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Mass Spectrometry & Spectroscopy

Sensitive Femtogram Determination of Aflatoxins B_1, B_2, G_1 and G_2 in Food Matrices using Triple Quadrupole LC/MS

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A simple and inexpensive sample cleanup procedure based on a dispersive solid phase adsorption approach (C18) is effective in removing background matrix contaminants for reliable determination of aflatoxins in food at the femtogram level by triple quadrupole LC/MS. This application demonstrates fast analysis time (< 6 min) with good chromatographic resolution and separation for all four aflatoxins. Standard curves for each aflatoxin analyte show good linearity (> 0.998) across a wide concentration range (0.1–100 µg/L). Recoveries using the dispersive solid phase adsorption approach were between 85–110% for each aflatoxin for all four spiked food matrices and were comparable to other widely used SPE routines. The limit of detection was determined to be < 0.15 µg/kg and the limit of quantitation < 0.5 µg/kg for all four sample matrices. Precision data was typically below 5% RSD for all analytes.

Introduction

Aflatoxins are a group of mycotoxins produced as metabolites by the fungi aspergillus flavus and aspergillus parasiticus [1]. They can be found in various foods including grains, nuts, and spices [2]. There are four major naturally occurring aflatoxins: B_1, B_2, G_1 and G_2 *(Figure 1)*. Exposure to them can cause cancer in humans and live stock, therefore reliable and sensitive analytical methods for the determination of aflatoxins are required to safeguard our food supply.

Experimental

These analyses were performed using an Agilent G6460A Triple Quadrupole LC/MS/MS System equipped with Agilent Jet Stream Technology [3] using an Agilent 1200 Series SL LC. The LC system consisted of a binary pump (G1312B), vacuum degasser (G1379B), a low carryover automatic liquid sampler (G1367D), thermostatted column compartment (G1316B) and MassHunter data system.

Aflatoxin standards and foods

Purified aflatoxin standards (B_1, B_2, G_1 and G_2) were obtained from Sigma-Aldrich. Aflatoxin-free corn flour, wheat, peanut and walnut samples obtained from a local grocery store were used for recovery studies.

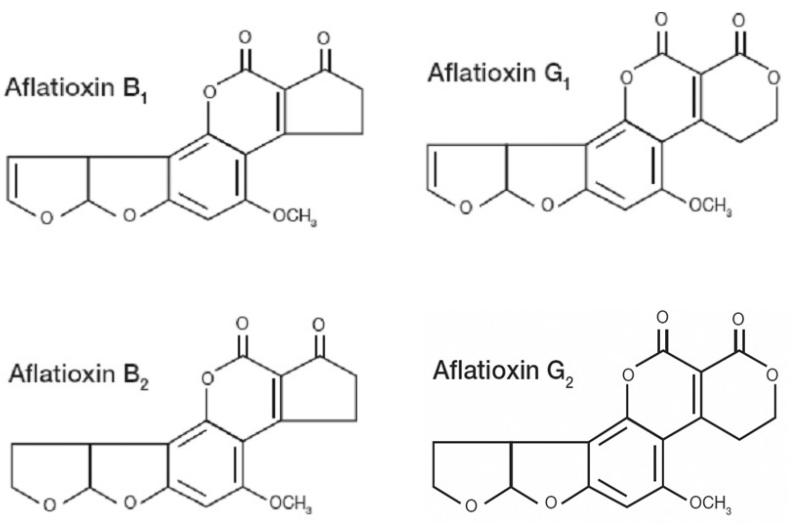

Figure 1. Structures of aflatoxins B1, B2, G1 and G2.

