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Determination of Mycotoxins in Milk-Based Products and Infant Formula Using Stable Isotope Dilution Assay and Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT: A stable isotope dilution assay and liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated for the determination of 12 mycotoxins, aflatoxins B_1, B_2, G_1, G_2, and M_1, deoxynivalenol, fumonisins B_1, B_2, and B_3, ochratoxin A, T-2 toxin, and zearalenone, in milk-based infant formula and foods. Samples were fortified with 12 ^{13}C uniformly labeled mycotoxins ([^{13}C]-mycotoxins) that correspond to the 12 target mycotoxins and prepared by dilution and filtration, followed by LC-MS/MS analysis. Quantitation was achieved using the relative response factors of [^{13}C]-mycotoxins and target mycotoxins. The average recoveries in fortified milk, milk-based infant formula, milk powder, and baby yogurt of aflatoxins B_1, B_2, G_1, and G_2 (2, 10, and 50 μg/kg), aflatoxin M_1 (0.5, 2.5, and 12.5 μg/kg), deoxynivalenol, fumonisins B_1, B_2, and B_3 (40, 200, and 1000 μg/kg), ochratoxin A, T-2 toxin, and zearalenone (20, 100, and 500 μg/kg), range from 89 to 126% with RSDs of <20%. The individual recoveries in the four fortified matrices range from 72% (fumonisin B_3, 20 μg/kg, milk-based infant formula) to 136% (T-2 toxin, 20 μg/kg, milk powder), with RSDs ranging from 2 to 25%. The limits of quantitation (LOQs) were from 0.01 μg/kg (aflatoxin M_1) to 2 (fumonisin B_1) μg/kg. Aflatoxin M_1 was detected in two European Reference materials at 0.127 ± 0.013 μg/kg (certified value = 0.111 ± 0.018 μg/kg) and 0.46 ± 0.04 μg/kg (certified value = 0.44 ± 0.06 μg/kg), respectively. In 60 local market samples, aflatoxins B_1 (1.14 ± 0.10 μg/kg) and B_2 (0.20 ± 0.03 μg/kg) were detected in one milk powder sample. Aflatoxin M_1 was detected in three imported samples (condensed milk, milk-based infant formula, and table cream), ranging from 0.10 to 0.40 μg/kg. The validated method provides sufficient selectivity, sensitivity, accuracy, and reproducibility to screen for aflatoxin M_1 at nanograms per kilogram concentrations and other mycotoxins, without using standard addition or matrix-matched calibration to compensate for matrix effects.

KEYWORDS: *mycotoxins, stable isotope dilution assay, LC-MS/MS, milk-based infant formula, milk*

INTRODUCTION

Mycotoxins are routinely screened for in milk, milk-based foods, and infant formula because dietary intake of these natural contaminants can pose a threat to consumers, especially children and infants. Mycotoxins are toxic metabolites generated by fungi growing in foods and animal feeds. Aflatoxin B_1 (Figure 1, **1**), a metabolite of *Aspergillus flavus* and *Aspergillus parasiticus*, is often found in animal feeds that contain corn, peanut meal, and other agricultural commodities. Aflatoxin B_1 in contaminated cattle feed can be converted to aflatoxin M_1 via oxidation metabolism[1,2] and subsequently excreted in milk by lactating cattle.[3] Previous studies have demonstrated the potential toxicity and carcinogenicity of aflatoxins B_1 and M_1 (Figure 1, **1** and **5**, respectively).[4−6]

Regulatory agencies have established regulatory limits or action levels for aflatoxin M_1 in milk products to ensure that milk products are safe and wholesome. According to U.S. FDA Compliance Policy Guidance, regulatory actions could be initiated if detected concentrations of aflatoxin M_1 in milk are >0.5 μg/kg (ppb).[7] The European Union (EU) maximum levels of aflatoxin M_1 are 0.05 μg/kg in milk and 0.025 μg/kg in infant formula.[8] Other mycotoxins, fumonisins, ochratoxin A, T-2 toxin, and zearalenone (Figure 1, **6**−**12**), could also be present in milk or milk-based products due to consumption of contaminated animal feeds or direct contamination of fungi such as pathogenic *Aspergillus*, *Penicillium*, or *Fusarium* species.[9−13]

Monitoring the concentrations of mycotoxins in milk, milk-based infant formula (milk as major ingredient), or milk-based foods can be achieved using enzyme immunoassay,[14] immunoaffinity column cleanup with thin layer chromatography (TLC),[15] liquid chromatography (LC)−fluorescence,[16,17] or liquid chromatography tandem mass spectrometry and high-resolution mass spectrometry (LC-MS/MS and LC-HRMS).[18−20] For regulatory applications, violative samples (samples containing toxins above the regulatory limit) analyzed by enzyme immunoassay, TLC, or LC-fluorescence often require confirmation by LC-MS methods.[7] Increasingly, LC-MS/MS and LC-HRMS methods have been developed, validated, and utilized due to their superior sensitivity, selectivity, and specificity. Following

