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Rapid Method for the Determination of Multiple Mycotoxins in Wines and Beers by LC-MS/MS Using a Stable Isotope Dilution Assay

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ABSTRACT: A "dilute and shoot" method for the liquid chromatography−tandem mass spectrometric (LC-MS/MS) determination of multiple mycotoxins (aflatoxins B1, B2, G1, G2, ochratoxin A (OTA), fumonisins (F) B1 and B2, zearalenone, deoxynivalenol, T-2 toxin, and HT-2 toxin) in wines and beers has been developed and validated. Separation was accomplished using ultrahigh-performance liquid chromatography (UHPLC) with <10 min analysis time. Mycotoxins were detected by dynamic multiple reaction monitoring (MRM) in positive electrospray ionization mode. Due to matrix effects, ^{13}C-uniformly labeled mycotoxins were added to the sample extracts prior to LC-MS/MS analysis. With external calibration, recoveries were 18−148% for white wines, 15−118% for red wines, and 20−125% for beers, at three spiking levels. The ^{13}C-labeled internal standards compensated for matrix effects effectively, with overall recoveries of 94−112% for white wines, 80−137% for red wines, and 61−131% for beers, with greater recoveries for FB1 and FB2, at three spiking levels. The relative standard deviation was <20% for all analytes in the wines and beers. This method was applied to a USDA-funded nationwide survey of domestic and imported wines and beers for the determination of OTA and extended to include other mycotoxins.

KEYWORDS: *multiple mycotoxins, beer, wine, internal standard, LC-MS/MS*

INTRODUCTION

Mycotoxins are natural, toxic chemicals (secondary metabolites) produced by molds that cause general toxic effects, immune system suppression, cancer, birth defects, liver damage, and nervous tissue damage in animals and humans.[1,2] The Food and Agriculture Organization (FAO) estimated that mycotoxins affect 25% of the world's food crops each year, resulting in annual losses of about 1 billion metric tons of food and food products. Economic costs due to mycotoxins in the United States and Canada are estimated to be about $5 billion per year.[3]

About 300−400 mycotoxins of vast structural diversity exist, which results in different chemical and physiochemical properties. Aflatoxins and ochratoxin, produced mainly by *Aspergillus* sp., and fumonisins, tricocethenes, and zearalenone, produced by *Fusarium* sp., cause the most public health concern due to their frequent occurrence and their severe impact on animal and human health.[1,2]

The chemical diversity of mycotoxins and their varying concentrations in a wide range of agricultural commodities, foods, and biological samples present a great challenge for sample preparation, extraction, cleanup, separation, and detection methods. Most methods target only individual mycotoxins or a group of closely related mycotoxins, which are based on tedious sample preparation methods followed by traditional chromatographic separation. Each group of mycotoxins requires a different cleanup method (e.g., immunoaffinity column, solid-phase extraction cartridge, Mycosep cartridge) and specific analytical separation conditions for liquid chromatography with different detectors (e.g., ultraviolet detector, fluorescence detector, and mass spectrometer).[4−6] To deal with the increasing number of sample matrices and mycotoxins of interest, fast and accurate analytical methods are needed for the simultaneous determination of multiple classes of mycotoxins in different commodities.

A quick "dilute and shoot" method was developed and validated for identifying and quantifying multiple mycotoxins in beers and wines using a LC-MS/MS system. Matrix effects can cause suppression or enhancement of the target analytes and may hamper accurate mass spectrometric quantification, leading to false results. Therefore, ^{13}C-labeled internal standards were used to compensate for matrix effects.[7,8] Stable isotopically labeled standards exhibit similar chemical and physical properties as the target analytes and are not present in naturally contaminated samples. Because the naturally abundant isotopic distribution of the analyte is diluted because of the addition of stable isotope-labeled standards, this procedure is often referred to as stable isotope dilution assay (SIDA). The

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Table 1. LC-MS/MS Acquisition Parameters for Mycotoxins

