

APPLICATIONS

Determination of Aflatoxin B₁, B₂, G₁ and G₂ in Grain using Solid Phase Extraction and LC-MS/MS

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Introduction

Produced by various fungi, mycotoxins are very harmful toxins found regularly in feed and grain products. Mycotoxins result in both acute and chronic health effects in humans and livestock when consumed. With the globalization of the food supply, mycotoxins from grain is a global concern for both developing and developed countries. In this study, we present a rapid and sensitive method for mycotoxins per the new China GB method GB2009.22-2016 that can be used on both HPLC and UHPLC platforms. Specifically, Aflatoxin B₁, B₂, G₁ and G₂ in grain are extracted using Strata[®]-X solid phase extraction (SPE) followed by a rapid LC-MS/MS method using a Kinetex[®] 1.7 μm C18 core-shell LC column. The SPE sample cleanup successfully removes interferences from the grain, resulting in great recoveries and meetings the assay acceptance criteria. Additionally, the high efficiency and selectivity of the core-shell column produced excellent baseline separation of the 4 aflatoxins for more accurate quantitation.

Materials

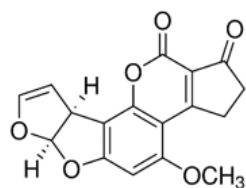
Aflatoxin (B₁, B₂, G₁ and G₂) mix, 20 μg/mL in acetonitrile (Sigma-Aldrich, P/N: 33415)

Aflatoxin B₁ – 13C17, 0.5 μg/mL in acetonitrile (Sigma-Aldrich, P/N: 32764)

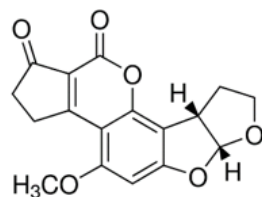
Aflatoxin G₁ – 13C17, 0.5 μg/mL in acetonitrile (Sigma-Aldrich, P/N: 32772)

All other reagents and chemicals were obtained from Sigma-Aldrich[®] (St. Louis, MO).

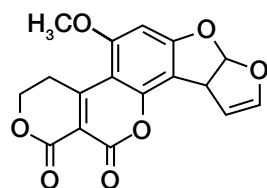
Structures:



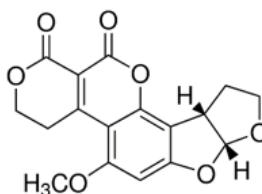
Aflatoxin B₁



Aflatoxin B₂



Aflatoxin G₁



Aflatoxin G₂

Experimental Conditions

Sample Pre-treatment

1. Grind the grain sample using a grinder until a homogeneous powder is formed
2. Sift the powder through a 2 mm sieve and store in a dark room at 4 °C
3. Weigh 2.5 g of sample powder to a 50 mL centrifuge tube
4. Add 30 μL working IS solution (Aflatoxin B₁ – ¹³C₁₇/ Aflatoxin G₁ – ¹³C₁₇, 100/100 ng/mL in acetonitrile)
5. Add 10 mL of 0.1% Formic Acid in 85:15 Acetonitrile/Water to the sample tube and mix for 1 min
6. Sonicate samples for 30 min under 30 °C.
7. Centrifuge sample tubes at 4000 rpm for 10 min
8. Transfer the sample supernatant to 20 mL glass vial
9. Aliquot 1 mL sample supernatant and dilute with 1 mL DI water

Solid Phase Extraction (SPE) Conditions

SPE Cartridge: Strata-X, 60 mg/3 mL
Part No.: 8B-S100-UBJ
Condition: 2 mL Methanol
Equilibrate: 2 mL Methanol/Water (10:90)
Load: 2 mL Diluted sample supernatant with DI water (1:1)
Wash: 1 mL Methanol/Water (20:80)
Elute: 1 mL 2% Formic Acid in Methanol
Dry: 40 °C under N₂
Reconstitute: 300 μL 0.1% Formic Acid in Acetonitrile/Water (5:95)
Filter: 0.2 μm Phenex[™] Syringe Filter*
Inject: 10 μL