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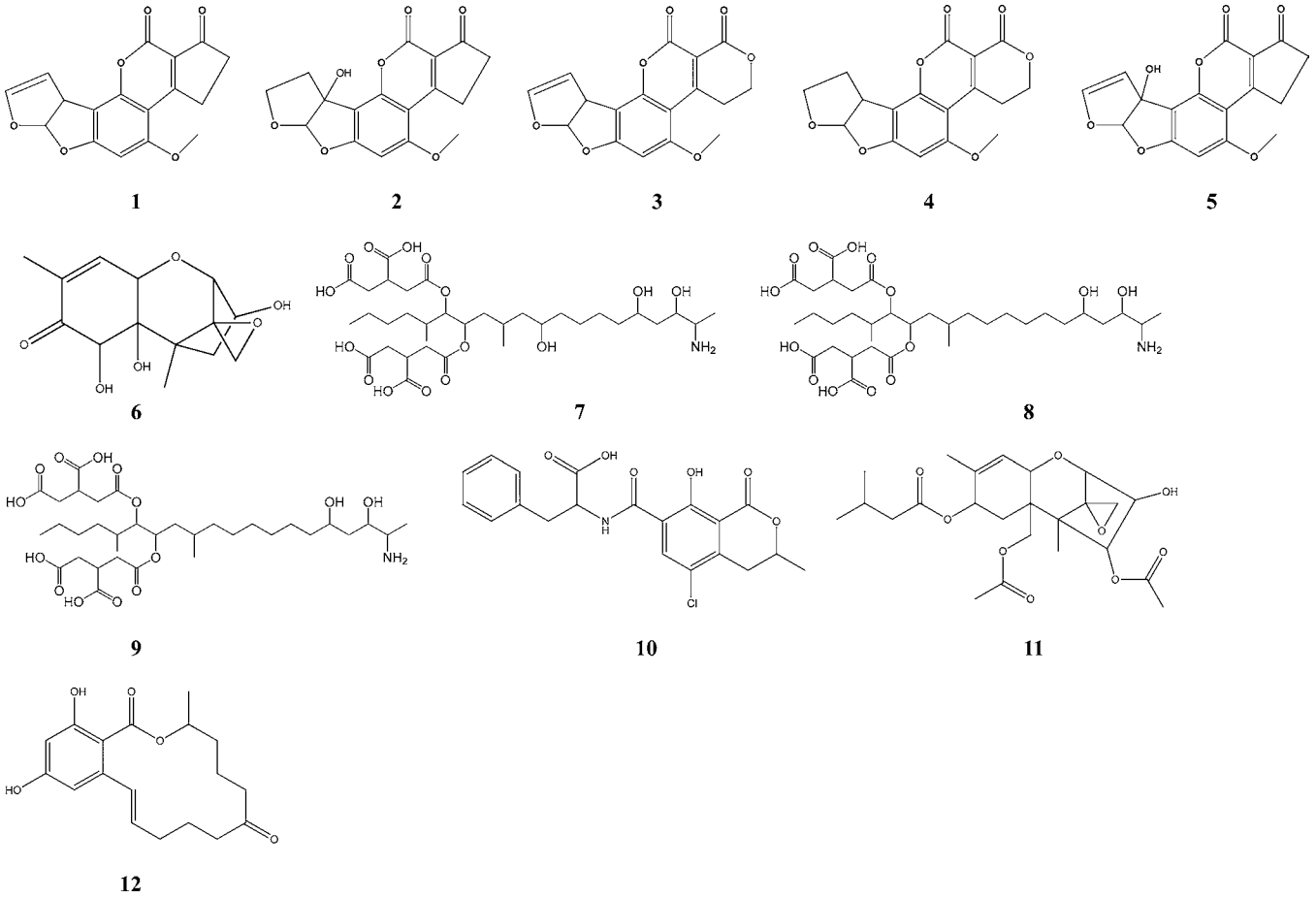

Figure 1. Structures of aflatoxins B_1 (**1**), B_2 (**2**), G_1 (**3**), G_2 (**4**), and M_1 (**5**), deoxynivalenol (**6**), fumonisins B_1 (**7**), B_2 (**8**), and B_3 (**9**), ochratoxin A (**10**), T-2 toxin (**11**), and zearalenone (**12**).

EU identification criteria,[21] a large number of mycotoxins could be identified, quantitated, and confirmed in a single LC-MS analysis.[22,23]

LC-MS is prone to matrix effects, which generally refers to the observation that ionization (signal intensity) of target analytes is affected by coeluted matrix components, resulting in different responses of the same analytes in calibration solvent and sample matrices at the same concentrations.[24] Matrix effects are dependent on analytes, sample matrices, and LC-MS conditions, as well as sample preparation. So far, there is no ideal approach to address this issue due to the unpredictable nature of matrix effects.[25] Researchers could use standard addition,[26] customized sample preparation,[27] matrix-matched calibration standards,[28] dilution,[29,30] and/or internal standards (IS)[31] to compensate for matrix effects. Standard addition becomes impractical and tedious when one has to quantitate multiple analytes at different concentrations in the same samples. Customized sample preparation requires knowledge of matrices prior to sample preparation and LC-MS analysis. It would be more time-consuming and costly to customize sample preparation procedures for different samples. Matrix-matched calibration, one of the most widely used approaches, does not always work effectively due to the lack of appropriate "blank matrix" or difference between selected "blank matrix" used for making matrix-matched calibration standards and real sample matrix.[28] Depending on the sample preparation procedure, preparation of matrix-matched calibration standards could be a demanding task for routine analysis, leading to lower sample throughput. Dilution (often referred to as "dilute-and-shoot") is preferable, but it requires sensitive instruments, and it is challenging to determine dilution factors for trace analysis of multiple analytes with different sensitivities in various matrices.[32] The dilute-and-shoot technique was demonstrated in two recent studies, one for pesticides, mycotoxins, plant toxins, and veterinary drugs in feed and food matrices[33] and the other for pesticides and veterinary drugs in honey.[34]

The use of internal standards , especially stable isotope dilution, can compensate for matrix effects in an efficient manner. Additionally, if internal standards are fortified prior to sample preparation, they also can offset the variation in signals of target analytes caused by sample preparation. The selection of the IS depends on molecular structures, physicochemical properties, availability, and cost of candidate compounds. For this study, we chose [^{13}C]-uniformly labeled mycotoxins ([^{13}C]-mycotoxins) as internal standards. Because [^{13}C]-mycotoxins have molecular structures and physicochemical properties almost identical to those of their native counterparts, they can closely replicate conditions undergone by target mycotoxins through the entire analytical procedure. By monitoring the relative response of [^{13}C]-mycotoxins and target mycotoxins, we can perform quantitation with less concern about potential signal suppression or the loss of target mycotoxins caused by sample preparation. Stable isotope dilution using [^{13}C]-mycotoxins would facilitate quantitation, simplify method development, and ensure quality for the analysis, provided that the affordability and availability of [^{13}C]-mycotoxins are not concerns.[35]

Although the application of isotope dilution assay on mycotoxin analysis can be traced back to the 1980s,[36] the application used to be limited because of the availability and costs of isotope-labeled mycotoxins. Using modern LC-MS with increasing sensitivity, one only needs to fortify each sample with a trace amount of [^{13}C]-mycotoxins, significantly decreasing the operation cost. More [^{13}C]-mycotoxins are also commercially available. Therefore, it is worthwhile to explore potential applications of stable isotope dilution assay for screening mycotoxins in foods. So far, only a handful of stable isotope dilution assays and LC-MS/MS have been developed for the determination of multiple mycotoxins in beers and wines,[37] maize,[38] or animal feeds.[39]