analyte	RT (min)	m/z precursor ion	m/z product ion	fragmentor (V)	collision energy (V)
aflatoxin B1	7.54	313.1	285, 241	190	20
[$^{13}C_{17}$]-aflatoxin B1	7.53	330.1	301.1	180	20
aflatoxin B2	7.21	315.1	287.1, 259	190	24
[$^{13}C_{17}$]-aflatoxin B2	7.2	332.2	303.1	190	24
aflatoxin G1	6.84	329.1	311, 243	190	20
[$^{13}C_{17}$]-aflatoxin G1	6.83	346.1	212	180	44
aflatoxin G2	6.36	331.1	313, 245.1	190	24
[$^{13}C_{17}$]-aflatoxin G2	6.35	348.1	259	175	28
deoxynivalenol	2.1	297.3	248.9, 203	90	4, 12
[$^{13}C_{15}$]-deoxynivalenol	2.1	312.1	262.9	90	4
fumonisin B1	8.87	722.4	352.3, 334.2	190	40
[$^{13}C_{34}$]-fumonisin B1	8.87	756.5	374.2	190	40
fumonisin B2	9.72	706.4	336.3, 318.3	260	40
[$^{13}C_{34}$]-fumonisin B2	9.71	740.5	358.4	210	36
HT-2 toxin	8.58	442.2	263.1, 215	110	8
[$^{13}C_{22}$]-HT-2 toxin	8.58	464.5	278.4	110	8
T-2 toxin	9.1	484.3	305, 215.2	110	8
[$^{13}C_{24}$]-T-2 toxin	9.09	508.3	322	110	8
ochratoxin A	7.67	404.1	358, 239, 193	140	8, 25, 45
[$^{13}C_{20}$]-ochratoxin A	9.5	424.2	250.2	120	24
zearalenone	9.55	319.2	301.1, 283.1	80	4
[$^{13}C_{18}$]-zearalenone	9.54	337.3	301.4	110	8

stable isotope dilution approach in the mycotoxin analysis of food overcomes the problem of ion suppression or enhancement.[7,8]

The aim of the present study was to develop a rapid UHPLC-MS/MS method for the simultaneous determination of aflatoxins B1, B2, G1, and G2, ochratoxin A, fumonisins B1 and B2, zearalenone, deoxynivalenol, T-2 toxin, and HT-2 toxin using a SIDA. This method was then applied to a USDA-funded nationwide survey of domestic and imported wines and beers for the determination of ochratoxin A and extended to include other mycotoxins.

MATERIALS AND METHODS

Standard Preparation. The following mycotoxin unlabeled standards were purchased from Sigma-Aldrich, St. Louis, MO, USA: Aflatoxin (AF) G1, 5 mg; AFG2, 5 mg; AFB1, 5 mg; AFB2, 5 mg; ochratoxin A (OTA), 5 mg; zearalenone (ZEN), 5 mg; deoxynivalenol (DON), 5 mg; T-2 toxin, 5 mg; HT-2 toxin, 5 mg; fumonisin mix, 50 μg/mL in acetonitrile/water (each of FB1 and FB2). The following mycotoxin internal standard (IS) mixes were purchased from Romer Laboratories, Vienna, Austria: Mix-11 (^{13}C aflatoxins) consisting of 500 ng/mL each of [$^{13}C_{17}$]- AFB1, [$^{13}C_{17}$]-AFB2, [$^{13}C_{17}$]-AFG1, and [$^{13}C_{17}$]-AFG2; Mix-12 (^{13}C fumonisins) consisting of 5 μg/mL each of [$^{13}C_{24}$]-FB1 and [$^{13}C_{24}$]-FB2; Mix-10 (^{13}C fusarium toxins) consisting of 10 μg/mL of [$^{13}C_{15}$]-DON, 10 μg/mL of [$^{13}C_{22}$]-HT-2 Toxin, 1 μg/mL of [$^{13}C_{24}$]-T-2 toxin, and 3 μg/mL of [$^{13}C_{18}$]-ZEN; [$^{13}C_{20}$]-OTA at 10 μg/mL.

Working Solution Preparation. A 10 μg/mL stock solution of the unlabeled mycotoxin stock solution was prepared in 5 mL of acetonitrile (Fisher Scientific, Hanover Park, IL, USA). The ^{13}C-labeled analogues were prepared as another stock solution in 1 mL of acetonitrile/water (30:70, v/v). A 1250 ng/mL working solution of the unlabeled mycotoxins was made, and dilutions were prepared at the following concentrations: 0.125, 0.625, 1.25, 6.25, 12.5, 62.5, and 125 ng/mL. For the preparation of the calibration standards, 80 μL of the standard solutions was transferred into HPLC vials with microinserts (VWR International, Batavia, IL, USA) and 20 μL of the ^{13}C-labeled stock solution mixture was added. This resulted in concentrations of 0.1L, 0.5, 1, 5, 10, 50, and 100 ng/mL for the mycotoxin calibration curve with the internal standards. The internal standard solutions had various final concentrations as follows: [$^{13}C_{17}$]-AFB1, 1 ng/mL; [$^{13}C_{17}$]-AFB2, 1.03 ng/mL; [$^{13}C_{17}$]-AFG1, 1.03 ng/mL; [$^{13}C_{17}$]-AFG2, 1.02 ng/mL; [$^{13}C_{34}$]-FB1, 10.04 ng/mL; [$^{13}C_{34}$]-FB2, 10.02 ng/mL; [$^{13}C_{15}$]-DON, 20.2 ng/mL; [$^{13}C_{22}$]-HT-2 toxin, 20.6 ng/mL; [$^{13}C_{24}$]-T-2 toxin, 2 ng/mL; [$^{13}C_{20}$]-OTA, 20 ng/mL; [$^{13}C_{18}$]-ZEN, 6.14 ng/mL. All stock and working standard solutions were stored in amber vials at −20 °C.