Instrumentation

Rapid Resolution HPLC Conditions and Configuration:

- Agilent 1200 Series Binary Pump SL (G1312B)
- High Performance WP Sampler SL Plus (G1367D) Sampler Thermostat (G1330B)
- Thermostatted Column Compartment SL, including 10P/Two-Position switching valve (G1316B with option #057)

Method Conditions:

Column:	Agilent ZORBAX Eclipse Plus C18, 2.1 x 50 mm,1.8 µm	
Column temperature:	40 °C	
Injection volume:	5 µL	
Autosampler temp:	4 °C	
Needle wash:	Flushport (100% methanol), 5 seconds	
Mobile phase:	A = 10 mM NH4 acetate in water	
	B = 100% methanol	
Gradient flow rate:	0.6 mL/min (no split)	
Gradient:	Time (min)	%B
	0	5
	5	100
	6	100
Analysis time:	6 min	
Equilibration time:	1.5 min	
Total run time:	7.5 min	

Mass Spectrometer Source Conditions and Configuration:

Agilent 6460 Triple Quadrupole LC/MS equipped with Agilent Jet Stream Technology.

Ion Source Conditions:

Ion Mode:	ESI/Agilent Jet Stream, Positive ionization
Capillary Voltage:	4000 V
Drying gas (nitrogen):	10 L/min
Drying gas temperature:	325 °C
Nebulizer gas (nitrogen):	50 psi
Sheath Gas temperature:	350 °C
Sheath Gas flow:	11 L/min
Nozzle Voltage:	0 V
Q1 and Q2 Resolution:	0.7 amu [autotune]
Delta EMV:	400V

The Triple Quadrupole MS MRM parameters are listed in *Table 1*. All fragmentor voltage (frag) settings and respective collision energies (CE) and the most abundant MS/MS product ions per analyte were determined automatically using the Agilent MassHunter Optimiser Software.

Table 1. MRM Transitions for Aflotoxins and Respective Internal Standards.

Name	Retention time (min)	Fragmentor voltage (V)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Aflatoxin B_1	4.68	130	313.1	241.1	35
				285.1	20
				269.1	25
Aflatoxin B_2	4.57	130	315.1	287.1	25
				259.1	25
				243.1	40
Aflatoxin G_1	4.40	130	329.1	243.1	25
				311.1	20
				283.1	20
Aflatoxin G_2	4.26	130	331.1	245.1	30
				285.1	25
				313.1	25
Isotope B_1	4.68	130	330.1	301.1	20
				255.1	40
Isotope B_2	4.57	130	332.1	303	25
				273.0	30
Isotope G_1	4.40	130	346.1	257.1	25
				299.1	25
Isotope G_2	4.26	130	348.1	330.1	25
				259.1	30

Sample Preparation and Recovery Studies

Corn flour, ground wheat, peanut and walnut samples (10g each) were spiked with a mixture of four aflatoxin standards, each at 5 and 25 ng/g. This was then extracted using 40 mL of acetonitrile-water (84:16, v/v) for 30 min with shaking at room temperature. The extract was cleaned up using both C18 powdered adsorbent material (ODS SPE bulk sorbent, Agilent p/n 5982-1182) and MycoSep 226 multifunctional SPE (Romer). Aliquots (0.4mL) of the cleaned up extracts were diluted with 0.6mL 10 mM ammonium acetate in water.

The sample was then centrifuged at 14,000 rpm for 3 minutes prior to LC/MS/MS analysis. Each food matrix and spike level was conducted in seven replicates to represent and maintain statistical integrity. A schematic of this sample preparation is illustrated in *Figure 2*.