In recent years, several incidences of natural occurrence of mycotoxins in addition to aflatoxin M_1 in milk or milk-based infant food have been reported. In Italy, a survey of zearalenone in infant food indicated that 17 (9%) milk samples were contaminatedwith zearalenone at a maximum of 0.76 μg/L.[40] In another study, ochratoxin A was detected in 133 (72%) infant formulas marketed in Italy with contamination levels ranging from 35.1 to 689.5 ng/L.[41] In China, ochratoxin A (1.43μg/L) and aflatoxins B_1 (0.25μg/L) and M_1 (0.57 μg/L were found in one of three milk samples.[42] In the United States, fumonisin B_1 was found in 1 of 165 milk samples at an unspecified low level.[43] Fumonisin B_1 was also detected in 8 of 10 milk samples analyzed in Italy.[44] Fumonisin B_1 levels ranged from 0.26 to 0.43 μg/kg. The presence of multimycotoxins in milk has not been assessed because of the lack of sensitive analytical techniques for this matrix. The purpose of this work is to develop a stable isotope dilution and LC-MS/MS method for the multimycotoxin analysis in milk-based infant formula, milk, milk powder, and baby yogurt. Twelve mycotoxins, aflatoxins B_1, B_2, G_1, G_2, and M_1, deoxynivalenol, fumonisins B_1, B_2, and B_3, ochratoxin A, T-2 toxin, and zearalenone, and 12 corresponding [^{13}C]-uniformly labeled IS ([^{13}C]-IS) were selected to evaluate the applicability of the method. Besides aflatoxin M_1, no other mycotoxin metabolites were included in this study because of the lack of commercially available labeled standards.

MATERIALS AND METHODS

Chemicals and Materials. Aflatoxins B_1, B_2, G_1, and G_2, deoxynivalenol, fumonisins B_1, B_2, and B_3, ochratoxin A, T-2 toxin, and zearalenone standards were purchased in neat form from Romer Laboratories, Inc. (Union, MO, USA). Aflatoxin M_1 (10 μg/mL) was purchased from Supleco (St. Louis, MO, USA). Stable isotope labeled IS, [$^{13}C_{17}$]-aflatoxin B_1 (500 ng/mL), [$^{13}C_{17}$]-aflatoxin B_2 (500 ng/mL), [$^{13}C_{17}$]-aflatoxin G_1(500 ng/mL), [$^{13}C_{17}$]-aflatoxin G_2 (500 ng/mL), [$^{13}C_{17}$]-aflatoxin M_1 (500 ng/mL), [$^{13}C_{15}$]-deoxynivalenol (25,000 ng/mL), [$^{13}C_{34}$]-fumonisin B_1 (25000 ng/mL), [$^{13}C_{34}$]-fumonisin B_2 (10000 ng/mL), [$^{13}C_{34}$]-fumonisin B_3 (10000 ng/mL), [$^{13}C_{20}$]-ochratoxin A (10000 ng/mL), [$^{13}C_{24}$]-T-2 toxin (25000 ng/mL), and [$^{13}C_{18}$]-zearalenone (25000 ng/mL) were purchased from Romer Laboratories, Inc. Molecular formulas and weights of mycotoxins and isotope-labeled IS are included in Table 1. Three European reference materials, ERM-BD282 (whole milk powder), ERM-283 (low level aflatoxin M_1 in whole milk powder), and ERM-284 (high level aflatoxin M_1 in whole milk powder), were purchased from Analytical Reference Materials International (Golden, CO, USA). Fresh milk, baby yogurt, milk powder, and milk-based infant formula samples were purchased from Washington, DC, metropolitan area or other commercially available sources. LC grade acetonitrile, methanol, water, and MS grade formic acid and ammonium formate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Amicon Ultra-4 centrifugal filters with Ultracel-3 membrane (3 kDa) were purchased from EMD Millipore (Billerica, MA, USA).

Stock and Working Solutions. Stock standard solutions of aflatoxins B_1, B_2, G_1, and G_2, deoxynivalenol, fumonisins B_1, B_2, and B_3, ochratoxin A, T-2 toxin, and zearalenone were prepared by dissolving 5.0 ± 0.1 mg of the mycotoxin in acetonitrile in 25 mL volumetric flasks. Three working standard solutions A, B, and C containing these mycotoxins were prepared. In working solution A, concentrations of these mycotoxins are prepared as follows: 250 ng/mL for aflatoxin M_1; 1000 ng/mL for aflatoxins B_1, B_2, G_1, and G_2; 20000 ng/mL for deoxynivalenol and fumonisins B_1, B_2, and B_3; and 10000 ng/mL for ochratoxin A, T-2 toxin, and zearalenone. Working solution B consists of 50 ng/mL for aflatoxin M_1; 200 ng/mL for aflatoxins B_1, B_2, G_1, and G_2; 4000 ng/mL for deoxynivalenol, and fumonisins B_1, B_2, and B_3; and 2000 ng/mL for ochratoxin A, T-2 toxin, and zearalenone. Working solution C consists of 10 ng/mL for aflatoxin M_1, 40 ng/mL for aflatoxins B_1, B_2, G_1, and G_2, 800 ng/mL for deoxynivalenol and fumonisins B_1, B_2, and B_3, and 400 ng/mL for ochratoxin A, T-2 toxin, and zearalenone. The three working solutions were prepared by diluting and pipetting the appropriate amount of each individual stock standard solution 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 this study were stored at −20 °C in the dark.

Calibration standards were prepared from the working standard solutions by the dilution of working solutions. The mixed isotope-labeled IS solution was prepared by mixing appropriate amount of each of [^{13}C]-IS in a 5 mL volumetric flask and bringing it up to volume with acetonitrile/water (50:50, v/v). The final concentrations are as follows: 50 ng/mL for [$^{13}C_{17}$]-aflatoxin B_1, [$^{13}C_{17}$]-aflatoxin B_2, [$^{13}C_{17}$]-aflatoxin G_1, [$^{13}C_{17}$]-aflatoxin G_2, and [$^{13}C_{17}$]-aflatoxin M_1; 2000 ng/mL for [$^{13}C_{15}$]-deoxynivalenol; 900 ng/mL for [$^{13}C_{34}$]-fumonisin B_1, [$^{13}C_{34}$]-fumonisin B_2, and [$^{13}C_{34}$]-fumonisin B_3; 500 ng/mL for [$^{13}C_{20}$]-ochratoxin A; 2000 ng/mL for [$^{13}C_{24}$]-T-2 toxin; and 3000 ng/mL for [$^{13}C_{18}$]-zearalenone .