Sample Preparation. Domestic and imported wines including red (n = 72) and white (n = 71) and domestic and imported beers (n = 76) were collected throughout the United States during March−June 2012. The wines were from the United States, Europe, South America, and Australia, whereas the beers were from breweries in the United States, Canada, Mexico, South America, and Australia. The vintage years of the white wines collected were from 2008 to 2010, and for red wines the vintage years were from 2003 to 2011.

Three milliliters of sample (beer/wine) was filtered through a 17 mm, 0.2 μm nylon syringe filter (Fisher Scientific). Beer samples were degassed prior to filtering by allowing dissolved CO_2 to dissipate overnight. Wine and beer samples were prepared at room temperature. A dilution of 1 to 5 was made by mixing 20 μL of sample, 20 μL of IS, and 60 μL of solvent mixture (30:70 acetonitrile/water, v/v) in a HPLC vial with a microinsert. Four replicate subsamples were prepared for each of the samples. A 10 μL injection of the diluted samples was made into the UHPLC-MS/MS system.

Method Validation. Recovery experiments were carried out with degassed/filtered beer (3 mL) and wine (3 mL) samples to ensure no particulates went into the LC-MS/MS. The matrices (1 mL) were spiked with unlabeled mycotoxins at three levels: 12.5, 25, and 50 ng/mL. The spiked samples were then prepared for analysis using the external standard calibration method and the internal standard calibration method.

For the external standard method, a dilution of 1 to 5 was made by mixing 20 μL of the spiked sample with 80 μL of the solvent mixture (30:70 acetonitrile/water, v/v) in a HPLC vial containing a microinsert. Four replicates were prepared for each of the samples. An external standard calibration curve containing unlabeled myctoxins was run with the spikes on the LC-MS/MS at concentrations of 0.1, 0.5, 1, 5, 10, 50, and 100 ng/mL.

For the internal standard method, a dilution of 1 to 5 was made by mixing 20 μL of the spiked wine and beer samples, 20 μL of the ^{13}C-labeled internal standards, and 60 μL of the solvent mixture (30:70

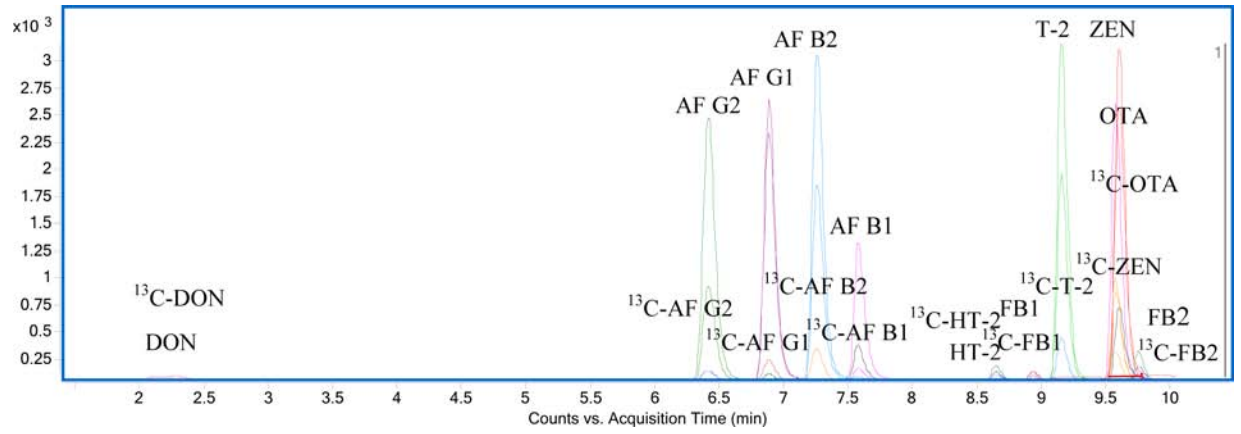

Figure 1. Extracted ion chromatogram of a European white wine spiked with unlabeled mycotoxins at a 12.5 ng/mL level. The ^{13}C-labeled internal standards are also shown.

acetonitrile/water, v/v) in a HPLC vial with a microinsert. Four replicates were prepared for each of the samples. An internal standard calibration curve containing unlabeled mycotoxins at concentrations of 0.1, 0.5, 1, 5, 10, 50, and 100 ng/mL was prepared with ^{13}C-labeled internal standards at various concentrations as discussed under Standard Preparation.

