.2	−5.6
GIPSA-MRM2010-010A	deoxynivalenol	wheat	1010 ± 60	1060 ± 150	+4.9
GIPSA-MRM2010-016B	deoxynivalenol	corn	1100 ± 50	1010 ± 110	−8.2
GIPSA-MRM2010-017	deoxynivalenol	corn	2030 ± 90	1900 ± 150	−6.4
GIPSA-MRM-2010-021	fumonisins[c]	corn	2310 ± 110	1962 ± 185	−6.7
GIPSA-MRM-2010-023	ochratoxin A	wheat	6.31 ± 0.31	5.0 ± 0.5	−20.8
GIPSA-MRM2010-025	zearalenone	corn	98.3 ± 9.9	93.5 ± 11.7	−4.9
GIPSA-MRM2011-004	deoxynivalenol	wheat	2060 ± 60	1970 ± 120	−4.4
Romer Labs 003010	aflatoxin B_1	corn	15.47 ± 3.93	14.46 ± 1.66	−6.5
Romer Labs 003019	zearalenone	corn	177.3 ± 64.8	155.2 ± 28.8	−12.5

[a]Detailed procedures and materials are described under Materials and Methods. % difference is calculated as the difference between the measured and certified concentrations (measured − certified) divided by the certified concentration and the result multiplied by 100%. n = 21 for GIPSA reference materials and minimum of n = 3 for Romer materials. [b]Total aflatoxins (B_1 + B_2 + G_1 + G_2). [c]Total fumonisins (B_1 (1470 ± 30) + B_2 (634 ± 20) + B_3 (208 ± 5) = 2310 ± 110 μg/kg) but only B_1 + B_2 were measured and % difference determined.

v/v; 5:1 solvent-to-sample ratio) and 30 min of shaking. After centrifugation and dilution, the extract was filtered using a 13 mm × 0.2 μm PTFE syringe filter directly into the autosampler vials for LC-MS/MS analysis.

Method Validation. *Recovery Studies.* Food commodities of low water content consisting of three grain (rice, wheat, and corn) and three nut matrices (peanut, pistachio, and almond) were selected for method validation. These six commodities are widely used consumer and animal feed commodities and have a high risk for mycotoxin contamination. Recovery studies were performed by the addition of fortification standards to blank grain and nut samples at three concentration levels, 10, 50, and 100 μg/kg and 100, 500, and 1000 μg/kg for group A and B mycotoxin mixes (n = 4 at each fortification level), respectively, as listed in Table 2 and quantitated using matrix-matched calibration curves (r^2 > 0.99) and the primary (target) product ion transition. The mean recoveries (n = 12, ± standard deviation) of each mycotoxin at the three fortification levels were 84 ± 6, 89 ± 6, 97 ± 9, 87 ± 12, 104 ± 16, and 92 ± 18% from samples of corn, rice, wheat, almond, peanut, and

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Table 6. Survey Results of Mycotoxins in Different Grain and Nut Commercial Samples

	rate[a] and range of contamination,[b] μg/kg					
mycotoxin	rice	wheat	corn	peanut	pistachio	almond
aflatoxin B_1				5/11[a] (0.3−0.6)[b]	2/10 (0.7, 1.4)	1/9 (0.3)
aflatoxin B_2					1/10 (0.6)	
aflatoxin G_1					1/10 (0.4)	
beauvericin		1/16 (1.8)	14/18 (0.5−130)	2/11 (0.9, 5.0)	1/10 (1.4)	
deoxynivalenol		2/16 (63, 88)	3/18 (78−134)			
ergocornine		2/16 (2.4, 3.8)				
ergocristine		2/16 (7.7, 8.8)				
ergocryptine		2/16 (5.6, 6.4)				
ergometrine		1/16 (3.1)				
ergosine		2/16 (1.4, 1.5)				
ergotamine		2/16 (8.3, 10.7)				
fumonisin B_1			11/18 (41−1143)			
fumonisin B_2			8/18 (25−937)			
neosolaniol					1/10 (58)	
ochratoxin A	1/6 (3.3)	5/16 (1.5−2.7)			3/10 (1.1−7.1)	
ochratoxin B					2/10 (0.6, 2.8)	
sterigmatosin	1/6 (0.9)	1/16 (1.1)				
T-2		1/16 (2.1)				
zearalenone			4/18 (115−339)			

