Application Note 00927

A Rapid LC/MS/MS Method for the Analysis of Aflatoxins in Complex Matrices With Immunoaffinity Clean-up

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Introduction

Aflatoxins are a group of structurally related carcinogenic mycotoxins produced by *Aspergillus sps*. Most commonly found aflatoxins in food and feed are aflatoxins B1, B2, G1 and G2. Aflatoxin B1 has been classified as Class 1 human carcinogen by the International Agency for Research on Cancer.

Globally, there are significant regulations on the presence of aflatoxins in food and feed. The US Federal Government action level for aflatoxins in food for human consumption or in dairy cow feed is 20 ppb. The European Commission has set very low action levels for aflatoxins in cereals intended for direct human consumption or for ingredients in foodstuffs. These action levels are 4 ppb total aflatoxins and 2 ppb for aflatoxin B1.

So far, these compounds have been extensively analyzed by HPLC. We developed a rapid and sensitive LC/MS/MS method for the detection and analysis of aflatoxins B1, B2, G1 and G2. We validated the method in sake and wheat beer matrices cleaned up using the AflaCLEAN™ immuno affinity column.

Instrumentation

- Varian 320-MS LC/MS/MS equipped with an ESI source
- Varian ProStar™ 210 solvent delivery system (2)
- Varian ProStar™ 430 AutoSampler

Materials and Reagents

All the reagents and aflatoxins were purchased from Sigma-Aldrich Co, St. Louis, MO. The AflaCLEAN immuno affinity columns used for sample clean up were provided by LC Tech, Germany.

Sample Preparation

Alcohol was removed from wheat beer and sake wine by sonication for 30 minutes at room temperature. 3 mL of wheat beer/sake was spiked with a known concentration of aflatoxins B1, B2, G1 and G2. This mix was diluted with 3 mL of 10% PBS in DI water. 6 mL of this mix was applied onto the AflaCLEAN immuno affinity column, washed twice with 3 mL of 10% PBS followed by a wash with 3 mL DI water.

The bound aflatoxins were collected in a silanized tube by eluting with 3 mL methanol (100%). This solution was further dried under nitrogen and reconstituted in a known volume of LC buffer A and analyzed by MS. The aflatoxins mix spiked in 10% PBS (no matrix) was run through the AflaCLEAN column using the same procedure above. This was used as a control. Unspiked sake wine and wheat beer run through the column served as blanks for the analysis. Zymark's automated sample prep work station was employed for the sample clean up and Zymark's TurboVap® was used to dry the purified samples.

HPLC Conditions

Column	Pursuit® XRs C18 3 µm, 50 x 2.0 mm ID (Varian Part No. A6001050X020)
Buffer A	1% acetic acid, 5 mM ammonium acetate in 10% methanol
Buffer B	1% acetic acid, 5 mM ammonium acetate in 100% methanol
Injection Solvent	Buffer A
Injection Volume	5 µL

LC Program

Time (min:sec)	%A	%B	Flow (mL/min)
0:00	60.0	40.0	0.2
4:00	40.0	60.0	0.2
4:01	60.0	40.0	0.250
8:00	60.0	40.0	0.250

API Conditions

Ionization Mode	ESI Positive
Collision Gas	1.8 mTorr Argon
API Drying Gas	30 psi at 300 ^{o}C
API Nebulizing Gas	50 psi
SIM Width	0.7 amu
Needle	4500 V
Capillary	Scanning (see Table 2)
Shield	600 V
Detector	1900 V

MRM Parameters

Name	Capillary (V)	Q1 (*m/z*)	Ion polarity	Q3 (1) (*m/z*)	CE1 (V)	Q3 (2) (*m/z*)	CE2 (V)	Ion ratio (%) Q3(1):Q3(2)	Dwell (sec)
Aflatoxin G2	90	331.0	[M+H]+	245.0	16.5	275.0	11.5	25.8	0.3
Aflatoxin G1	90	329.0	[M+H]+	283.0	25.5	243.0	25.5	38.7	0.3
Aflatoxin B2	63	315.0	[M+H]+	287.0	21.5	259.0	10.0	23.1	0.3
Aflatoxin B1	63	313.0	[M+H]+	285.0	22.0	241.0	33.0	83.2	0.3

Table 2 MRM parameters of the four aflatoxins.