One milliliter portions of a solvent mixture (30:70 acetonitrile/water, v/v) were spiked with different concentrations of unlabeled mycotoxins (n = 5) ranging from 0.01 to 10 ng/mL for the determination of the limits of detection (LODs) and limits of quantitation (LOQs) for each of the mycotoxins in solvent solutions. Similarly, filtered wines and beers (1 mL) were spiked with different concentrations of unlabeled mycotoxins (n = 5) also ranging from 0.01 to 10 ng/mL for the determination of the LODs and LOQs for each of the mycotoxins in the three matrices (white wines, red wines, and beers).

A reference material from FAPAS.com, T17102QC-ochratoxin A in wine, was tested for OTA to confirm the trueness of the method. A reference material was purchased only for OTA because this was the major mycotoxin of concern for the USDA study.

LC-MS/MS Conditions. An Agilent 1260 Infinity HPLC system equipped with an Agilent Zorbax Poroshell 120 EC-C18 (100 mm × 2.1 mm, 2.7 μm) column was used for chromatographic separation of the mycotoxins. Mobile phase A was composed of 0.1% formic acid in water, and mobile phase B was composed of 0.1% formic acid in methanol; both eluents contained 5 mM ammonium formate. The column temperature was kept at 35 °C, and the flow rate was 0.3 mL/min. The gradient conditions were as follows: initial time, 30% B; 0.5 min, 30% B; 8 min, 100% B; 10 min, 100% B; and re-equilibration to 12 min.

Mycotoxins were analyzed using an Agilent 6460 triple-quadrupole LC-MS/MS with jet stream technology. They were identified by dynamic multireaction monitoring (DMRM) in positive electrospray ionization mode (ESI+). The gas temperature was 300 °C, and the flow rate was 10 L/min. The nebulizer was set at 45 psi, and sheath gas temperature and flow rate were 350 °C and 11 L/min, respectively. The capillary and nozzle voltages were 3500 and 0 V, respectively. The LC-MS/MS acquisition parameters that were used for the analysis of mycotoxins in wines and beer are shown in Table 1.

Data Analysis. Mycotoxin identification and quantitation analyses in beers, red wines, and white wines were performed using Agilent's Mass Hunter Qualitative Analysis and Quantitative Analysis Software (version B.04.00). Statistical analysis was performed to calculate the means, standard deviations, and relative standard deviation using Microsoft Excel 2010.

RESULTS AND DISCUSSION

Method Validation. The Optimization tool in Mass Hunter Acquisition software was used to determine LC-MS/MS MRM transitions, fragmentor voltages, and collision energies for the mycotoxins. Two to three mass transitions with the highest abundances were selected. One quantifier ion and two qualifier ions were monitored for each of the unlabeled mycotoxins, whereas only one quantifier ion was chosen for each of the ^{13}C-labeled internal standards. Figure 1 shows the overlay of the MRM transitions for unlabeled mycotoxins spiked into a European white wine at 12.5 ng/mL before extraction and the isotopically labeled internal standards.

The concentration of mycotoxins in wines and beers and for spike recoveries were calculated using $1/x$ weighted calibration curves for each analyte by plotting the relative response versus the analyte concentration. The relative response was the peak area of the analyte signal divided by the peak area of the corresponding internal standard. For determining spike recoveries using the external standard calibration method, the mycotoxin concentrations were calculated by plotting the area response versus the analyte concentration using $1/x$ weighted calibration curves.

Matrix effects can cause suppression or enhancement of the analyte signal. A predominant cause of matrix effect is the presence of endogeneous or interfering components that coelute in the chromatographic separation and change the ionization process. These can be components of the sample, compounds released during the extraction/sample cleanup process, or reagents added to the mobile phase to improve chromatographic separation.[9]

In these experiments, responses for the target analytes in the spiked sample were reduced or enhanced, compared to those spiked into solvent. When matrix effects were observed for beer samples, it was noted that all mycotoxins were affected by ion suppression, except for FB1, which showed some ion enhancement. It was found that OTA, ZEN, FB1, FB2, and T-2 toxin were affected by ion enhancement in white wines, and the other mycotoxins were affected by ion suppression. DON and AFG2 showed ion suppression in red wines. Ion suppression can reduce the ion intensity as well as affect the reproducibility and accuracy of the assay. The chemical properties of the target compound, matrix type, and the matrix to analyte concentration ratio can play a role. Also, sample preparation (extraction process, cleanup), chromatographic conditions, mass spectrometric instrumentation (i.e., design of the ion source), and ionization conditions influence the extent and nature of the matrix effects.[9] One way to deal with matrix effects is by introducing stable isotopically labeled standards