[a]Number of samples contaminated with the specified mycotoxin/number of certain matrix samples analyzed. [b]The range of contamination levels of each mycotoxin and each concentration listed is an average of three replicates (n = 3).

pistachio, respectively. The mean recoveries of each mycotoxin at the different fortification levels for the six different matrices were compared and subjected to one-way ANOVA ($p < 0.05$). The results indicate a statistical difference between group A (10, 50, and 100 μg/kg) and the six different matrices, whereas there was no significant difference observed with the group B (100, 500, and 1000 μg/kg) mycotoxins. A possible explanation is that the group B mycotoxins were fortified at the higher concentration levels and less susceptible to signal or matrix background observed at the lower levels. For nut products, the peanut matrix seemed to have an effect on the recovery at the 100 μg/kg for group A mycotoxins when compared to that of almond and pistachio, whereas both peanut and pistachio matrices were shown to exert their effects on the recoveries of the group B mycotoxins at the 100 and 500 μg/kg levels. The three nut matrices also showed a decreased recovery at the three fortified levels (100, 500, and 1000 μg/kg). Because both grain and nut commodities have similar water contents, the higher lipid content in nut products can be the factor contributing to the differences in the mycotoxin recoveries between the two commodity types. Several attempts have been made to remove the lipid content from matrices such as nuts using hexane rinses, hydrophobic sorbents such as octadecyl-linked silica or hydrophobic ligand-linked polymer sorbents, or immunoaffinity sorbents to selectively bind the myctotoxin to separate the mycotoxin from the matrix components. These attempts did little to improve the performance and would have increased the labor by the additional defatting steps as well as the costs of using expensive cleanup columns and related consumables. The low recoveries of the acidic fumonisins compared to the other mycotoxins in multi-mycotoxin procedures have been observed from other studies.[28,31] Spanjer et al. reported recoveries <40% of fumonisins in peanut matrices,[31] and Sulyok et al. reported that this is a common observation when relatively polar compounds such as fumonisins are extracted when a higher composition of acetonitrile is used in acetonitrile/water extraction processes.[28]

Limits of Detection and Quantitation. The limit of detection (LOD) was determined by calculating the matrix-dependent instrument detection limit (MD-IDL) using the EPA's protocol,[40] and the matrix-dependent instrument quantitation limit (MD-IQL) was calculated by 3.3 × MD-IDL values. The MD-IDL and MD-IQL results were based on the secondary transition of the mycotoxin for the grain and nut matrices and are provided in Table 3. The EPA protocol presents a consistent metric for other laboratories to carry out follow-up experiments for comparison purposes because statistical calculation rather than estimation of signal-to-noise ratios is used to determine the detection limits. The MD-IDL and MD-IQL results indicate fusarenon-X, 15-acetyldeoxynivalenol, and verrucarin A were consistently higher than the other mycotoxins in the six matrices. Aflatoxins, beauvericin, diacetoxyscirpenol, ergot alkaloids, ochratoxin, sterigmatocystin, and T-2 have MD-IQLs < 1 μg/kg), whereas 15-acetyldeoxynivalenol, citrinin, deoxynivalenol, fumonisins, fusarenon-X, HT-2, neosolaniol, verrucarin A, and zearalenone have MD-IQLs < 12.5 μg/kg for the six matrices evaluated. The MD-IQLs of aflatoxin B_1, ochratoxin A, T-2 toxin, HT-2 toxin, and fumonisin B_1 in wheat and aflatoxin B_1, ochratoxin A, deoxynivalenol, and zearalenone in corn are consistent with other studies.[31−33,35−37] The values for nuts (almond, peanut, and pistachio) were similar to the grain products and also consistent with peanut and pistachio results determined by Spanjer et al.[34] MRLs of deoxynivalenol and fumonisins determined by most countries and the EU are in the 500−1000 μg/kg range, whereas aflatoxins and ochratoxin A limits are set at <20 and <50 μg/kg, respectively, in grain and nut products,[9] indicating the detection and quantitation limits obtained in this study are well below the published regulation requirements. The results obtained from the extracts without any prior cleanup for LC-MS/MS analysis are more than adequate for the analysis of mycotoxins in grain and nut matrices.

Matrix Effects. Isobaric interference and ion suppression/enhancement were assessed because these effects can affect the quantitation of mycotoxins in different agricultural commodities.