* Filtering sample is optional depending on the sample matrix

LC-MS/MS Conditions

Column: Kinetex 1.7 μm C18
Dimensions: 100 x 3.0 mm
Part No.: 00D-4475-YO
Guard Column: SecurityGuard[™] ULTRA Cartridges
Part No.: AJ0-8775
Mobile Phase: A: 5 mM Ammonium acetate in Water
 B: Acetonitrile/Methanol (50:50)
Gradient:

Time (min)	%B
0.01	40
0.50	40
3.00	70
4.00	70
4.20	100
5.00	100
5.01	40
7.00	40

Flow Rate: 0.3 mL/min
Col. Temp.: 40 °C
Inj. Volume: 10 μL
Detector: SCIEX Triple Quad[™] 4500, ESI+



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Standard and Quality Control sample preparation scheme in spiking solution and grain sample

STDs in spiking solution and grain matrix

STD (ng/mL)	Spiking Solution ID	Spiking Solution Volume (µL)	Dilution Solvent (40:60 Acetonitrile/Water) (µL)	Spiking Solution Concentration (ng/mL)	STD Volume Spiking into Matrix (µL)	Final Conc. in Matrix (ng/mL)	Matrix Weight (g)
STD 6	20 µg/mL	50	1950	500	50	10	2.5
STD 5	20 µg/mL	12.5	987.5	250	50	5	2.5
STD 4	STD 6	200	800	100	50	2	2.5
STD 3	STD 6	100	900	50	50	1	2.5
STD 2	STD 6	50	950	25	50	0.5	2.5
STD 1	STD 6	10	990	5	50	0.1	2.5

STDs QCs in spiking solution and grain matrix

QCs in spiking solution and grain matrix

QC (ng/mL)	Spiking Solution ID	Spiking Solution Volume (µL)	Dilution Solvent (40:60 Acetonitrile/Water) (µL)	Spiking Solution Concentration (ng/mL)	STD Volume Spiking into Matrix (µL)	Final Conc. in Matrix (ng/mL)	Matrix Weight (g)
QC H	20 µg/mL	12.5	987.5	250	50	5	2.5
QC L	STD 6 (500 ng/mL)	50	950	25	50	0.5	2.5

Stock solution of aflatoxin mix at 20 µg/mL is in acetonitrile
 Dilution Solvent: 40:60 Acetonitrile/Water
 Matrix: Grain sample