Sample Preparation and Recovery Studies. In general, sample preparation consisted of three steps: dilution, centrifugation, and filtration. Samples (0.5 ± 0.01 g of milk, milk-based infant formula, milk powder, or baby yogurt) were weighed out in 15 mL disposable glass vials and then fortified by 25 μL of IS solution and vortexed for 30 s. After the addition of 5.0 mL of acetonitrile/water (50:50, v/v), the glass vials were capped and placed on a shaker with pulsation (Glas-Col, Terre Haute, IN, USA) and shaken for 10 min at a speed set to 50 and pulser frequency set at 30−35 pulsations/min. An aliquot of 2.0 mL of each sample was transferred to an Amicon Ultra-4 centrifugal filter with Ultracel-3 membrane (molecular weight cutoff value of 3 kDa) and centrifuged for 30 min at 4500 rpm (4200g) using a centrifuge. The resulting filtrates were pipetted in autosampler vials for LC-MS/MS analysis. Recovery studies were carried out using whole milk, milk-based infant formula, whole milk powder (ERM-BD282), and baby yogurt at three fortification levels (Table 3). The prepared working solution A, B, or C (25 μL) and IS solution (25 μL) were added to 0.5 g of milk powder, milk-based infant formula, milk, or yogurt and then prepared following the dilution, centrifugation, and filtration procedures described above. At each fortification level, samples were prepared in quadruplicates. No mycotoxins were detected in blank samples used for recovery studies.

LC-MS/MS Analysis. A Shimadzu Prominence/20 series (Columbia, MD, USA) liquid chromatograph coupled with an Applied Biosystems (Forest City, CA, USA) 4000 or 6500 quadruple linear ion trap (QTrap) mass spectrometer equipped with an electrospray ionization (ESI) interface source were evaluated and compared for instrumental performance in terms of sensitivity and linear range. The 6500 QTrap was chosen and employed for sample analysis. The column used was a 100 mm × 2.1 mm i.d., 2.6 μm, Phenomenex Kinetex XB-C18, with a 10 mm × 2.1 mm i.d. guard cartridge (Torrance, CA. USA). The LC mobile phases consist of 10 mM ammonium formate/0.1% formic acid/water (A) and 10 mM ammonium formate/0.1% formic acid/methanol (B). Gradient elution at 0.3 mL/min flow rate was begun at 5% B, ramped to 40% B in 2 min via linear gradient mode and then to 100% B by 9 min via exponential gradient mode (pump B curve 3 to 6), held for 2.5 min, and changed to 5% B at 12 min. Total run time was 15 min

Table 1. Name, Molecular Formula, Molecular Weight (Da), Retention Time (RT), MRM Transition, Declustering Potential (DP), Collision Energy (CE), Entrance Potential (EP), and Exit Potential (EXP) of Native Mycotoxins and [^{13}C]-IS

[^{13}C]-IS	mol formula	mol wt	RT (min)	MRM transition	DP (eV)	EP (eV)	CE (eV)	CXP (eV)	mycotoxin	mol formula	mol wt	RT (min)	MRM transition	DP (eV)	EP (eV)	CE (eV)	CXP (eV)
[$^{13}C_{17}$]-aflatoxin B_1	$^{13}C_{17}H_{12}O_6$	329.1	7.0	**330.1→255.2**[a]	86	10	57	14	aflatoxin B_1	$C_{17}H_{12}O_6$	312.1	7.0	**313.1→241.0**	86	10	55	14
			7.0	330.1→301.0	106	10	37	8				7.0	313.1→285.0	106	10	37	8
[$^{13}C_{17}$]-aflatoxin B_2	$^{13}C_{17}$ $H_{14}O_6$	331.1	6.5	**332.0→303.2**	91	10	39	16	aflatoxin B_2	C_{17} $H_{14}O_6$	314.1	6.5	**315.2→287.1**	91	10	39	16
			6.5	332.0→273.1	91	10	45	14				6.5	315.2→259.1	91	10	45	14
[$^{13}C_{17}$]-aflatoxin G_1	$^{13}C_{17}$ $H_{12}O_7$	345.1	5.9	**345.8→257.1**	86	10	41	14	aflatoxin G_1	C_{17} $H_{12}O_7$	328.1	5.9	**328.8→243.2**	86	10	41	12
			5.9	345.8→124.2	86	10	99	10				5.9	328.8→115.1	86	10	99	20
[$^{13}C_{17}$]-aflatoxin G_2	$^{13}C_{17}$ $H_{14}O_7$	347.1	5.4	**348.0→330.0**	111	10	36	18	aflatoxin G_2	C_{17} $H_{14}O_7$	330.1	5.4	**331.2→313.0**	111	10	36	18
			5.4	348.0→259.0	111	10	49	20				5.4	331.2→245.0	111	10	49	20
[$^{13}C_{17}$]-aflatoxin M_1	$^{13}C_{17}$ $H_{12}O_7$	345.1	5.5	**346.1→288.1**	81	10	37	16	aflatoxin M_1	C_{17} $H_{12}O_7$	328.1	5.5	**329.1→273.0**	81	10	37	16
			5.5	346.1→242.2	81	10	59	14				5.5	329.1→229.2	81	10	59	12
[$^{13}C_{15}$]-deoxynivalenol	$^{13}C_{15}H_{20}O_6$	311.2	3.2	**312.0→263.0**	71	10	17	44	deoxynivalenol	$C_{15}H_{20}O_6$	296.1	3.2	**297.0→249.0**	71	10	17	44
			3.2	312.0→345.2	61	10	21	22				3.2	297.0→231.2	61	10	21	22
[$^{13}C_{34}$]-fumonisin B_1	$^{13}C_{34}H_{59}NO_{15}$	755.6	9.3	**756.4→374.5**	111	10	53	10	fumonisin B_1	$C_{34}H_{59}NO_{15}$	721.4	9.3	**722.5→352.5**	111	10	53	10
			9.3	756.4→356.4	111	10	57	54				9.3	722.5→334.5	111	10	57	54
[$^{13}C_{34}$] fumonisin B_2	$^{13}C_{34}H_{59}NO_{14}$	739.5	9.9	**740.3→358.4**	106	10	55	10	fumonisin B_2	$C_{34}H_{59}NO_{14}$	705.4	9.9	**706.3→336.3**	106	10	55	10
			9.9	740.3→340.5	106	10	59	20				9.9	706.3→318.3	106	10	59	20
[$^{13}C_{34}$]- fumonisin B_3	$^{13}C_{34}H_{59}NO_{14}$	739.5	9.7	**740.5→358.4**	106	10	55	10	fumonisin B_3	$C_{34}H_{59}NO_{14}$	705.4	9.7	**706.3→336.3**	106	10	55	10
			9.7	740.5→340.4	106	10	59	20				9.7	706.3→318.3	106	10	59	20
[$^{13}C_{20}$]-ochratoxin A	$^{13}C_{20}H_{18}ClNO_6$	423.1	9.5	**424.1→250.1**	66	10	41	16	ochratoxin A	$C_{20}H_{18}ClNO_6$	403.1	9.5	**404.0→239.0**	66	10	41	16
			9.5	424.1→110.1	66	10	101	16				9.5	404.0→102.0	66	10	101	16
[$^{13}C_{24}$]-T-2 toxin	$^{13}C_{24}H_{34}O_9$	490.3	9.4	**508.3→229.2**	56	10	29	17	T-2 toxin	$C_{24}H_{34}O_9$	466.2	9.4	**484.3→215.2**	57	10	29	17
			9.4	508.3→198.2	56	10	33	11				9.4	484.3→185.1	57	10	33	11
[$^{13}C_{18}$]-zearalenone	$^{13}C_{18}H_{22}O_5$	336.1	9.8	**337.2→138.1**	71	10	79	8	zearalenone	$C_{18}H_{22}O_5$	318.1	9.8	**319.2→128.0**	71	10	79	8
			9.8	337.2→199.3	71	10	31	14				9.8	319.2→187.2	71	10	31	14

[a]MRM in bold was used for quantitation.