The following expression, based on the slope ratios of matrix-matched and solvent-only calibration curves of the mycotoxin analyte, was used to quantitatively evaluate the signal suppression/enhancement (SSE) for each mycotoxin:

$$SSE = 100 \times (slope_M/slope_S - 1)\%$$

$Slope_M$ and $slope_S$ are the slopes of the matrix-matched and solvent-only calibration curves, respectively. Signal suppression or enhancement appears if the value is <0% or >0%, respectively, and a value of 0% indicates there is no absolute matrix effect. The SSE results for each mycotoxin in the presence of the six matrices are provided in Table 4. The ranges of how the matrix exerts its effect on the mycotoxin's LC-MS/MS responses are −5 to −24%, −2 to −19%, −4 to −25%, +57 to −39% %, −12 to −56%, and +26 to −42% for corn, rice, wheat, almond, peanut, and pistachio, respectively. Signal suppression was present for all of the mycotoxin in grains, whereas both signal suppression and enhancement were present in nuts. The absolute average SSE values, indicating a deviation from a 0% baseline (no matrix effect), were 13, 11, and 16% for corn, rice, and wheat compared to 23, 32, and 21% for almond, peanut, and pistachio, respectively. Suppression of deoxynivalenol and verrucarin A by as much as −54 and −34%, respectively, was determined in peanut, yet enhancement of the same mycotoxins was determined to be as much as +45 and +43%, respectively, in almond. The results indicate that the signal enhancement/suppression responses are dependent on the matrix because no correlation could be established between individual mycotoxin and the six different matrices studied.

Trueness. Eleven corn and wheat reference materials from USDA-GIPSA and Romer Labs were analyzed (n = 3) to demonstrate the validity of the method with results shown in Table 5. Results from the analysis of the reference materials ranged from concentration levels of 6.31 ± 0.31 μg/kg (n = 21) of ochratoxin A in wheat to as high as 2310 ± 110 μg/kg (n = 21) of fumonisins in corn. There was no statistical difference between the certified and measured values of these reference materials, with the exception of ochratoxin A in wheat. The percent difference ranged from +4.9% (deoxynivalenol in wheat) to −20.8% (ochratoxin A in wheat) with 9 of the 11 reference materials <10% (absolute % difference), despite the reference values of the certified materials being analyzed by different procedures and instrumentation. The analytical variability as discussed by Whitaker[45,46] was shown to contribute least to the overall variability associated with mycotoxin test procedures. The experimentally determined concentrations of target analytes were within the satisfactory range for the tested mycotoxins (aflatoxins, DON, fumonisins, ochratoxin A, zearalenone), indicating this is a reliable method based on the evaluation and results in these reference materials.

Application of the Validated Method to the Analysis of Finished Grain and Nut Products. The validated method was used to analyze finished cereal and nut products purchased from local markets and online stores in the United States from August 2011 to January 2012. A total of 70 commercial products were analyzed, which included 6 rice, 16 wheat, 18 corn, 11 peanut, 10 pistachio, and 9 almond finished samples. The frequency of mycotoxin presence and the range of their concentrations in these finished products are summarized in Table 6 (additional and detailed information of these products and mycotoxin concentrations are provided in the Supporting Information).