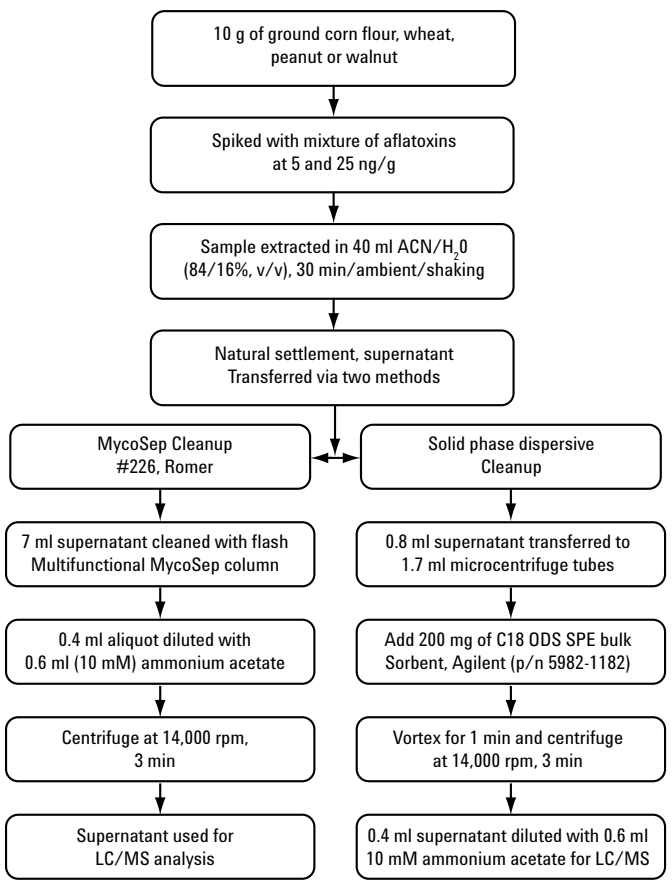

Figure 2. Schematic matrix sample preparation workflow showing the dispersive solid phase adsorption approach versus a widely used SPE approach.

Results and Discussion

The rapid chromatography conditions as outlined in the experimental section yielded good chromatographic resolution for each aflatoxin analyte and each analysis was completed in six minutes. A typical chromatogram is shown in *Figure 3a*, which illustrates 1 ppb concentration level of each aflatoxin together with the corresponding isotopically labelled internal standards at a concentration level of 2.5 ppb *(Figure 3b)*. These chromatograms show overlaid extracted ion chromatograms (EICs.)

Standard curves for aflatoxins B_1, B_2, G_1 and G_2 all showed a good linearity through the concentration range 0.1 to 100 ppb each with a linear correlation (R2) of greater than 0.999. *Figure 4* illustrates an overlay of each standard curve on the same scale, but without internal standard correction. The use of internal standards effectively adjusted for matrix differences, as shown in *Figure 5*.

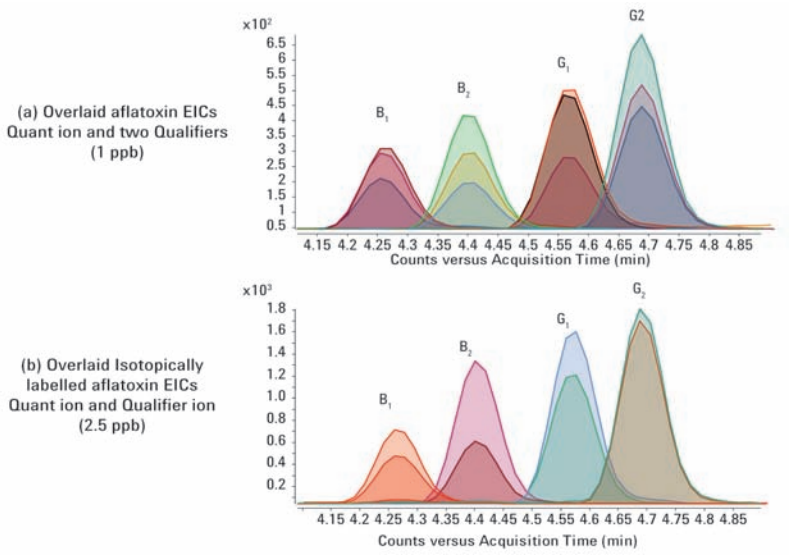

Figure 3. LC/MS/MS chromatogram of aflatoxin B_1, B_2, G_1 and G_2 standards at 1 ppb with corresponding isotopically labelled internal standards at 2.5 ppb.