Results & Discussion

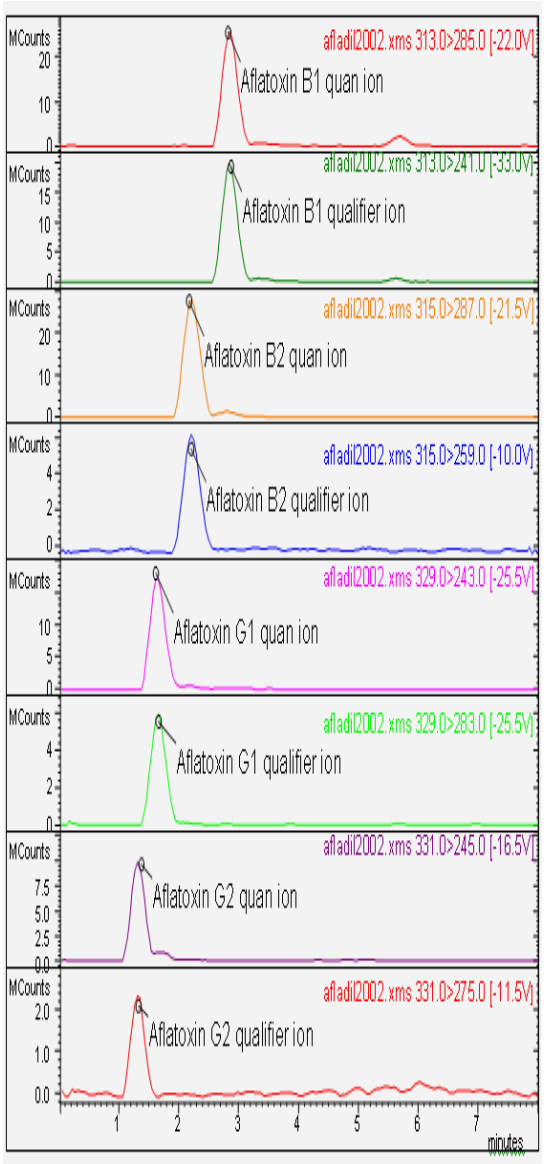

Figure 1 MRM chromatogram of aflatoxins B1, B2, G1 and G2 (50 pg/µl Conc., 5 µl injection) on the 320-MS.

All four aflatoxins were separated and detected in less than eight minutes. Two transitions were monitored for each toxin: one for quantitation and the other for confirmation. Ion ratios were measured. Figure 1 gives the MRM chromatogram of four aflatoxins with two transitions (quantitation and qualifier ion) for each analyte.

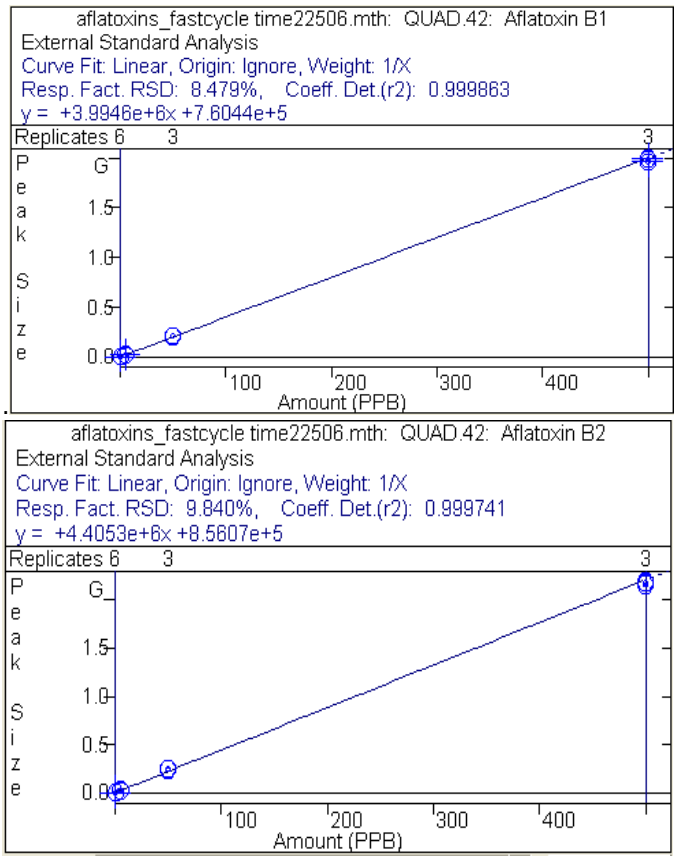

Figure 2 Calibration curves of the four aflatoxins (continued on page 3).

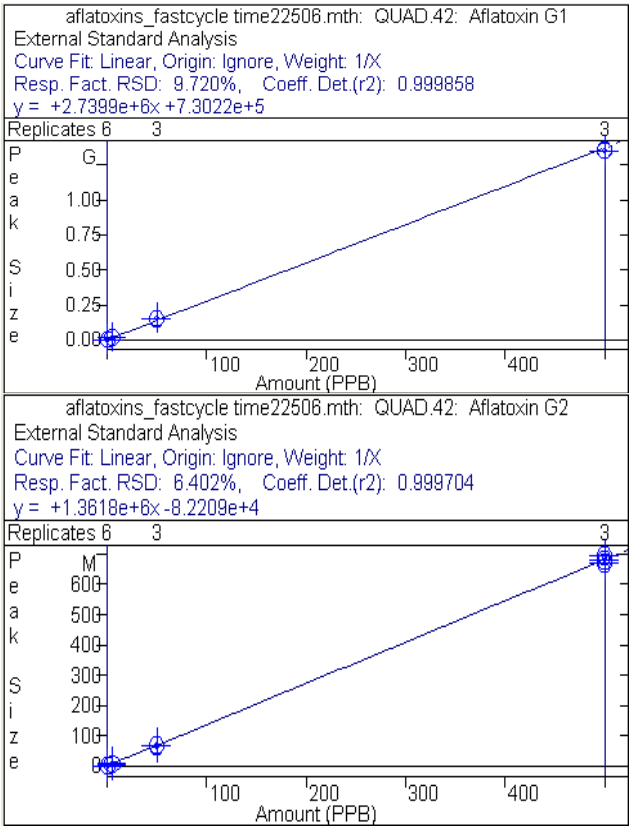

Figure 2 (continued) Calibration curves of the four aflatoxins.