Table 2. Method Performance and Matrix Effects

		beer		white wine		red wine	
mycotoxin	spike level (ng/g)	rec external ± RSD	rec internal ± RSD	rec external ± RSD	rec internal ± RSD	rec external ± RSD	rec internal ± RSD
DON	12.5	24.06 ± 14.16	61.03 ± 4.27	17.64 ± 3.52	111.95 ± 7.86	19.86 ± 5.06	83.55 ± 13.65
	25	21.31 ± 12.55	84.15 ± 11.03	17.70 ± 2.27	105.5 ± 9.30	21.02 ± 11.62	118.8 ± 19.88
	50	20.67 ± 7.45	89.87 ± 13.44	18.41 ± 6.42	99.54 ± 6.98	14.99 ± 4.11	80.24 ± 2.27
AFB1	12.5	51.59 ± 2.39	103.87 ± 7.11	112.27 ± 2.56	93.91 ± 6.09	87.1 ± 3.81	90.94 ± 7.43
	25	52.23 ± 1.63	111.76 ± 6.57	113.01 ± 7.20	101.31 ± 1.92	83.94 ± 2.38	124.65 ± 17.77
	50	51.09 ± 0.52	108.02 ± 7.34	98.49 ± 2.40	99.49 ± 1.89	96.06 ± 0.73	94.29 ± 2.19
AFB2	12.5	53.93 ± 4.89	109.19 ± 9.63	111.27 ± 1.74	100.23 ± 2.45	86.97 ± 3.91	90.05 ± 6.33
	25	53.39 ± 2.74	115.83 ± 5.07	112.73 ± 8.37	103.41 ± 6.38	84.23 ± 1.47	118.30 ± 15.26
	50	52.09 ± 0.518	109.65 ± 6.29	97.25 ± 2.82	96.33 ± 0.46	94.76 ± 1.70	87.79 ± 3.30
AFG1	12.5	57.38 ± 1.80	109.67 ± 9.06	111.78 ± 2.07	98.89 ± 4.64	84.17 ± 2.73	93.82 ± 5.92
	25	57.61 ± 1.91	118.28 ± 7.31	114.13 ± 9.16	104.94 ± 2.44	81.80 ± 1.24	124.84 ± 14.87
	50	55.12 ± 1.54	108.57 ± 5.78	97.94 ± 3.44	101.63 ± 2.68	96.88 ± 1.65	92.36 ± 1.94
AFG2	12.5	59.76 ± 1.62	105.19 ± 6.13	98.50 ± 3.15	98.11 ± 2.02	70.72 ± 1.64	91.54 ± 8.11
	25	57.62 ± 1.16	119.63 ± 7.18	100.07 ± 9.10	100.17 ± 0.59	68.29 ± 1.37	119.00 ± 15.10
	50	56.34 ± 0.791	106.46 ± 5.18	85.54 ± 4.02	104.2 ± 8.63	69.31 ± 0.17	88.75 ± 2.60
OTA	12.5	77.49 ± 3.99	104.27 ± 7.27	136.79 ± 3.16	95.09 ± 2.93	106.65 ± 3.34	104.08 ± 7.5
	25	84.51 ± 4.78	107.47 ± 7.23	139.42 ± 6.70	100.10 ± 2.63	103.01 ± 3.99	135.12 ± 17.26
	50	83.21 ± 2.74	102.18 ± 5.96	118.05 ± 3.71	96.56 ± 2.27	102.67 ± 6.52	97.33 ± 2.88
ZEN	12.5	68.16 ± 0.461	125.00 ± 12.03	136.57 ± 1.82	107.52 ± 6.79	100.36 ± 4.53	100.13 ± 8.01
	25	64.70 ± 4.23	122.24 ± 10.39	134.69 ± 8.02	106.92 ± 2.83	100.96 ± 1.76	137.78 ± 19.26
	50	61.99 ± 1.31	120.78 ± 10.46	111.93 ± 3.82	101.09 ± 2.59	98.98 ± 6.02	104.39 ± 1.03
FB1	12.5	121.66 ± 9.381	202.70 ± 12.08	148.12 ± 7.19	101.15 ± 18.05	117.18 ± 13.51	155.10 ± 9.30
	25	124.80 ± 4.58	184.93 ± 13.71	140.68 ± 5.05	94.49 ± 9.39	118.40 ± 7.79	157.99 ± 15.06
	50	117.83 ± 7.82	200.25 ± 10.64	120.22 ± 8.98	102.95 ± 4.89	108.15 ± 2.04	152.41 ± 8.68
FB2	12.5	110.67 ± 2.65	149.24 ± 10.37	144.24 ± 11.59	95.90 ± 6.44	91.62 ± 6.78	144.68 ± 9.69
	25	85.01 ± 10.64	157.08 ± 6.12	148.65 ± 12.94	104.12 ± 6.62	96.05 ± 11.66	177.15 ± 2.75
	50	96.33 ± 4.51	146.60 ± 7.75	127.06 ± 6.22	96.9 ± 4.66	99.34 ± 2.43	135.44 ± 7.09
HT-2 toxin	12.5	85.68 ± 3.39	101.39 ± 7.74	111.64 ± 6.83	103.45 ± 5.92	87.46 ± 6.37	96.47 ± 9.67
	25	86.09 ± 2.10	109.32 ± 7.32	107.70 ± 5.34	102.74 ± 2.83	80.30 ± 1.69	135.98 ± 15.90
	50	86.72 ± 3.42	103.29 ± 3.98	95.16 ± 8.34	104.12 ± 6.33	90.15 ± 1.37	101.68 ± 5.68
T-2 toxin	12.5	76.63 ± 2.53	126.42 ± 6.33	131.85 ± 1.25	98.34 ± 4.32	105.93 ± 3.13	92.68 ± 7.13
	25	79.58 ± 3.00	131.39 ± 8.28	135.16 ± 8.04	104.06 ± 3.2	104.87 ± 1.34	125.66 ± 17.84
	50	77.71 ± 2.28	125.9 ± 5.0	116.00 ± 3.83	100.69 ± 1.89	114.88 ± 0.63	91.45 ± 3.15