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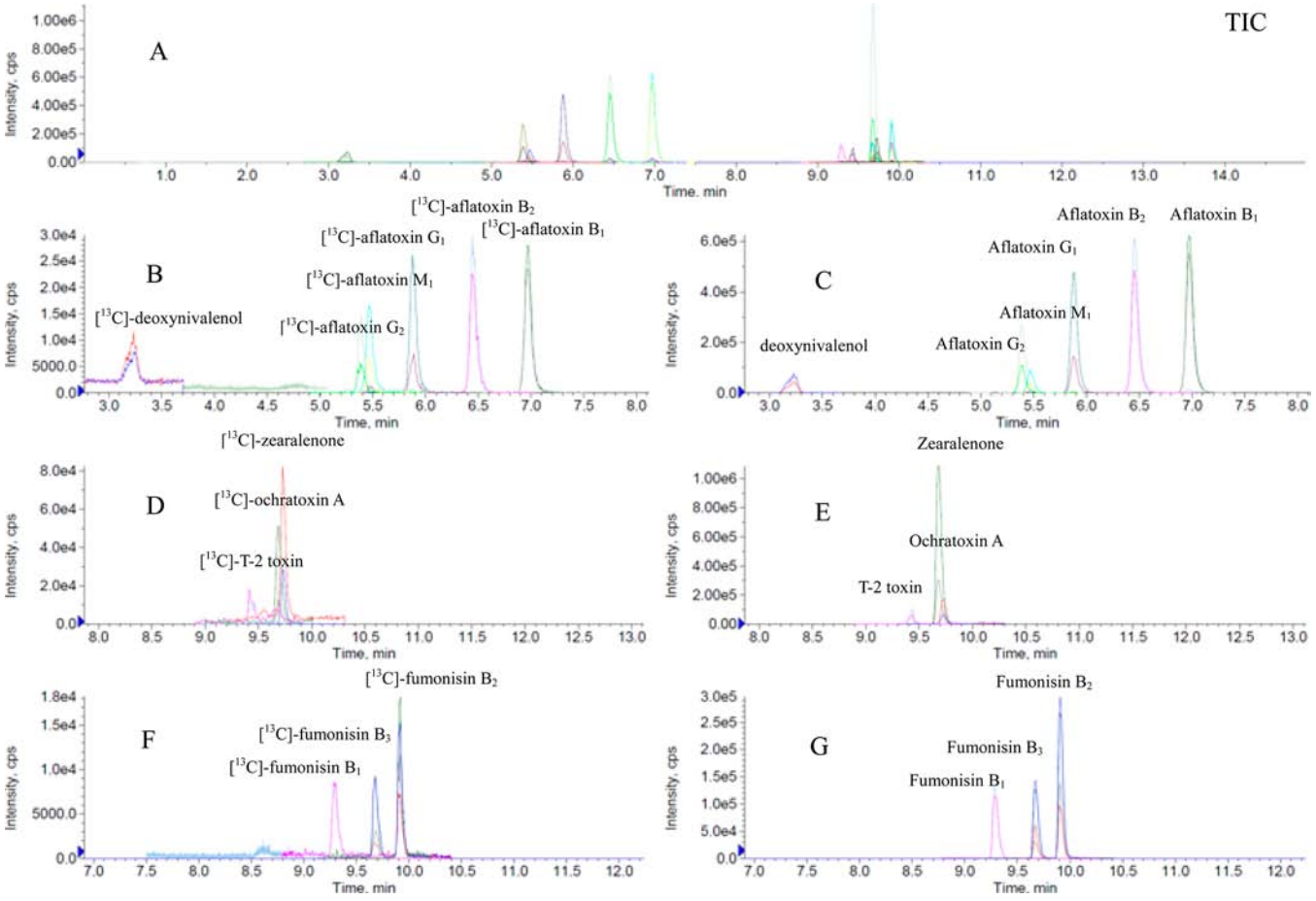

Figure 2. Extracted ion chromatogram of native mycotoxins and [^{13}C]-IS fortified milk sample: (A) full extracted ion chromatogram; (B) region between 2.5 and 8 min ([^{13}C]-aflatoxins and [^{13}C]-deoxynivalenol); (C) region between 2.5 and 8 min (native aflatoxins and deoxynivalenol); (D) region between 8 and 13 min ([^{13}C]-ochratoxin A, [^{13}C]-T-2 toxin, and [^{13}C]-zearalenone); (E) region between 8 and 13 min (native ochratoxin A, T-2 toxin, and zearalenone); (F) region between 7 and 12 min ([^{13}C]-fumonisins); (G) region between 7 and 12 min (native fumonisins).

including 3 min of column conditioning time. The injection volume was 5 μL, and the column temperature was set at 40 °C.

Two MRM transitions of each [^{13}C]-labeled or native mycotoxin were generated using direct infusion on the 4000 QTrap and then transferred to the 6500 QTrap. Because compound-dependent parameters such as declustering potential (DP) and collision energy (CE) could be transferred between the two instruments, the infusion experiments were not repeated. The same mycotoxin calibration standards were analyzed using identical LC conditions with the identical MS/MS transitions for the two instruments so that the sensitivity, linear dynamic range, and linearity of the two instruments could be compared. The instrumental limit of quantitation (LOQ) of each mycotoxin was determined as the lowest concentration at which the S/N of the weaker MRM transition is ≥10. The 4000 and 6500 QTrap were operated in scheduled multiple reaction monitoring (sMRM) mode. All native and [^{13}C]-uniformly labeled mycotoxins were monitored in positive ionization mode. Scan time was set to 0.5 s, and sMRM window was set to 60 s. Nitrogen gas of 99% purity 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) and FDA criteria.[21,45] The mycotoxins were quantitated using the relative response factor between target mycotoxins and their [^{13}C]-uniformly labeled IS fortified to the samples. Ionization source-dependent parameters in positive ionization mode were set as follows: curtain gas (CUR), 30 psi; ion spray voltage, 5500 V; nitrogen collision gas (CAD), medium; source temperature (TEM), 450 °C; ion source gases 1 and 2 (GS1 and GS2), each at 60 psi. Resolution at Q1 and Q3 were set to unit. Retention time, values of DP, EP, CE, and CXP, and the two specific, selected MRM transitions are listed in Table 1 and used for sMRM data acquisition. Analyst 1.6 and MultiQuan 2.0 (Applied Biosystems) were used for data processing. Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) was used to calculate recoveries and RSDs.