Figure 1.
Grain sample at 2 ng/mL

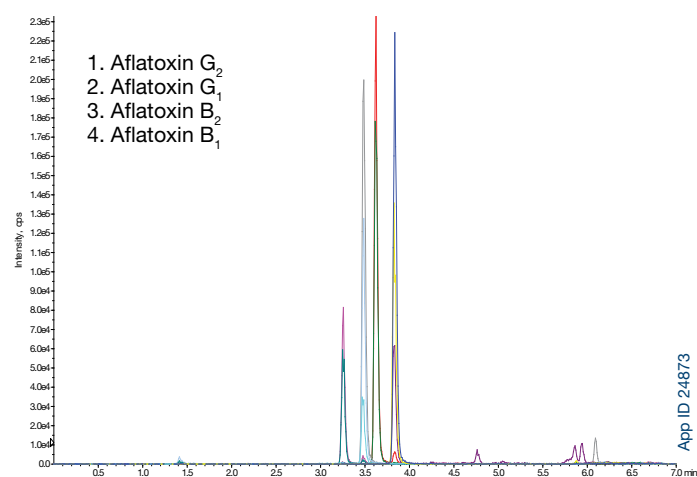


Figure 2.
LLOQ in grain sample at 0.1 ng/mL

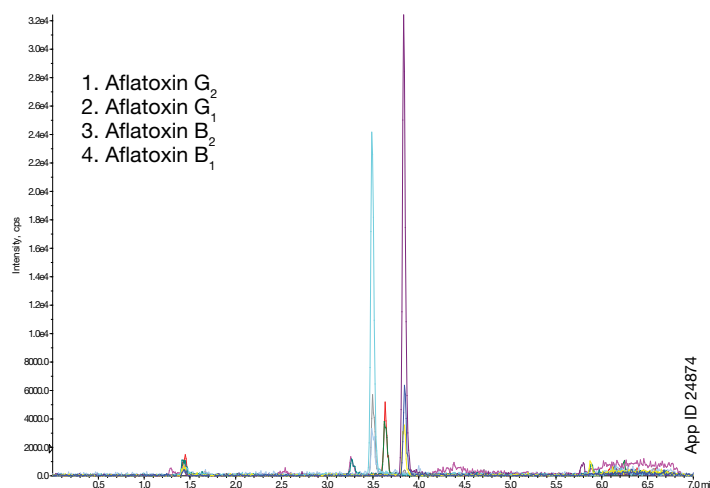
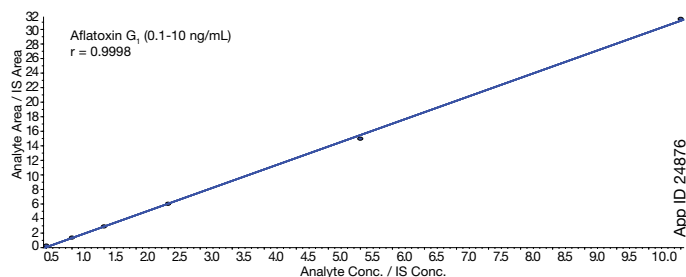
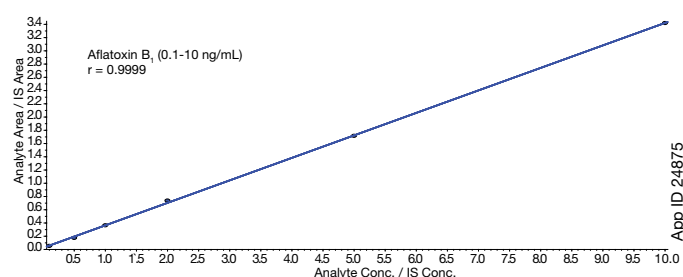


Figure 3.
Linearity Curves



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Table 1.
MRM Transitions

ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	CE
AFB ₁ 1	313.2	241.2	50	51
AFB ₁ 2	313.2	269.1	50	45
AFB ₂ 1	315.2	287.2	50	36
AFB ₂ 2	315.2	259.2	50	44
AFG ₁ 1	329.2	243.1	50	38
AFG ₁ 2	329.2	215.2	50	45
AFG ₂ 1	331.2	245.2	50	42
AFG ₂ 2	331.2	217.2	50	47
AFB ₁ - ¹³ C ₁₇	330.2	285.2	50	35
AFG ₁ - ¹³ C ₁₇	346.2	228.2	50	40

Table 2.
Accuracy and Precision

Sample ID	QCL (n=3)	QCH (n=3)
Nominal Concentration (ng/mL)	0.500	5.00
Aflatoxin B₁		
1	0.443	4.99
2	0.442	5.26
3	0.449	5.43
Mean	0.445	5.23
S.D.	0.00	0.22
% CV	0.85	4.25
% Theoretical	88.9	105
Aflatoxin B₂		
1	0.416	4.96
2	0.448	5.04
3	0.450	5.56
Mean	0.438	5.19
S.D.	0.02	0.33
% CV	4.36	6.28
% Theoretical	87.6	104
Aflatoxin G₁		
1	0.557	4.67
2	0.529	4.98
3	0.543	4.90
Mean	0.543	4.85
S.D.	0.01	0.16
% CV	2.58	3.32
% Theoretical	109	97.0
Aflatoxin G₂		
1	0.467	4.72
2	0.448	5.21
3	0.529	5.32
Mean	0.481	5.08
S.D.	0.04	0.32
% CV	8.80	6.28
% Theoretical	96.3	102

Figure 4.
Sample concentration calculation formula

$$X = \frac{A \times V \times f \times 1000}{m \times 1000}$$

Where,

X is the content of aflatoxins in the sample (µg/kg)

A is the calculated concentration in the sample (ng/mL)

V is the constant volume (mL), in this method V= 0.3 mL

f is the times diluted, in this method f=10

m is the weight of sample (g)

Results and Discussion

Demonstrated is an LC-MS/MS method for the determination of Aflatoxin B₁, B₂, G₁ and G₂ in whole grain using a Phenomenex Strata-X SPE and a Kinetex 1.7 µm C18 LC column. The assay shows acceptable accuracy and precision in triplicate samples which is presented in **Table 2**. **Table 1** shows all mass transitions that were used in the study. The assay linearity was evaluated with 0.1-10 ng/mL with 1/X² regression and r > 0.9998 value for all compounds (**Figure 3**). **Figure 4** is the actual sample concentration calculation formula from the grain sample.