into sample extracts. These compounds' chemical properties and chromatographic elution are so similar to the analytes that they suffer the same matrix effects but are separated by their mass difference and do not interfere.

Used as internal standards, the stable isotopes efficiently compensated for matrix effects, especially for DON in all wine and beer matrices where spike recoveries for all matrices were between 61 and 118%, whereas for external calibration, the spike recoveries for DON ranged between 18 and 24% (Table 3). With external calibration, recoveries were 18−148% for white wines, 15−118% for red wines, and 20−125% for beers, at three spiking levels. The ^{13}C-labeled internal standards compensated for matrix effects effectively with overall recoveries of 94−112% for white wines, 80−137% for red wines, and 61−131% for beers, with greater recoveries for FB1 and FB2, at three spiking levels, 12.5, 25, and 50 ng/mL (Table 2). Most, but not all, spike recoveries fell between 70 and 120%. Table 3 compares the recoveries and standard deviations for each mycotoxin for each of the matrices using both the external standard calibration method and the internal standard calibration method.

The LOD for each mycotoxin analyzed by LC-MS/MS was determined to be the amount of mycotoxin that would produce at least a 3:1 signal-to-noise in the standards for the quantifier ion. As the quantifier ion is the most abundant fragment, the qualifier ion fragments cannot be detected at the LOD and the identity of the analyte cannot be confirmed. Most of the LOD values for mycotoxins in solvent solutions ranged from 0.01 to 1 ng/mL, compared with 0.05−0.5 ng/mL for mycotoxins in

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Table 3. Linearity, Limits of Detection, and Limits of Quantitation

		standard solution		red wine		white wine		beer	
mycotoxin	linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)
AFB1	0.1−100	0.025	0.1	0.25	1	0.25	0.5	1	5
AFB2	0.1−100	0.01	0.05	0.05	0.25	0.05	0.25	0.1	0.5
AFG1	0.1−100	0.01	0.05	0.25	1	0.1	0.5	0.25	1
AFG2	0.1−100	0.01	0.05	0.25	1	0.1	0.3	0.25	1
DON	0.5−100	0.1	0.5	1	5	1	5	10	50
HT-2 toxin	1−100	0.1	1	2.5	5	2.5	5	2.5	10
T-2 toxin	0.1−100	0.01	0.05	0.1	0.25	0.1	0.3	0.1	1
FB1	1−100	0.5	1.5	1	10	1	10	2.5	10
FB2	0.5−100	0.125	0.25	1	5	1	5	2.5	10
OTA	0.5−100	0.1	0.5	0.1	0.5	0.1	0.5	0.1	1
ZEN	0.1−100	0.025	0.1	0.5	2.5	0.5	2.5	2.5	10