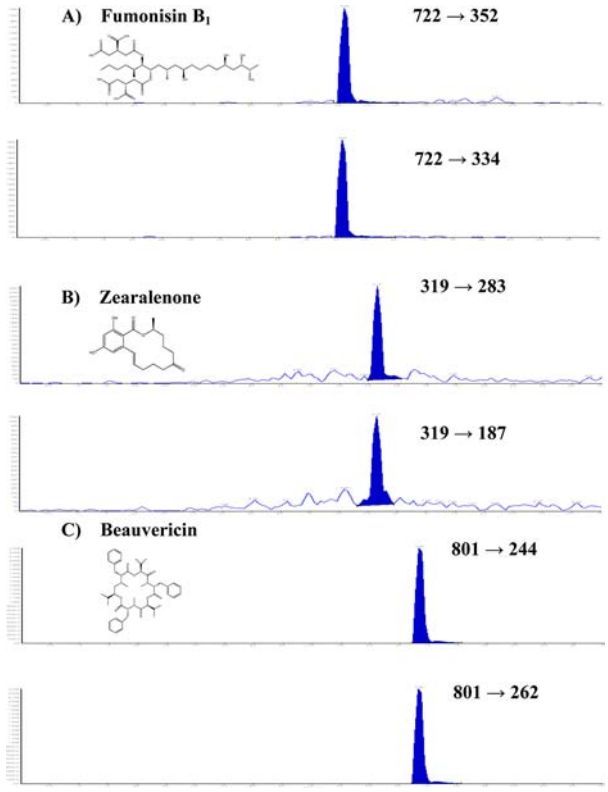

Figure 1. LC-MS/MS chromatograms of an incurred corn sample containing three mycotoxins as quantitated and identified by LC-MS/MS: (A) fumonisin B_1 (426 μg/kg) as identified by the 722 → 352 and 722 → 334 transitions and retention time of 5.7 min; (B) zearalenone (339 μg/kg) as identified by the 319 → 283 and 319 → 187 transitions and retention time of 6.2 min; (C) beauvericin (130 μg/kg) as identified by the 801 → 244 and 801 → 262 MS/MS transitions and retention time of 6.9 min. Quantitation was determined by the primary transition (top panel of the two transitions of the mycotoxin found). The ion ratios of the two precursor-to-product ion transitions were compared to matrix-matched standards for identification.

One rice flour sample was found to contain 0.9 ± 0.2 and 3.3 ± 0.3 μg/kg (n = 3) sterigmatocystin and ochratoxin A, respectively. Wheat products (wheat flour and dried pasta) were found to contain mycotoxins (deoxynivalenol, sterigmatocystin, ochratoxin A, T-2 toxin, ergometrine, ergosine, ergocornine, ergocryptin, ergotamine, ergocristine, and beauvericin) at concentration levels ranging from 1.1 ± 0.2 to 88 ± 10 μg/kg (n = 3), consistent with mycotoxin type and concentrations found in other studies. Ochratoxin A was present in five wheat samples at concentrations <3 μg/kg, below the 3−50 μg/kg maximum tolerance levels set by most governments. Figure 1 illustrates a LC-MS/MS chromatogram of a contaminated corn sample obtained using the validated method to quantitate and identify the mycotoxins beauvercin (130 μg/kg), zearalenone (339 μg/kg), and fumonisin B_1 (426 μg.kg). Among the commercial grains analyzed, 16 of the 18 corn samples were shown to be contaminated with one or more mycotoxins: ochratoxin A, T-2 toxin, beauvericin, fumonisin B_1, fumonisin B_2, zearalenone, and deoxynivalenol, ranging in concentrations from 0.6 ± 0.1 (beauvericin, n = 3) to 1143 ± 87 μg/kg (fumonisin B_1, n = 3). (Complete results of mycotoxin presence and concentrations in each corn sample are provided in the Supporting Information.) Levels were below the maximum levels of 2000−4000 μg/kg

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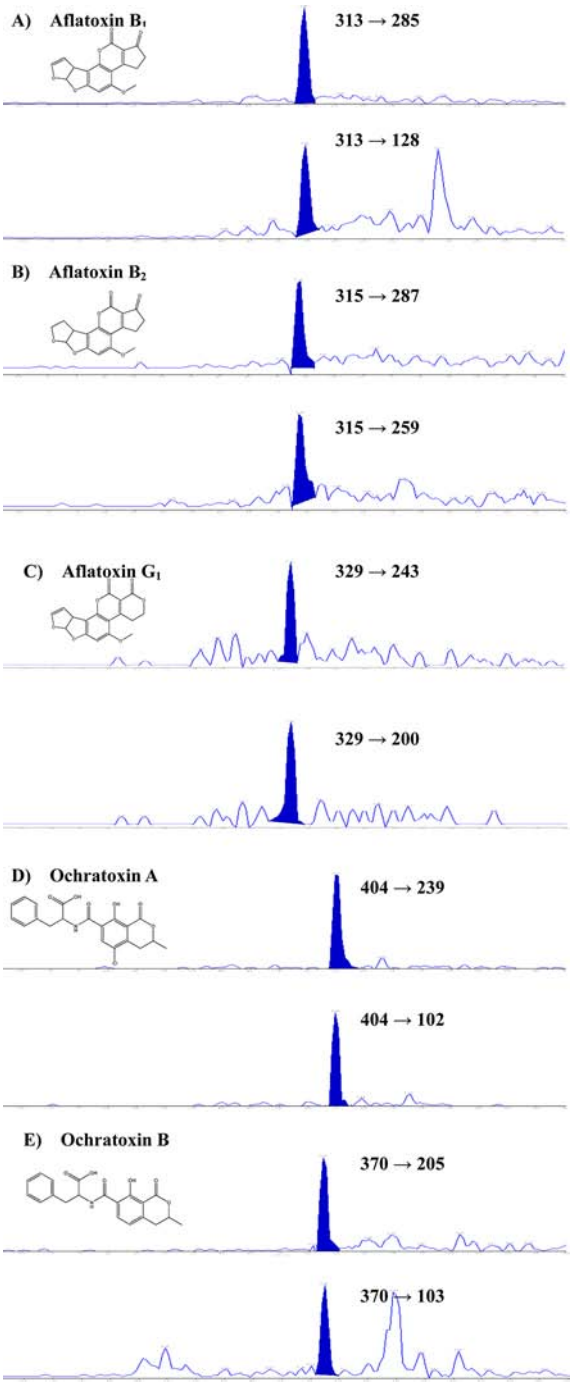

