Quantitation of Mycotoxins in Four Food Matrices Comparing Stable Isotope Dilution Assay (SIDA) with Matrix Matched Calibration Methods by LC-MS/MS

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Abstract & Introduction

Mycotoxins are secondary fungal metabolites produced by mold that may be found in food or feed. They can cause severe health problems in humans and animals, and can result in significant economic losses. Among the hundreds of toxic mycotoxins, aflatoxins, fumonisins, deoxynivalenol, ochratoxin A, HT-2 toxin, zearalenone, and T-2 toxin are considered as a major concern for corn, wheat, peanuts and other agricultural products. LC-MS has become the standard and is now widely used for routine mycotoxin analysis or identification. One of the challenges faced by LC-MS techniques is the matrix effect caused by the use of electro-spray ionization (ESI). Generally, sample preparation, chromatographic and calibration techniques are the common strategies for reducing the negative effects of matrix effects. Standard addition, matrix matching, and SIDA are all possible calibration solutions.

In this work, a quick "dilute-filter-shoot" method was used for sample preparation. A 7-min. LC-MS/MS method using a biphenyl column was developed and verified for quantifying 12 mycotoxins in 4 matrices (corn, peanut butter, brown rice, and corn/wheat mix). Both SIDA and matrix matched calibration methods were applied, compared, and evaluated in terms of recovery, efficiency, advantages, and limitations.

Methods

Table 1: Instrument and Sample Preparation Methods

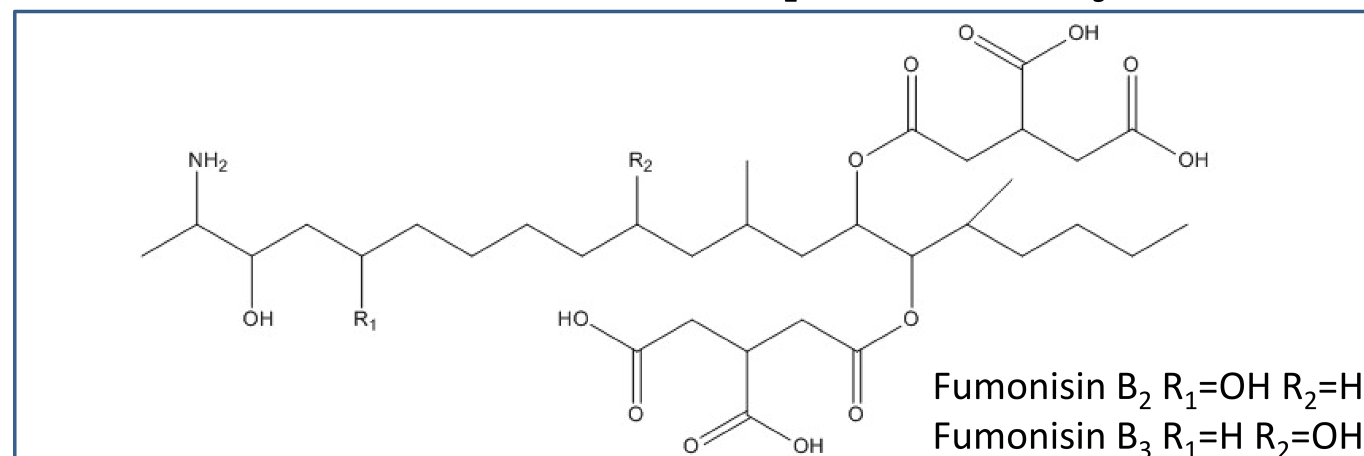
Analytical Column:	Raptor Biphenyl 2.7 µm, 50 mm x 2.1 mm (Restek Part No. 9309A52)	
Instrument:	Shimadzu Nexera X2 UHPLC	
Mobile Phase A:	2 mM ammonium formate with 0.1% formic acid in water	
Mobile Phase B:	2 mM ammonium formate with 0.1% formic acid in methanol	
Gradient:	Time (min)	%B
	0.60	30
	0.70	50
	3.00	70
	4.50	75
	5.00	90
	5.20	90
	5.21	75
	6.00	75
	6.01	30
	7.00	30
Injection Volume:	5 µL	
Column Temp.:	40 °C	
Detector:	Shimadzu LCMS-8060	
Ion Mode:	ESI+	

The twelve mycotoxins and six ^{13}C uniformly labeled mycotoxins were purchased from Romer Laboratories Inc. Corn flour, peanut powder, brown rice flour, and corn with wheat flour were purchased from commercially available sources. Two maize reference materials (TET017RM and T04301Q) were obtained from Fapas.