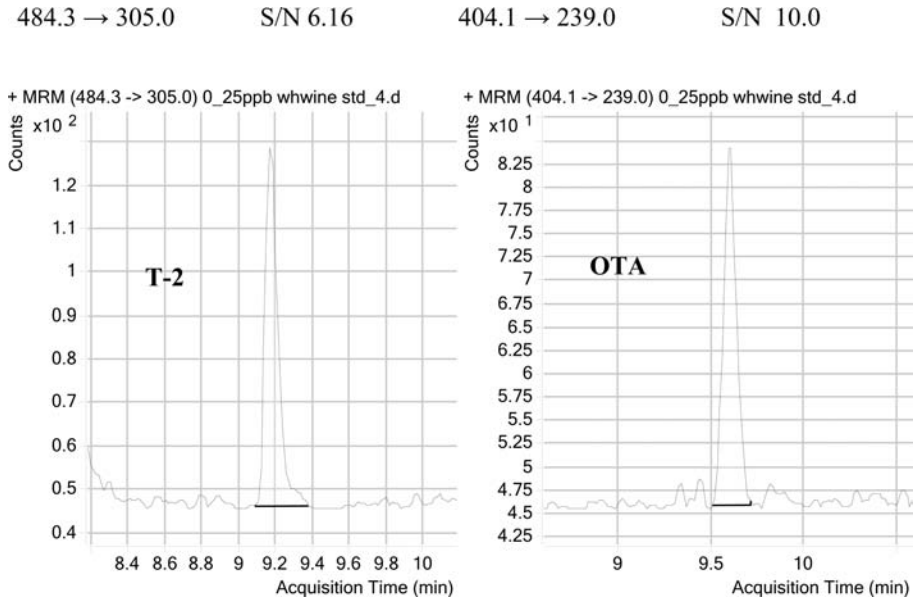

Figure 2. Extracted ion chromatogram of T-2 and OTA of a white wine spiked with 0.25 ng/mL and the corresponding signal-to-noise value for the mass transition.

red wine, 0.05−2.5 ng/mL for mycotoxins in white wine, and 0.1−10 ng/mL for beer (Table 3).

The LOQ for each mycotoxin analyzed by LC-MS/MS was determined to be the amount of mycotoxin that would produce at least a 10:1 signal-to-noise in the standards for the quantifier ion. Most of the mycotoxins were confirmed by the qualifier ion, where the intensity was at least 30% of the quantifier ion. Most of the LOQ values for mycotoxins in solvent solutions ranged from 0.05 to 1.5 ng/mL, compared with 0.25−10 ng/mL for mycotoxins in red wine, 0.3−10 ng/mL for mycotoxins in white wine, and 1−50 ng/mL for beer (Table 3). Figure 2 shows the chromatogram as well as the calculated signal-to-noise ratios for a spiking level of 0.25 ng/mL for T-2 and OTA.

The measured value fits the assigned value closely, 89% recovery, for the reference material, T17102QC-ochratoxin A in wine, which confirmed the trueness of the method.

Survey of 11 Mycotoxins in Commercial Wines and Beers. One hundred and forty-three red and white wines including 33 red organic and 32 white organic wines from 4 major regions were analyzed for 11 mycotoxins (Table 4). The vintage years of the white wines collected were from 2008 to 2010, and for red wines the vintage years were from 2003 to 2011. Seventy-six beers from seven major regions were also analyzed (Table 4). T-2 toxin was identified in 11% of white wines above the LOD of 0.1 ng/mL, but below the LOQ of 0.3 ng/mL. Concentrations between 0.23 and 0.29 ng/mL could be clearly detected, but not quantified or confirmed. T-2 toxin was quantifiable for 11% of white wines: two U.S. (0.76 ± 0.07 ng/mL; 0.66 ± 0.05 ng/mL), two European (0.58 ± 0.09 ng/mL; 0.67 ± 0.025 ng/mL), two Australian (0.71 ± 0.09 ng/mL; 0.67

Table 4. Description of Domestic and Imported Wines and Beers Analyzed

country of origin	white wines	red wines	beers
United States	22	20	12 (large brewery)
			11 (small brewery)
Europe	19	17	11
Australia	13	14	10
Canada			10
Mexico			10
South America	17	21	10
Asia			2
total	71	72	76

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± 0.04 ng/mL), and two South American (0.40 ± 0.08 ng/mL; 0.52 ± 0.05 ng/mL). In 8.3% of the red wines, OTA was detected above the LOD of 0.1 ng/mL, but below the LOQ of 0.5 ng/mL, at concentrations ranging from 0.11 to 0.43 ng/mL. No other mycotoxins were detected in red or white wine samples. Mycotoxins were not detected in any of the beer samples.