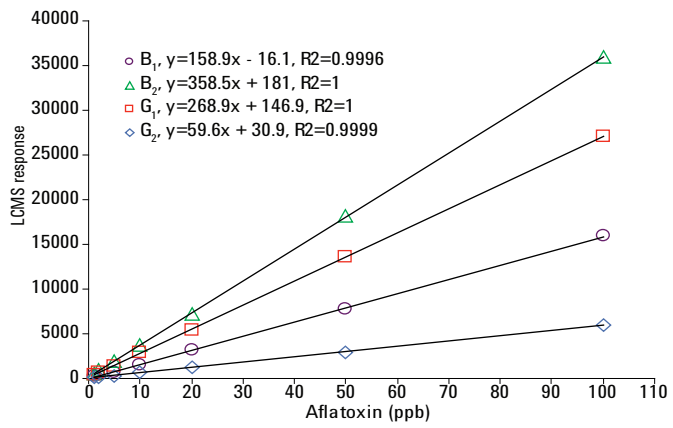

Figure 4. Overlaid standard curves for aflatoxins B_1, B_2, G_1 and G_2.

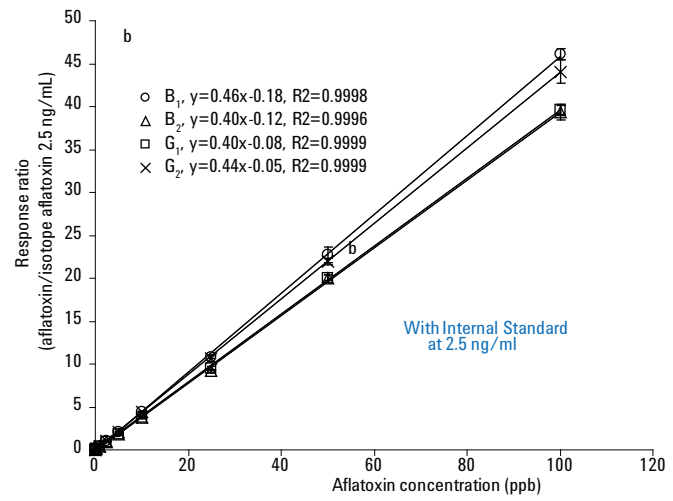

Figure 5. Overlaid standard curves for aflatoxins B_1, B_2, G_1 and G_2 with internal standard correction.

In order to determine the limits of detection (LOD) and reporting (LOR), seven separate and mutually exclusive batches of each food matrix were tested via the two sample preparation protocols and analytical methodology outlined previously. The results outlined in this document are derived from the average values across the seven batches of each matrix (N=7.) Limits of detection were determined using the protocol of chromatographic signal-to-noise ratio of above 3/1 (peak to peak.) Limits of reporting were determined using the protocol of chromatographic signal-to-noise ratio above 10/1 (peak to peak.)

Table 2 details the observed LODs and LORs for each aflatoxin across the series of four food matrices. The limit of detection overall was determined to be < 0.15 µg/kg and the limit of quantitation < 0.5 µg/kg for all four sample matrices and both sample preparation routines. *Tables 3* and *4* summarise the LOD data obtained across the seven batches. *Table 3* data is presented with respect to dispersive solid phase adsorption only, and *Table 4* data using the Mycosep SPE sample preparation only.

Sample Preparation Approach

Recovery studies were extensively undertaken for both sample cleanup techniques in parallel across the four matrices and across the seven batches for each aflatoxin analyte. *Figures 6* (a) and (b) graphically depict the recovery trends across the four food matrices for the dispersive C18 cleanup approach and the Mycosep SPE cleanup, respectively. As illustrated, the recoveries for both sample cleanup procedures were between 85-110 % for each of the aflatoxins for all four spiked food matrices, with the MycoSep cleanup method only marginally better than the C18 one for walnut samples .