For matrix matched calibration curve and QC samples, 1.00 ± 0.02 g of the homogenized sample was mixed with 2 mL water and 4 mL of extraction solvent (water:acetonitrile, 50:50, v/v). After vortex and centrifugation, a 475 µL of the supernatant was transferred and filtered using a Thomson SINGLE StEP Filter Vial with a 0.2 µm PTFE filter. A volume of 250 µL of the filtered matrix extract was then combined with one of the nine calibration standards prepared in extraction solvent.

For SIDA technique, a calibration curve was constructed using calibration standards in extraction solvents as well as their uniformly ^{13}C-labeled internal standards (ISs). Samples of the Fapas reference materials were only evaluated using the SIDA calibration technique

Figure 1 : Chemical Structure of Fumonisin B_2 and Fumonisin B_3



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Results and Discussion

All 12 mycotoxins studied were analyzed in 5.5 minutes, as shown in Figure 2. This list includes two isobaric mycotoxins, fumonisins B_2 and B_3 (Figure 1), which were fully resolved chromatographically.

Figure 2 : Chromatogram of Mycotoxins with ISs in Solvent

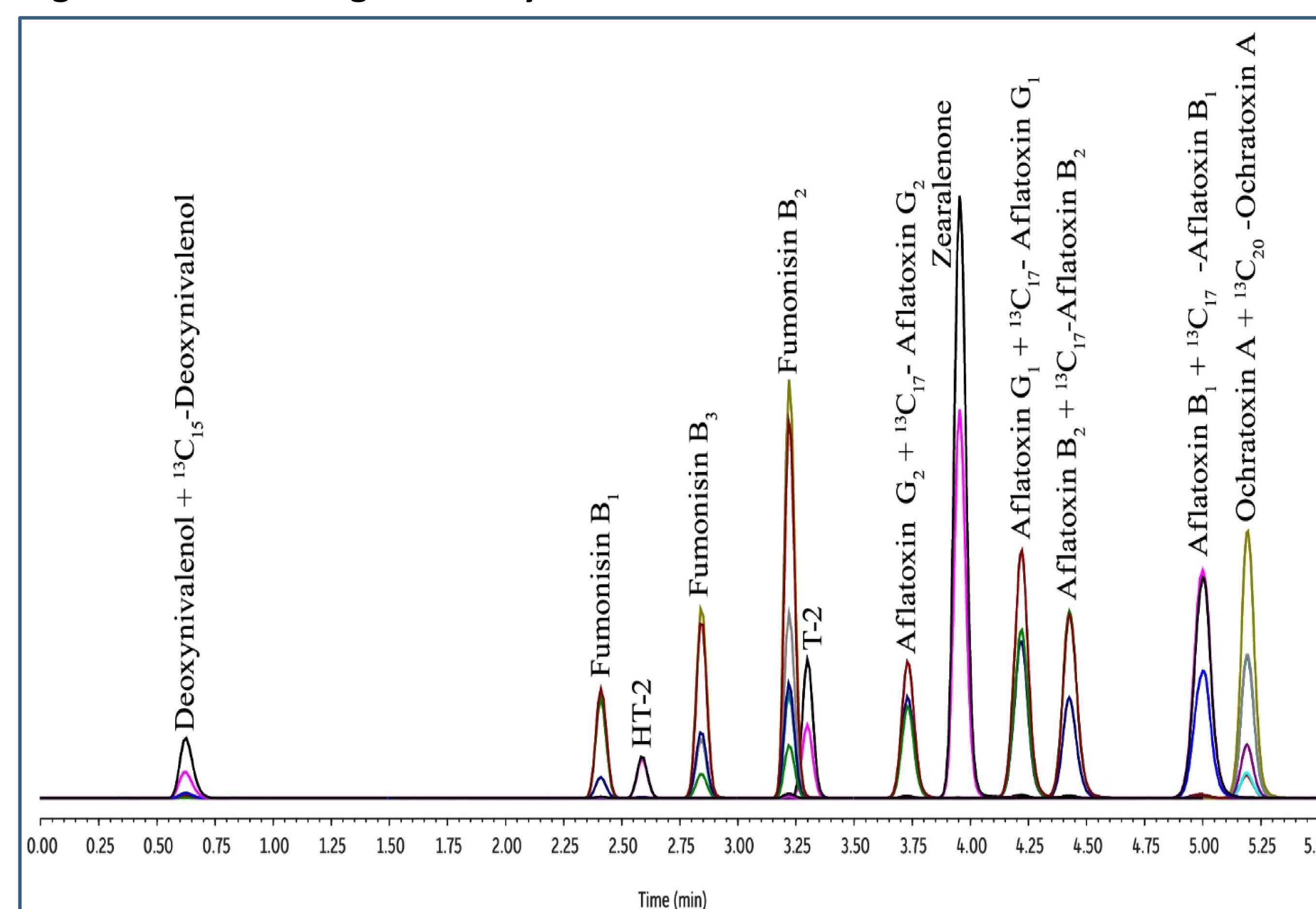
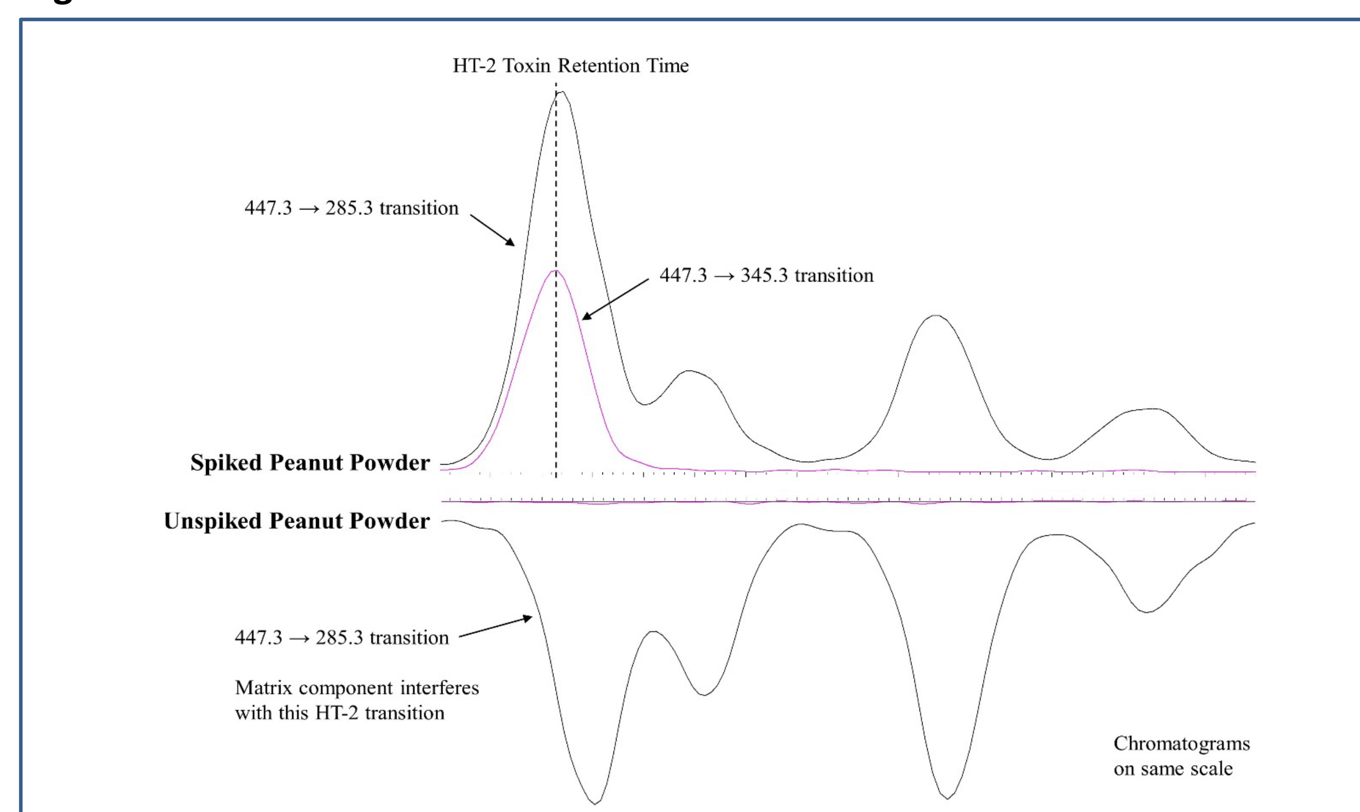


Figure 3 : Interference of MRM Transitions for HT-2 Toxin in Peanut Powder



A matrix-related interference was observed for the primary MRM transition for HT-2 toxin (447.3–>285.3) in peanut powder (Figure 3). This interference resulted in the need to select another, less abundant MRM transition for quantification (447.3–>345.3). As a result, the lowest level calibration point (5.00 ng/mL) was not observed.