The method was optimized with baseline separation of all compounds using a Kinetex 1.7 µm C18, 100 x 3.0 mm LC column (**Figure 1**) under LC-MS/MS conditions. The method can also be adapted for Fluorescence Detection (FLD). The 3.0 mm analytical LC column diameter was used to reduce the LC system back pressure significantly and reach an assay LLOQ at 0.1 ng/mL (**Figure 2**). The assay ruggedness was also increased using 3.0 mm ID column which improves the productivity and cost saving in the lab.

A SCIEX Triple Quad™ 4500 mass spectrometer or similar ion source designed of mass spectrometers are recommend for the assay to minimize the ion in-source saturation, which will affect the Aflatoxin B₁ and B₂ as quadratic curves, not linear curve.

The whole grain sample was obtained from a local Whole Foods Market®, the possible background from blank matrix was monitored after the Strata-X SPE extraction, there is no interference/ endogenous peak appearing near all the expected retention times of compounds.

Conclusion

A LC-MS/MS method for the determination of Aflatoxin B₁, B₂, G₁ and G₂ in whole grain using a Phenomenex Strata-X SPE and a Kinetex 1.7 µm C18 column is presented in this tech note. The SPE extraction procedure successfully removes interferences from the grain, resulting in great recoveries. Additionally, the high efficiency and selectivity of the core-shell column produced excellent baseline separation of the 4 aflatoxins for more accurate quantitation. The assay is not limited to only LC-MS/MS and can be adapted for FLD detector use. The method can be used in other samples in the food testing industry such as milk, honey and nuts.



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Ordering Information Kinetex[®] Core-Shell LC Columns

2.6 µm Minibore Columns (mm)						SecurityGuard [™] ULTRA Cartridges [†]
Phases	30 x 2.1	50 x 2.1	75 x 2.1	100 x 2.1	150 x 2.1	3/pk
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJO-8782

for 2.1 mm ID

2.6 µm MidBore [™] Columns (mm)						SecurityGuard [™] ULTRA Cartridges [†]
Phases	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	3/pk
C18	00A-4462-YO	00B-4462-YO	00C-4462-YO	00D-4462-YO	00F-4462-YO	AJO-8775

for 3.0 mm ID

2.6 µm Analytical Columns (mm)						SecurityGuard [™] ULTRA Cartridges [†]
Phases	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	3/pk
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AJO-8768

for 4.6 mm ID

1.7 µm Minibore Columns (mm)						SecurityGuard [™] ULTRA Cartridges [†]
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1		3/pk
C18	00A-4475-AN	00B-4475-AN	00D-4475-AN	00F-4475-AN		AJO-8782

for 2.1 mm ID

1.7 µm MidBore Columns (mm)					SecurityGuard [™] ULTRA Cartridges [†]
Phases	30 x 3.0	50 x 3.0	100 x 3.0		3/pk
C18	—	00B-4475-YO	00D-4475-YO		AJO-8775

for 3.0 mm ID

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Strata[®]-X Polymeric SPE Tubes

Tubes	1 mL (100/box)		3 mL (50/box)
	30 mg		60 mg
Strata-X	8B-S100-TAK	8B-S100-UBJ	
Strata-X-C	8B-S029-TAK	8B-S029-UBJ	
Strata-X-CW	8B-S035-TAK	8B-S035-UBJ	
Strata-X-A	8B-S123-TAK	8B-S123-UBJ	
Strata-X-AW	8B-S038-TAK	8B-S038-UBJ	
Strata-XL	8B-S043-TAK	8B-S043-UBJ	
Strata-XL-C	8B-S044-TAK	8B-S044-UBJ	
Strata-XL-CW	8B-S052-TAK	8B-S052-UBJ	
Strata-XL-A	8B-S053-TAK	8B-S053-UBJ	
Strata-XL-AW	8B-S051-TAK	8B-S051-UBJ	

[†]SecurityGuard Ultra Cartridges require holder, Part No.: AJO-9000

^{*}PREP SecurityGuard Cartridges require holder, Part No. AJO-8223

^{**}PREP SecurityGuard Cartridges require holder, Part No. AJO-8277



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