Table 2. Limits of Detection and Reporting Observed for Aflatoxins B_1, B_2, G_1 and G_2 Across Four Food Matrices via Dispersive SPA and Mycosep SPE Sample Preparation Approaches

		Mycosep (#226, Romer)		Dispersive C18 ODS SPE Bulk Sorbent, Agilent (p/n 5982-1182)	
Food Matrix	Aflatoxin	LOD ng/g (S/N>3)	LOR ng/g (S/N>10)	LOD ng/g (S/N>3)	LOR ng/g (S/N>10)
Corn	B_1	0.047	0.16	0.060	0.20
(Ave 7-batches)	B_2	0.036	0.12	0.085	0.28
	G_1	0.08	0.28	0.10	0.35
	G_2	0.046	0.15	0.033	0.11
Wheat	B_1	0.068	0.23	0.012	0.042
(Ave 7-batches)	B_2	0.11	0.36	0.037	0.12
	G_1	0.14	0.47	0.15	0.50
	G_2	0.038	0.13	0.11	0.36
Peanut	B_1	0.051	0.17	0.056	0.19
(Ave 7-batches)	B_2	0.045	0.15	0.069	0.23
	G_1	0.07	0.23	0.05	0.15
	G_2	0.052	0.17	0.14	0.45
Walnut	B_1	0.12	0.41	0.093	0.31
(Ave 7-batches)	B_2	0.035	0.12	0.098	0.33
	G_1	0.03	0.10	0.12	0.40
	G_2	0.047	0.16	0.04	0.13

Table 3. LOD Results Observed for Aflatoxins B_1, B_2, G_1 and G_2 via the Dispersive SPA

Food Matrix	B_1 LOD (ng/g)	B_2 LOD (ng/g)	G_1 LOD (ng/g)	G_2 LOD (ng/g)
Corn	0.060	0.085	0.100	0.033
Wheat	0.012	0.037	0.150	0.110
Peanut	0.056	0.069	0.050	0.140
Walnut	0.093	0.098	0.120	0.040
Average	0.055	0.072	0.105	0.080
Mass On-Column (fg)	275	360	525	400

Table 4. LOD Results Observed for Aflatoxins B_1, B_2, G_1 and G_2 via the Mycosep, #226 SPE Sample Preparation Approach

Food Matrix	B_1 LOD (ng/g)	B_2 LOD (ng/g)	G_1 LOD (ng/g)	G_2 LOD (ng/g)
Corn	0.047	0.036	0.080	0.046
Wheat	0.068	0.110	0.140	0.038
Peanut	0.051	0.045	0.070	0.052
Walnut	0.120	0.035	0.030	0.047
Average	0.072	0.057	0.080	0.046
Mass On-Column (fg)	360	283	400	229

Aflatoxin analyte recovery data for each separate food matrix is detailed in *Tables 5* through *8* and was undertaken at two concentration spiked levels of 5 ng/g and 25 ng/g. Each sample batch tested was split and divided between the two cleanup procedures outlined in this document following the natural settlement and supernatant transfer step outlined in *Figure 2(a)*.

Table 5. Spiked Corn Samples – Recovery Studies (% Recovery, ± RSD, N=7)

	Aflatoxin	Corn spiked at 5 ng/g C18 cleanup	Corn spiked at 25 ng/g C18 cleanup	Corn spiked at 5 ng/g Mycosep#226	Corn spiked at 25 ng/g Mycosep#226
No Internal Standard	B_1	101.7 ± 3.7	95.7 ± 3.0	107.8 ± 2.8	105.4 ± 2.8
	B_2	95 ± 6.3	95.4 ± 1.4	103.0 ± 5.7	105.3 ± 2.0
	G_1	102.7 ± 7.1	96.8 ± 1.75	110.2 ± 7.9	103.7 ± 3.0
	G_2	107.9 ± 3.5	97.8 ± 0.88	108.1 ± 5.4	104.3 ± 2.4
Internal Standard	B_1	102.3 ± 2.9	100.1 ± 2.4	108.2 ± 5.1	97.8 ± 3.3
	B_2	100.0 ± 7.9	94.0 ± 3.1	101.7 ± 4.7	92.8 ± 3.5
	G_1	107.3 ± 3.5	97.0 ± 6.0	110.3 ± 3.6	102.5 ± 1.8
	G_2	101.3 ± 5.6	100.4 ± 3.8	104.9 ± 5.4	97.2 ± 6.3