One challenge using matrix matched calibration is the presence of incurred mycotoxins in matrix blanks. In this study, mycotoxins were observed in the commercially purchased commodities, therefore, the SIDA calibration technique was applied for calculation (Table 2).

Table 2: Concentrations of Incurred Mycotoxins using SIDA Calibration

Matrix	Back-Calculated Incurred Mycotoxin Concentrations (ng/g)					
	DON	AFG_2	AFG_1	AFB_2	AFB_1	Och A
Corn Flour	103	0.870	n.d.	n.d.	n.d.	n.d.
Peanut Powder	103	1.18	n.d.	1.62	4.38	n.d.
Brown Rice Flour	n.d.	0.720	n.d.	n.d.	n.d.	n.d.
Corn/Wheat Flour Mix	121	0.680	n.d.	n.d.	n.d.	n.d.

Evaluation of Recoveries using Matrix Matched Calibration

A calibration curve was constructed with a 100-fold dynamic range and excellent linearity ($r^2 \geq 0.9950$) for all matrices. The true recovery and precision were assessed using the two QC sample concentrations (Table 3). Six replicates of each QC level were analyzed. The majority of the recoveries range between 27.7% and 204% with RSDs of < 11%. Since a single extraction method was used, various extraction efficiencies of each mycotoxin were observed. Matrix matching relies on the ability of the matrix used to make the calibration standards being a very close approximation to the actual sample. It is possible that the matrix matched calibration cannot fully account for varying degrees of incurred mycotoxins and/or matrix interferences that result in signal suppression or enhancement in complex samples.

Table 3: Average Recoveries (n=6) and Relative Standard Deviations (RSDs) at Two Fortification Levels using Matrix Matched Calibration

	Average Recovery, % (RSD, %)							
	Corn		Peanut Powder		Brown Rice		Corn/Wheat Mix	
Conc. (ng/g)	100	200	100	200	100	200	100	200
Deoxynivalenol	73.3 (3)	70.8 (2)	110 (8)	97.0 (10)	123 (2)	121 (1)	152 (6)	128 (11)
Fumonisin B_1	NA*	150 (2)	101 (2)	99.0 (1)	142 (2)	144 (2)	164 (9)	177 (1)
HT-2 toxin	102 (5)	111 (4)	87.2 (4)	91.6 (4)	104 (9)	116 (3)	156 (9)	158 (7)
Fumonisin B_3	156 (2)	129 (2)	79.2 (3)	78.2 (1)	139 (3)	135 (2)	118 (2)	125 (5)
Fumonisin B_2	204 (2)	124 (2)	79.9 (3)	76.2 (1)	123 (3)	127 (2)	123 (7)	126 (3)
T2 toxin	164 (9)	321 (4)	201 (4)	192 (2)	150 (3)	158 (3)	37.7 (1)	34.4 (5)
Zearalenone	84.2 (2)	78.1 (1)	93.8 (4)	89.0 (3)	118 (3)	118 (1)	82.3 (6)	66.2 (4)
	Corn		Peanut Powder		Brown Rice		Corn/Wheat Mix	
Conc. (ng/g)	10.0	20.0	10.0	20.0	10.0	20.0	10.0	20.0
Aflatoxin G_2	91.9 (5)	86.4 (2)	69.8 (10)	66.0 (2)	106 (5)	103 (2)	79.2 (8)	78.8 (6)
Aflatoxin G_1	96.8 (2)	95.4 (1)	83.8 (1)	81.8 (2)	94.7 (2)	95.8 (1)	87.2 (3)	87.7 (2)
Aflatoxin B_2	91.8 (3)	88.3 (2)	84.8 (2)	89.6 (2)	94.9 (3)	97.8 (2)	87.7 (6)	93.8 (6)
Aflatoxin B_1	96.7 (2)	87.3 (2)	85.1 (3)	86.3 (2)	27.7 (7)	28.2 (3)	46.7 (7)	62.2 (10)
Ochratoxin A	96.6 (1)	94.2 (1)	112 (5)	114 (4)	98.1 (2)	99.6 (1)	77.8 (1)	71.1 (10)

Evaluation of the Accuracy using SIDA Calibration

A wide dynamic range (1000-fold) and excellent linearity ($r^2 \geq 0.9996$) was observed using the SIDA calibration technique, as shown in Table 4. Linearity was determined by analysis of a 9-point calibration curve (2 replicates each) using 1/x weighted fit of relative instrument response ratio to relative concentration.