Limit of Quantitations (LOQs) of this method were estimated. The peak-to-peak (PP) signal-to-noise (S/N) ratio was measured at the lowest point of the calibration curve (0.5 ppb). The PP S/N ratios were 10 and above for all the four aflatoxins indicating that the LOQ's of this method are less than or equal to 0.5 ppb. The LOQ's of this method were compared to the European Union (EU) action levels for aflatoxins (Table 3).

Name	LOQ	EU Action Levels
Aflatoxin B1	<0.5 ppb	2 ppb
Aflatoxin B2	<0.5 ppb	4 ppb
Aflatoxin G1	<0.5 ppb	4 ppb
Aflatoxin G2	=0.5 ppb	4 ppb

Table 3 LOQ's of aflatoxins B1, B2, G1 and G2 in comparison with the EU action levels

As seen from Table 3, the LOQ's are far less than the action levels set by the EU.

Validation of the method

This method was validated in two complex matrices: wheat beer and rice wine. These samples were pre-spiked with aflatoxins at two different concentrations, 2 ppb and 20 ppb, corresponding to their respective EU and US Federal Government action levels. Samples were cleaned up using the AflaCLEAN™ immuno affinity cartridge. The eluate collected was analyzed using the above LC/MS/MS method. The recoveries obtained are reported in Table 4.

Matrix	Aflatoxin Conc.	Recovery % ± RSD %			
		Afla. B1	Afla. B2	Afla. G1	Afla. G2
Sake	20 ppb	83.1 ±5.0	91.0 ±1.8	113.0 ±3.0	116.9 ±3.6
Wheat beer	20 ppb	65.4 ±1.9	62.0 ±3.0	86.9 ±2.6	93.2 ±3.6
Sake	2 ppb	92.9 ±3.8	115.5 ±5.0	107.8 ±11.7	108 .0 ±17.0
Wheat beer	2 ppb	70.9 ±2.53	74.8 ±9.9	83.6 ±5.68	82.0 ±6.13

Table 4 Aflatoxin recoveries obtained from wheat beer and sake (n=3).

Recoveries of aflatoxins obtained using the AflaCLEAN column ranged from 65% to 116%. Recoveries of aflatoxins extracted from wheat beer were found to be less than sake at the two levels tested. Relative Standard Deviations (RSDs) obtained were less than 20%.

MRM chromatograms of aflatoxins extracted from wheat beer using the AflaCLEAN immuno affinity column at 2 ppb concentration is given in Figure 3. Both the qualifier and quantitation ions for all four aflatoxins were separated and detected, enabling quantification at these low levels. This column helps eliminate ion suppression caused by the matrix thus allowing the trace level analysis of these compounds.

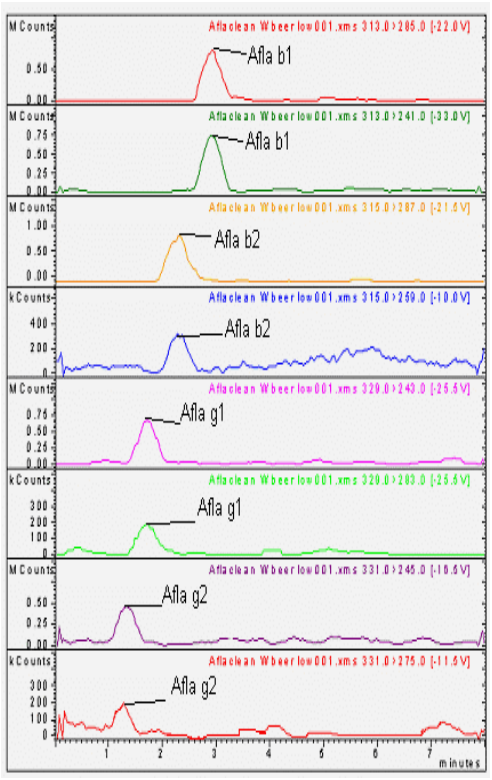

Figure 3 MRM of aflatoxins B1, B2, G1 and G2 at 2 ppb concentration cleaned up using the AflaCLEAN immuno affinity column.

Conclusion

The rapid LC/MS/MS method presented is a sensitive and specific method that fulfills ion ratio requirements for the detection and analysis of aflatoxins in complex matrices.

The AflaCLEAN™ immuno affinity column is a useful tool for the extraction of aflatoxins present in complex matrices prior to LC/MS/MS analysis**.**

References

1. Commission regulation (EC) No 2174/2003 / (EC) No 466/2001 as regards aflatoxins.
2. U.S. Food and Drug Administration Industry Activities Staff Booklet August 2000.
3. European Commission Council directive 96/23/EC/SANCO/1805/2000.

These data represent typical results.
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