RESULTS AND DISCUSSION

Instrument Evaluation. Incurred mycotoxins in milk, milk-based infant formula, or milk products often include aflatoxins, fumonisins, and ochratoxin A. FDA action or advisory levels of these mycotoxins range from 0.5 μg/kg (aflatoxin M_1) to 2000 μg/kg (fumonisins).[7] To analyze these mycotoxins in a single instrumental analysis, selected analytical instruments should have a wide linear dynamic range and sufficient sensitivity. We compared sensitivity and the linear dynamic range of two LC-MS systems, the 4000 QTrap and the 6500 QTrap.

The same mycotoxin calibration standards were analyzed using identical LC conditions with identical MS/MS transitions for the two instruments. For the 12 mycotoxins, the 6500 QTrap outperforms the 4000 QTrap with 3−50 times better sensitivity and 1−2 orders of magnitude more in linear dynamic range. For example, on the 6500 QTrap, the LOQ of aflatoxin M_1 is 0.01 ng/mL, 50 times lower than that (0.5 ng/mL) on the 4000 QTrap. Using the 6500 QTrap, one can obtain a linear calibration curve (coefficient of correlation, r^2 = 0.995) of fumonisin B_1 with concentrations ranging from 2 to 4000 ng/mL, whereas on the

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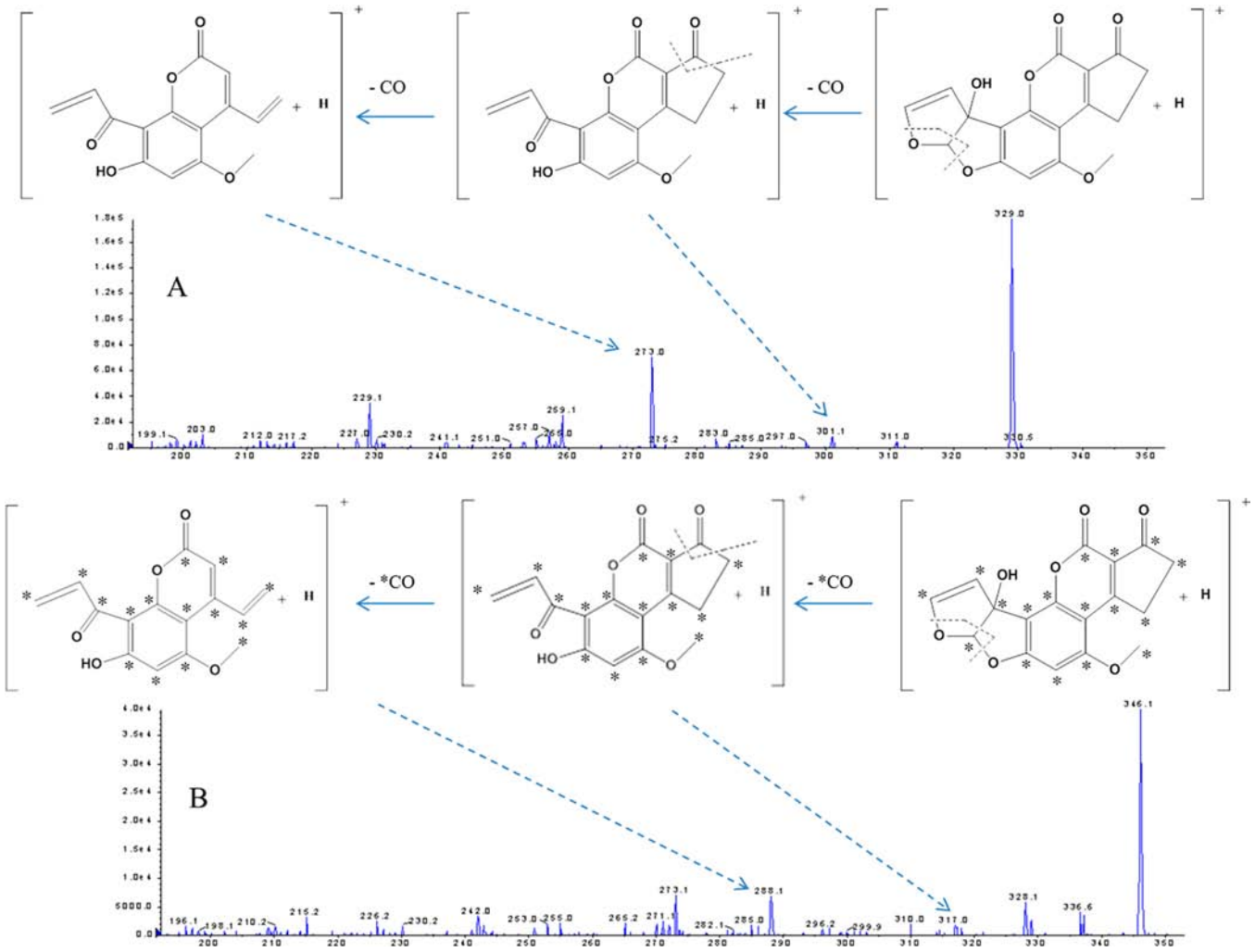

Figure 3. Proposed fragmentation pathways of aflatoxin M_1 (A) and [^{13}C]-aflatoxin M_1 (B) based on product ion spectra (∗ = ^{13}C).

4000 QTrap, the linear range is 10−500 ng/mL with a linearity r^2 = 0.991. The sensitivity and linear range of the 6500 QTrap make it possible to greatly simplify sample preparation, especially with milk, milk powder, or milk-based infant formula fortified at concentrations ranging from 0.5 μg/kg (for aflatoxin M_1) to 1000 μg/kg (for deoxynivalenol and fumonisins B_1, B_2, and B_3) followed by dilution in the recovery studies and sample analysis. Therefore, the 6500 QTrap was employed throughout the study.