Table 4: The Linear Range, Linearity (r^2) and Accuracy Using SIDA Method.

				Average Accuracy, % (RSD, %)							
	IS	Linear range (ng/mL)	r^2	Corn		Peanut		Brown Rice		Corn/Wheat Mix	
				100	200	100	200	100	200	100	200
DON	$^{13}C_{15}$-DON	1-1000	0.9999	78.6 (4)	106 (2)	102 (16)	109 (9)	102 (5)	99.0 (3)	99.1 (4)	112 (4)
				Corn		Peanut		Brown Rice		Corn/Wheat Mix	
				10.0	20.0	10.0	20.0	10.0	20.0	10.0	20.0
AFG_2	$^{13}C_{17}$-AFG_2	0.1-100	0.9996	97.2 (4)	90.7 (5)	98.2 (6)	96.6 (7)	92.3 (7)	92.9 (1)	94.1 (7)	96.1 (4)
AFG_1	$^{13}C_{17}$-AFG_1	0.1-100	0.9999	101 (4)	95.8 (4)	101 (2)	103 (2)	100 (4)	97.8 (2)	96.8 (8)	106 (5)
AFB_2	$^{13}C_{17}$ AFB_2	0.1-100	0.9997	100 (3)	95.2 (3)	100 (3)	96.8 (6)	96.7 (7)	98.6 (6)	97.0 (7)	97.8 (6)
AFB_1	$^{13}C_{17}$ AFB_1	0.1-100	0.9999	96.0 (3)	91.8 (3)	94.5 (3)	93.1 (6)	96.1 (5)	98.1 (5)	90.7 (6)	96.1 (5)
OchA	$^{13}C_{20}$-OchA	0.1-100	0.9998	99.0 (5)	93.1 (5)	96.9 (4)	93.6 (1)	94.6 (6)	93.3 (3)	91.0 (8)	88.6 (5)

Evaluation of the Accuracy using SIDA Calibration

Table 5 summarizes the assigned values as well as the standard deviations for the mycotoxin concentrations according to Fapas. However, only three of the four spiked mycotoxins had corresponding ^{13}C-IS, so a true SIDA calibration was only performed for those three. For zearalenone, which did not have a labeled IS in this study, the closely eluting $^{13}C_{17}$-aflatoxin G_1 was used as the analogue IS to determine if it could serve as a substitute. The accuracies for those mycotoxins with labeled ISs were between 91.4% and 98.6% with relative standard deviations from 2% to 7%. The results from using a non-matched labeled IS to quantify a given mycotoxin were not acceptable, despite the similar chromatographic retention.

Table 5: Analysis of Reference Material (maize) and Comparison Between the Assigned Value and Measured Concentrations

		Concentration, ng/g		
Reference Material	Analyte	Measured, n=3	Assigned Value	% Accuracy (% RSD)
TET017RM	Deoxynivalenol	1867.9 ± 37.4	1971 ± 195	94.8 (2)
TET017RM	Aflatoxin B_1	8.68 ± 0.430	9.49 ± 0.850	91.4 (5)
TET017RM	Ochratoxin A	4.48 ± 0.130	4.81 ± 0.750	93.2 (3)
TET017RM	Zearalenone	*31.26 ± 2.19	231 ± 25.0	*13.5 (7)
T04301Q	Deoxynivalenol	639.7 ± 19.19	649 ± 222	98.6 (3)
T04301Q	Aflatoxin B_1	8.69 ± 0.350	9.21 ± 4.05	94.4 (4)
T04301Q	Ochratoxin A	2.81 ± 0.200	3.03 ± 1.33	92.6 (7)
T04301Q	Zearalenone	*16.2 ± 0.81 0	138.5 ± 59.6	*11.7 (5)

* The results using a non-matched labeled ISs

Conclusions

A chromatographic method that efficiently separated the 12 mycotoxins studied was developed and successfully used in the analysis of four different commercially available matrices.

The preferred calibration approach when testing for mycotoxins in complex matrices is the SIDA technique. For labs unable to use the SIDA calibration approach, matrix matched calibration standards is a common alternative. In this study, we showed acceptable recoveries, but without the normalizing power of an IS, the matrix matching can only account for some of the matrix effects that ultimately influence instrument response for a particular mycotoxin in a given matrix.

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