Figure 2. LC-MS/MS chromatograms of an incurred pistachio sample containing five mycotoxins as quantitated and determined and identified by LC-MS/MS: (A) aflatoxin B_1 (1.4 μg/kg) as identified by the 313 → 285 and 313 → 128 transitions and retention time of 5.5 min; (B) aflatoxin B_2 (0.6 μg/kg) as identified by the 315 → 287 and 315 → 259 transitions and retention time of 5.4 min; (C) aflatoxin G_1 (0.4 μg/kg) as identified by the 329 → 243 and 329 → 200 transitions and retention time of 5.2 min; (D) ochratoxin A (7.1 μg/kg) as identified by the 404 → 239 and 404 → 102 transitions and retention time of 6.0 min; (E) ochratoxin B (2.8 μg/kg) as identified by the 370 → 205 and 370 → 103 MS/MS transitions and retention time of 5.8 min. Quantitation was determined by the primary transition (top panel of the two transitions of the mycotoxin found). The ion ratios of the two precursor-to-product ion transitions were compared to matrix-matched standards for identification.

for the fumonisins established by the U.S. Food and Drug Administration for degermed or partially degermed dry-milled corn products.[7,9]

The frequency of detection of the mycotoxins was less and in lower concentrations in finished nut products compared to grain products. Five of the 11 peanut products (peanut flour, peanut butter, and raw peanut) surveyed were contaminated with aflatoxin B_1 at an average concentration of 0.42 ± 0.16 μg/kg, whereas two of the peanut products also contained beauvericin at concentrations of 5.0 ± 0.7 and 0.9 ± 0.4 μg/kg (n = 3, each). Pistachio products (roasted pistachio, pistachio butter, and pistachio flour) were found to contain a total of seven mycotoxins distributed among 4 of the 10 samples studied. One pistachio product was cocontaminated with five mycotoxins, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, ochratoxin A, and ochratoxin B. All of these were detected and identified in one LC-MS/MS analysis as shown in Figure 2. The highest concentration found in the pistachio samples was that of neosolaniol at 58 ± 2 μg/kg; this mycotoxin has seldom been analyzed and reported in the literature.

The validated method used to analyze the samples for monitoring purposes was shown to be easy, efficient, and rugged for routine sample mycotoxin analysis in finished wheat and nut products and could be modified and expanded to include other mycotoxins and fungal metabolites if certified standards become available. Future directions would include expanding and modifying the method and testing other detection systems to screen and analyze more finished grain and nut products, fresh and dried plant foods, dairy foods (milk, cheeses, etc.), and other relevant food and feed commodities susceptible to mycotoxin contamination.

Conclusion. The occurrence of mycotoxins in finished grain and nut products is well established; therefore, a multi-mycotoxin LC-MS/MS method for the simultaneous analysis of 26 mycotoxins in grains and nuts was developed and validated. The sample preparation procedure followed by LC-MS/MS is efficient and easy to use. The validation results obtained from six grain and nut matrices showed acceptable linearity (r^2 > 0.99), recoveries, LOD/LOQ, and trueness. Minimal to moderate matrix effects were found in different matrices but were more pronounced in nut products. A total of 70 commercial products were analyzed using this method. Contamination of more than one mycotoxin was found in the limited number of wheat, corn, and pistachio samples. The contamination concentrations of commercial finished products were below the regulation limits established by several government agencies.

■ ASSOCIATED CONTENT

Supporting Information

Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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