Sample Preparation, Stable Isotope Dilution, and Recovery Studies. Compared to potential interferences such as proteins and fats in milk-based infant formula, milk, or milk-based products, the targeted mycotoxins are chemical contaminants with much smaller molecular weights (<800 Da). Separation of mycotoxins from these matrix components could be achieved using filtration membranes with molecular weight cutoff features. Our test portions were first dissolved or diluted 10× times using acetonitrile/water (50:50, v/v) and then centrifuged and filtered through membranes with molecular weight cutoff at 3 kDa. Similar approaches have been used to prepare milk samples for the analysis of veterinary drugs, but the filtration and dilution could not eliminate matrix suppression resulting from coextracted components passed through the membranes.[46,47] Given the fact that matrix suppression is still present and no additional cleanup is preferred, one could facilitate quantitation using dilution, standard addition, or matrix-matched calibration. All of these choices have limitations as previously discussed.

A stable isotope dilution assay was used to correct for the effects of suppression. Every sample was fortified with the 12 [^{13}C]-uniformly labeled mycotoxins ([^{13}C]-IS) that correspond to the 12 target mycotoxins. These [^{13}C]-IS have chromatographic and ionization properties identical to those of the corresponding target mycotoxins so they can closely replicate what the corresponding native mycotoxins would physically and chemically go through in the course of sample preparation and instrumental analysis. Figure 2 shows the identical retention times of target mycotoxins and their [^{13}C]-IS. Figure 3 illustrates that under the same mass spectrometric conditions MRM transitions of aflatoxin M_1 (329 → 273) and [^{13}C]-labeled aflatoxin M_1 (346 → 288) would be generated via the same fragmentation pathways. By monitoring the relative response factors of the MRM transitions of native mycotoxins and their [^{13}C]-IS, one can easily offset the signal suppression, compensate for the loss of target mycotoxins caused by sample preparation, correct volume change due to water content in samples, and eliminate the impact of inconsistent instrumental performance. Furthermore, as long as the fortified [^{13}C]-IS are detected by the instrument, one would know the instrument and sample preparation are working, providing quality assurance without additional efforts.

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Table 2. Relative Response Factors (RRF) of Native Mycotoxins and [^{13}C]-IS

mycotoxin/[^{13}C]-IS	av RRF[a]	RSD (%), n = 9	concn range of target mycotoxin (ng/mL)	concn of fortified [^{13}C]-IS (ng/mL)
aflatoxin B_1/[$^{13}C_{17}$]-aflatoxin B_1	1.15	3	0.08−50.00	0.25
aflatoxin B_2/[$^{13}C_{17}$]-aflatoxin B_2	0.99	5	0.08−50.00	0.25
aflatoxin G_1/[$^{13}C_{17}$]-aflatoxin G_1	0.91	4	0.08−50.00	0.25
aflatoxin G_2/[$^{13}C_{17}$]-aflatoxin G_2	0.94	6	0.08−50.00	0.25
aflatoxin M_1/[$^{13}C_{17}$]- aflatoxin M_1	0.90	5	0.02−12.50	0.25
deoxynivalenol/[$^{13}C_{15}$]-deoxynivalenol	1.32	8	1.60−500.00	10.00
fumonisin B_1/[$^{13}C_{34}$]-fumonisin B_1	0.90	10	1.00−500.00	4.50
fumonisin B_2/[$^{13}C_{34}$]-fumonisin B_2	1.21	11	1.00−500.00	4.50
fumonisin B_3/[$^{13}C_{34}$]-fumonisin B_3	1.75	8	1.00−500.00	4.50
ochratoxin A/[$^{13}C_{20}$]-ochratoxin A	1.43	5	0.80−500.00	2.50
T-2 toxin/[$^{13}C_{24}$]-T-2 toxin	0.90	9	0.80−500.00	10.00
zearalenone/[$^{13}C_{18}$]-zearalenone	0.91	6	0.80−500.00	15.00

[a]Equation 1.

The relative response factor (RRF) of each target mycotoxin was calculated using eq 1, and quantitation was achieved using eq 2.

$$\text{relative response factor (RRF)} = \frac{\text{concn}_{\text{13C-myco}} \times A_{\text{native myco}}}{\text{concn}_{\text{native mcyo}} \times A_{\text{13C-myco}}} \quad (1)$$

$\text{Concn}_{\text{13C-myco}}$ is the concentration of a [^{13}C]-labeled mycotoxin used as internal standard in a calibration standard; $\text{concn}_{\text{native myco}}$ is the concentration of a native mycotoxin within a selected calibration range; $A_{\text{13C-myco}}$ is the peak area of the quantitation ion of the [^{13}C]-labeled mycotoxin measured in a calibration standard; and $A_{\text{native myco}}$ is the peak area of the quantitation ion of the native mycotoxin measured in a calibration standard,

$$\text{concn in sample} = \frac{\text{concn}_{\text{13C-myco}} \times A_{\text{native myco}}}{\text{ARRF} \times A_{\text{13C-myco}}} \quad (2)$$

ARRF is the average relative response factor (average RRFs calculated using eq 1 from multiple calibration levels); $\text{concn}_{\text{13C-myco}}$ is the concentration of a [^{13}C]-labeled mycotoxin used as IS in a sample; concn in sample is the calculated concentration of a native mycotoxin detected in a sample; $A_{\text{13C-myco}}$ is the peak area of the quantitation ion of the [^{13}C]-labeled mycotoxin measured in a sample; and $A_{\text{native myco}}$ is the peak area of the quantitation ion of the native mycotoxin measured in a sample.

Because the fortified concentrations of [^{13}C]-IS remain the same in each sample or calibration standard, it is important to determine the concentration ranges of native mycotoxins within which RRF would be consistent.[48] Table 2 lists the average RRFs, RSDs, concentration ranges of target mycotoxins, and fortified concentrations of [^{13}C]-IS. Within the defined concentration range, the average RRF of each pair of target mycotoxin and [^{13}C]-IS was calculated using RRFs at nine different concentrations. The calculated average RRFs range from 0.90 (T-2 toxin/[^{13}C]-T-2 toxin) to 1.75 (fumonisin B_3/[^{13}C]-fumonisin B_3) with RSDs of <11%.

To test the applicability of the stable isotope dilution and LC-MS/MS method, recovery studies were conducted in four selected matrices: whole milk, milk-based infant formula, milk powder, and baby yogurt. Recoveries of each target mycotoxin were calculated on the basis of average RRFs and eq 2. The majority of the recoveries in the four tested matrices ranged from 70 to 120% with RSDs of <20%, except the recovery and RSD (136%, 25%) of T-2 toxin in milk powder. Average recoveries of each mycotoxin in the four matrices were calculated to demonstrate the ruggedness of the method at each fortification level. The average recoveries of aflatoxins B_1, B_2, G_1, and G_2 (2, 10, and 50 μg/kg) range from 90 to 103% with RSDs of ≤10%, those of aflatoxin M_1 (0.5, 2.5, and 12.5 μg/kg) from 92 to 101% with RSDs of ≤10%, those of deoxynivalenol and fumonisins B_1, B_2, and B_3 (40, 200, and 1000 μg/kg) from 93 to 114% with RSDs of ≤20%, and those of ochratoxin A, T-2 toxin, and zearalenone (20, 100, and 500 μg/kg) from 89 to 126% with RSDs of ≤20% (Table 3). The data demonstrate the method can be used to screen and quantitate the 12 target mycotoxins in milk-based infant formula, milk, and milk-based food.