The European Union established a maximum permitted limit of 2.0 μg/kg for OTA in wine.[10] No other regulations were set for other mycotoxins in wine. The concentration of OTA is usually correlated with color and geographical region. OTA levels usually decrease from red through rosé to white wines.[4,6,11−14] Our results of OTA in wines are in agreement with these studies. However, one study showed that the incidence and concentration of OTA in red wines were similar (70% positive; 0.2 μg/L) to those of white (63% positive; 0.21 μg/L), whereas rosé had a lower concentration (60% positive; 0.09 μg/L).[15] The greater contamination of red versus white wines may be due to the longer processing time of red wines. White grapes are immediately pressed after being harvested and the juice is fermented. Red wine grapes are mashed, and the skin and juice are set aside for several days. During this processing stage of pureeing for the red wines, aerobic conditions and high temperatures can favor the growth of molds and cause OTA production.[12−16]

In the majority of surveys, the higher prevalence and concentration of OTA in wines originated with increasing latitude of the producing countries.[11−13,17] Labrinea et al.[15] observed increasing concentrations of ochratoxin A from north to south and west to east in their survey of Greek wines. Wine samples collected from northern, western, and central Greece showed 22 contaminated samples of 43 tested, whereas southern Greece and the Aegean Islands had 54 positive of 77 samples and 28 positive of 30 samples, respectively.[15] Brera et al.[18] also observed a gradually increasing mean concentration from northern (0.05 ng/mL) to southern Italy (0.54 ng/mL). This may be due to the hotter and more humid conditions that can favor better the growth of the *Aspergillus carbonarius* and the consequent production of OTA.[15,18] With regard to the production year, Labrinea et al.[15] observed that 1999 was the year with the highest incidence and contamination of OTA in Greece, whereas 2000 recorded the least contamination. This was due to increased rainfall and relative humidity during the harvesting period, which favors growth of OTA-producing fungi.[15] Brera et al.[18] showed that the correlation between the harvest years and OTA concentration in wine production in Italy for the years 1997, 2002, and 2004 reported higher results with higher standard deviation values. This may be attributed to the very hot and humid conditions that occurred in the harvest season for those years.[18]

Zimmerli et al.[19] first detected the presence of OTA in 123 commercial wines in 1996 with the following median concentrations: white, 3 ng/mL; rosé, 19 ng/mL; red, 13 ng/mL; and dessert, 337 ng/mL. Since then, much research and many surveys have been conducted on the occurrence of ochratoxin A in wine in North and South America, Europe, Africa, and Australia.[13,15,17−19] Soleas et al.[4] surveyed 40 U.S. white wines and 71 U.S. red wines and did not detect OTA in the white wines and but did detect OTA in about 11% of the red wines, which is consistent with our results. Of 942 wines from Europe, Canada, South Africa, Australia, South America, New Zealand, and the United States surveyed for OTA, 3.9% of the white and 16.2% of the red wines had concentrations >0.05 ng/mL.[4] Shephard et al.[6] found detectable OTA levels (>0.01 μg/L) in all 24 South African wines tested (15 white and 9 red), with a mean of 0.16 μg/L in the white wines and a mean of 0.24 μg/L in the red wines.

Cereal grains (barley, wheat, corn, rice) are frequently contaminated with mycotoxins such as aflatoxins, fumonisins, ochratoxin A, and tricothecenes (deoxynivalenol, T-2, and HT-2 toxin). Because cereals are raw materials of beer, these mycotoxins can also be detected in beer because they survive the brewing process.[20,21] At present, the European Union has set a maximum allowable limit for OTA of 0.2 μg/kg in beer with <6% alcohol content.[10] OTA occurrence has been related to the contamination of malt barley with ochratoxigenic species, especially *Penicillum verrucosum*. Ochratoxin A has not been detected at >1 ng/mL in beer.[20] Between 13 and 32% of the OTA present in the original malt can be found in the beer.[22] High concentrations of aflatoxins and zearalenone have been found in local beers brewed in Africa, but aflatoxins have not been detected in European beers. DON, which survives the brewing process, has been found in Canadian and European beers, with concentrations of >200 ng/mL in many German beers. Fumonisins B1 and B2 are not frequently detected in beer.[20] Romero-González et al.[21] detected HT-2 toxin at 0.9 μg/L, FB2 at 2.8 μg/L, T-2 at 1.0 μg/L, and AFB1 at 0.6 μg/L in Spanish beer. Tamura et al.[5] analyzed 24 commercial beer-based drinks from Japan and detected nivalenol, deoxynivalenol, and fumonisins in several sample, but concentrations were under the limit of quantification (<5 ng/mL).

The dilute and shoot method for the preparation of wines and beer for multiple mycotoxin determination using UHPLC-MS/MS allows for a rapid analysis of analytes with different chemical and physical properties. This study showed that internal standards efficiently compensate for matrix effects. Application of this method to the USDA-funded nationwide survey of domestic and imported wines showed that T-2 toxin was detected in 11% of the white wines at levels above the LOD and another 11% above the LOQ. OTA was found in 8.3% of red wines above the LOD. There were no mycotoxins detected in beers. This study suggests that human exposure to mycotoxins from wines and beers is low.

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