Table 6. Spiked Wheat Samples – Recovery Studies (% Recovery, ± RSD, N=7)

	Aflatoxin	Corn spiked at 5 ng/g C18 cleanup	Corn spiked at 25 ng/g C18 cleanup	Corn spiked at 5 ng/g Mycosep#226	Corn spiked at 25 ng/g Mycosep#226
No Internal Standard	B_1	100.1 ± 4.4	96.6 ± 2.9	113.5 ± 5.9	100.6 ± 1.8
	B_2	98.2 ± 6.9	96.4 ± 2.6	105.1 ± 4.5	102.1 ± 4.4
	G_1	100.5 ± 5.5	105.4 ± 3.8	111.5 ± 10.4	106.1 ± 4.0
	G_2	104.9 ± 3.2	106.7 ± 1.3	108.6 ± 5.2	103.7 ± 2.9
Internal Standard	B_1	100.9 ± 3.6	109.3 ± 4.7	107.5 ± 4.8	111.7 ± 4.9
	B_2	85.2 ± 7.7	99.8 ± 2.8	92.4 ± 6.3	101.0 ± 4.0
	G_1	110.6 ± 7.8	112.8 ± 1.8	117.6 ± 7.7	109.3 ± 5.7
	G_2	108.4 ± 6.2	108.3 ± 3.9	115.6 ± 7.1	109.8 ± 3.6

Table 7. Spiked Peanut Samples – Recovery Studies (% Recovery, ± RSD, N=7)

	Aflatoxin	Corn spiked at 5 ng/g C18 cleanup	Corn spiked at 25 ng/g C18 cleanup	Corn spiked at 5 ng/g Mycosep#226	Corn spiked at 25 ng/g Mycosep#226
No Internal Standard	B_1	96.7 ± 3.4	97.0 ± 4.6	112.0 ± 8.4	104.9 ± 1.7
	B_2	98.3 ± 4.7	97.4 ± 2.9	108.0 ± 4.6	104.5 ± 2.0
	G_1	95.0 ± 5.6	95.0 ± 4.9	109.9 ± 2.1	105.7 ± 3.4
	G_2	100.0 ± 2.3	100.0 ± 2.0	114.7 ± 3.2	106.3 ± 1.1
Internal Standard	B_1	101.8 ± 3.6	96.1 ± 2.0	100.0 ± 6.8	103.0 ± 3.5
	B_2	102.5 ± 5.5	100.2 ± 5.0	99.4 ± 4.1	102.9 ± 2.7
	G_1	105.7 ± 7.3	99.2 ± 2.2	105.2 ± 4.3	101.7 ± 5.2
	G_2	107.5 ± 10.9	104.9 ± 6.7	109.3 ± 8.7	102.4 ± 3.1

Table 8. Spiked Walnut Samples – Recovery Studies (% Recovery, ± RSD, N=7)

	Aflatoxin	Corn spiked at 5 ng/g C18 cleanup	Corn spiked at 25 ng/g C18 cleanup	Corn spiked at 5 ng/g Mycosep#226	Corn spiked at 25 ng/g Mycosep#226
No Internal Standard	B_1	84.9 ± 3.7	85.2 ± 2.2	101.4 ± 3.2	101.0 ± 2.3
	B_2	91.5 ± 3.9	89.8 ± 2.8	104.2 ± 7.9	106.3 ± 2.9
	G_1	89.4 ± 4.4	86.7 ± 1.5	103.9 ± 5.9	101.7 ± 4.2
	G_2	84.0 ± 4.0	83.1 ± 1.3	109.9 ± 3.4	106.3 ± 1.5
Internal Standard	B_1	106.5 ± 4.9	98.9 ± 4.1	93.8± 1.4	100.2 ± 2.9
	B_2	99 ± 5.4	96.5 ± 3.5	92.4 ± 2.7	98.7 ± 4.4
	G_1	103.2 ± 5.9	94.9 ± 2.5	102.8 ± 9.0	102.1 ± 3.9
	G_2	100.2 ± 6.2	97.5 ± 4.6	99.5 ± 6.8	101.2 ± 3.8