Analysis of Reference Materials and Market Samples. The performance of the stable isotope dilution and LC-MS procedure was tested using three European Reference Materials (whole milk powders) and 60 samples collected from local stores. In ERM-BD282, aflatoxin M_1 was not detected (certified value < 0.02 μg/kg); in BD283, aflatoxin M_1 was detected at 0.127 ± 0.013 μg/kg, n = 4 (certified value = 0.111 ± 0.018 μg/kg); in BD284, aflatoxin M_1 was detected at 0.46 ± 0.04 μg/kg, n = 4 (certified value = 0.44 ± 0.06 μg/kg). Our results were in good agreement with the certified values. This was a good indication that 0.5 g sample size was sufficient for the homogeneous powder infant formula, liquid milk, milk powder, and baby yogurt. The 60 samples analyzed included 4 cream, 18 milk-based infant formula, 27 milk, 8 milk powder, and 3 baby yogurt samples, among which 15 samples are imported products. In an imported milk powder, aflatoxins B_1 and B_2 were detected at 1.14 ± 0.10 and 0.20 ± 0.03 μg/kg, respectively. Aflatoxin M_1 was detected in three imported condensed milk, milk-based infant formula, and table cream samples at 0.41 ± 0.04, 0.19 ± 0.04, and 0.10 ± 0.01 μg/kg, respectively. No mycotoxins were detected in the other 56 samples. The sampling size of this study is small, so these results should not be interpreted as representative information for the local markets.

This study shows that stable isotope dilution assay and LC-MS/MS can efficiently screen for multiple mycotoxins in milk-based infant formula and milk-based foods in a single and simple sample preparation and instrumental analysis. Additionally, it is worth noting that method validation is simplified using this approach. When analyzing uncommon food matrices, one could use stable isotope dilution to facilitate method development and sample analysis. Despite the high initial instrumentation cost, with more [^{13}C]-IS becoming commercially available and cost-effective and the advent of sensitive LC-MS systems,

Table 3. Recoveries of Native Mycotoxins in Fortified Milk-Based Infant Formula, Milk, Milk Powder, and Baby Yogurt

		recovery (RSD)%, [b] $n = 4$																
mycotoxin	concn[a] (ng/g)	infant formula	milk	milk powder	yogurt	av (RSD)[c] $n = 16$	concn (ng/g)	infant formula	milk	milk powder	yogurt	av (RSD) $n = 16$	concn (ng/g)	infant formula	milk	milk powder	yogurt	av (RSD) $n = 16$
aflatoxin B_1	2	93 (2)	98 (3)	108 (4)	106 (3)	101 (7)	10	88 (5)	105 (3)	90 (2)	105 (2)	97 (9)	50	90 (7)	100 (2)	96 (10)	105 (2)	98 (8)
aflatoxin B_2	2	94 (2)	97 (3)	120 (4)	101 (3)	103 (10)	10	91 (4)	106 (2)	100 (5)	102 (4)	100 (7)	50	91 (3)	102 (2)	106 (9)	101 (4)	100 (8)
aflatoxin G_1	2	90 (5)	95 (2)	115 (1)	104 (5)	101 (10)	10	86 (4)	104 (5)	93 (3)	101 (3)	96 (8)	50	86 (6)	99 (3)	104 (11)	103 (4)	98 (10)
aflatoxin G_2	2	94 (5)	104 (3)	96 (11)	106 (8)	100 (8)	10	86 (5)	104 (4)	83 (4)	98 (4)	93 (10)	50	87 (5)	95 (5)	82 (5)	96 (7)	90 (8)
aflatoxin M_1	0.5	98 (8)	97 (11)	101 (7)	107 (11)	101 (9)	2.5	85 (2)	99 (4)	83 (5)	100 (1)	92 (9)	12.5	85 (6)	97 (5)	87 (7)	104 (4)	93 (10)
deoxynivalenol	40	89 (5)	95 (5)	111 (8)	101 (8)	99 (11)	200	84 (5)	103 (4)	94 (2)	98 (6)	95 (9)	1000	86 (7)	97 (5)	96 (8)	101 (6)	95 (8)
fumonisin B_1	40	110 (9)	99 (9)	111 (10)	98 (14)	105 (11)	200	78 (7)	97 (3)	106 (8)	102 (11)	96 (13)	1000	83 (8)	101 (7)	103 (4)	94 (12)	95 (11)
fumonisin B_2	40	115 (11)	104 (6)	117 (8)	108 (16)	111 (11)	200	76 (12)	98 (1)	102 (1)	106 (7)	95 (13)	1000	81 (6)	98 (2)	101 (3)	94 (20)	94 (13)
fumonisin B_3	40	115 (13)	108 (9)	117 (17)	117 (17)	114 (13)	200	72 (7)	91 (9)	110 (8)	102 (19)	94 (19)	1000	84 (7)	100 (3)	106 (5)	80 (26)	93 (16)
ochratoxin A	20	88 (3)	97 (7)	121 (5)	111 (4)	104 (13)	100	82 (10)	105 (1)	95 (3)	103 (2)	96 (10)	500	90 (8)	101 (4)	101 (9)	106 (2)	99 (8)
T-2 toxin	20	132 (13)	114 (9)	136 (25)	121 (18)	126 (18)	100	101 (10)	133 (10)	99 (20)	101 (2)	109 (17)	500	101 (16)	110 (13)	100 (16)	112 (12)	106 (14)
zearalenone	20	114 (22)	94 (17)	98 (14)	107 (13)	103 (17)	100	82 (10)	105 (1)	95 (3)	103 (2)	99 (20)	500	90 (8)	101 (4)	101 (9)	106 (2)	89 (17)

[a]Fortified concentration. [b]Recovery and corresponding relative standard derivation (RSD, $n = 4$) of a mycotoxin in one of the fortified matrices at a fortified concentration. [c]Average recovery and corresponding relative standard derivation (RSD, $n = 16$) of a mycotoxin in the four matrices at a fortified concentration.

stable isotope dilution will be widely used for routine mycotoxin analysis in the future.

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Notes

The authors declare no competing financial interest.

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