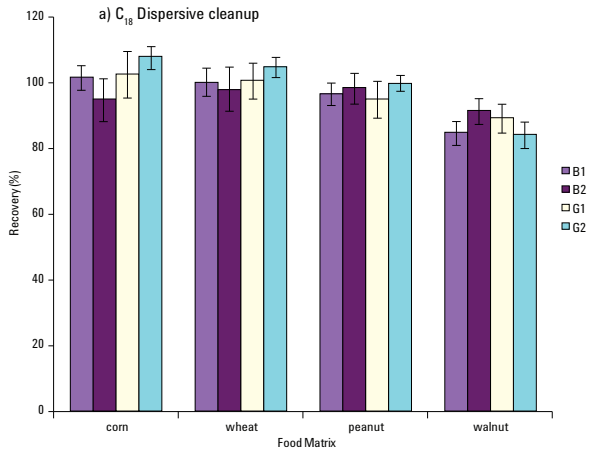

Figure 6(a). Recovery of aflatoxin B1, B2, G1 and G2 from food matrices using C18 dispersive cleanup.

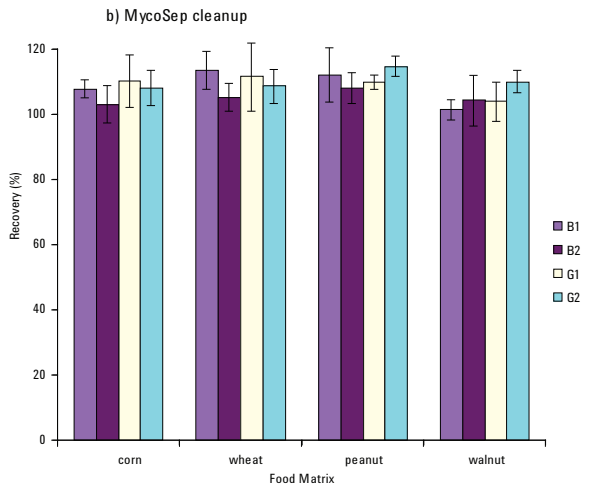

Figure 6b. Recovery of aflatoxin B_1, B_2, G_1 and G_2 from food matrices using Mycosep, #226 SPE cleanup.

Conclusions

An inexpensive and rapid LC/MS/MS method has been developed for the analysis and confirmation of aflatoxins B_1, B_1, G_2 and G_2 in cereals and nuts, with a detection limit of less than 1 ppb. This method is inclusive of sample preparation using a dispersive C18 solid phase adsorption approach (Agilent bulk sorbent p/n 5982-1182.) The performance of this simple sample cleanup procedure was comparable to that of a widely used and generally accepted SPE approach in terms of matrix cleanup and aflatoxin recoveries.

Aflatoxin limits of detection were determined to be less than 0.15 µg/kg and aflatoxin limits of reporting were all less than 0.5 µg/kg for all four sample matrices (corn, wheat, peanut and walnut.)

Standard curves for aflatoxins B_1, B_2, G_1 and G_2 showed a good linearity through the concentration range of 0.1 to 100 ppb with a linear correlation (R_2) of greater than 0.999 for all analytes.

Aflatoxin recoveries were between 85-110% for each of the aflatoxins for all four spiked food matrices using the dispersive C18 solid phase adsorption approach.

References:

1. *Microhim Acts 153, 2006, 101-108*
2. *Rapid Commun. Mass Spectrum, 2009; 23: 3-11*
3. *"Agilent Jet Stream Thermal Gradient Focussing Technology" Agilent Technologies Publication 5990-3494EN.*

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