APPLICATION NOTE

Liquid Chromatography / Mass Spectrometry

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Analysis of Aflatoxins in Milk by HPLC Using Kobra Cell and Fluorescence Detection

Introduction

Aflatoxins are some of the most carcinogenic mycotoxins known and include aflatoxins B1, B2, G1 and G2. They are produced by toxigenic strains of *Aspergillus flavus, Aspergillus nominus,* and *Aspergillus parasiticus* fungi after feed, crop or harvest exposure to moisture or warm temperatures. Aflatoxin B1 is considered to be the most genotoxic of the mycotoxins, and, when ingested by cows, is converted to aflatoxin M1. Though less potent than B1, M1 has been shown to cause liver cancer in certain animals[1].

Milk is especially vulnerable to aflatoxin contamination, as it can be easily ingested and concentrated during a cow's grazing/feeding. With this in mind, and as M1 is considered the primary aflatoxin expected to be found in milk, the European Union (EU) has established a stringent control limit for M1, set at 0.05 ppb in milk[2]. This is currently the strictest global control limit in this regard, setting a significantly lower level than the Food and Drug Administration's (FDA's) limit of 0.5 ppb[1].

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For aflatoxin analysis, HPLC with fluorescence (FL) detection is commonly used along with online post-column derivatization. As B1 and G1 do not natively fluoresce as well as the other aflatoxins, a Kobra® cell (R-Biopharm Rhône, Ltd) is typically used to electrochemically brominate these two aflatoxins, greatly enhancing their detectability at very low levels. Using this approach, we describe an HPLC method for monitoring B1, B2, G1, G2 and M1 aflatoxins in whole and 1% milk at ppb/ppt levels. This procedure incorporates simple immunoaffinity solid phase extraction (SPE) methodology for initial sample preparation and clean-up.

Experimental

Hardware/Software

A PerkinElmer Altus™ HPLC system was used, including the A-10 Sampling/Solvent Delivery Module, Column Heater and A-10 FL detector (PerkinElmer, Shelton, CT, USA). For online post-column derivatization, a Kobra® cell (R-Biopharm Rhône, Ltd) was used. A PerkinElmer Brownlee C18 3 µm, 4.6 x 100-mm column was used for all determinations (PerkinElmer, Shelton, CT, USA). All instrument control, analysis, and data processing was performed via Waters® Empower® 3 CDS software.

Method Parameters

The HPLC method parameters are shown in Table 1.

Table 1. HPLC Method Parameters.

HPLC Conditions	
Column	PerkinElmer Brownlee C18, 3 µm, 4.6 x 100-mm (Part# N9303507)
Immunoaffinity Column	AflaPure™ immunoaffinity column, 3 mL (BioScientific)
Mobile Phase	17:23:60 acetonitrile/methanol/water
Analysis Time	10 min
Flow Rate	1.1 mL/min. (~5000 psi maximum pressure)
Oven Temp.	30 °C
FL Detection	Excitation (Ex): 362 nm, Emission (Em): 435 nm
Injection Volume	100 µL
Sampling (Data) Rate	5 pts./sec
Diluent	Water

Solvents, Standards and Samples

All solvents and diluents used were HPLC grade and filtered via 0.45-µm filters.

A 20-µg/mL (20-ppm) aflatoxin B1, B2, G1 and G2 standard stock solution in acetonitrile was obtained from Sigma-Aldrich, Inc® (Allentown, PA). A 10-µg aflatoxin M1 standard was also obtained from Sigma-Aldrich and taken up in water to make a 10-µg/mL (10-ppm) stock solution. A 10-ppb aflatoxin working standard was then prepared by transferring 50 µL of the B1/B2/G1/G2 stock solution and 100 µL of the M1 stock solution to a 100-mL volumetric flask and diluting to the mark with water. For calibration, aflatoxin concentrations of 3.82, 0.764, 0.382, 0.0765 and 0.0382 ppb were prepared via serial dilution with water.

Whole and 1% milks were obtained from a local grocery store. Each milk was run as a duplicate set. In a 50-mL centrifuge tube, 40 mL of each milk was spiked with 800 µL of 3.82-ppb aflatoxin calibrant to 0.0765 ppb and vortexed for one minute. To prepare the milk extracts, the spiked milks were each diluted 10-fold by transferring 2.5 mL of each sample to another 50-mL tube and adding 22.5 mL of water. 0.6 g of sodium chloride was added to each tube and the tubes were then well shaken. The samples were again vortexed for one minute and centrifuged for 10 minutes at 5500 rpm. A 20.0 mL aliquot of each defatted supernatant was then loaded onto individual AflaPure™ 3-mL immunoaffinity SPE columns at a rate of one drop/second. (Note: one should not let the column packing dry out at this point.) Subsequently, the columns were washed with 10 mL 0f 90:10 water/methanol, at a rate of 1-2 drops/second, until air was seen passing through the column.

For the spiked 1% milk sample, aflatoxin elution was performed by next passing 800 µL of methanol through the SPE column, at a rate of one drop/second, and captured in a 15-mL conical-bottomed centrifuge tube. Again, one should wait until air is seen passing through the column. The eluent was then topped off to 2.0 mL with 70:30 water/methanol. 1 mL of this solution was then transferred to an autosampler vial and injected (100-µL injection).

For the spiked whole milk sample, aflatoxin elution was performed by passing 2.5 mL of methanol through the SPE column, at a rate of 1 drop/second, and captured in a 15-mL conical-bottomed centrifuge tube. The eluent was then dried down to approximately 0.25 mL and brought back to 2.0 mL with 70:30 water/methanol. 1 mL of this solution was then transferred to an autosampler vial and injected (100-µL injection).

Results and Discussion

Figure 1 shows chromatograms of the Level-3 (0.382 ppb) aflatoxin calibrant (A) and the Level-1 (0.0382 ppb) aflatoxin calibrant (B), demonstrating all five analytes isocraticly separated in under six minutes.

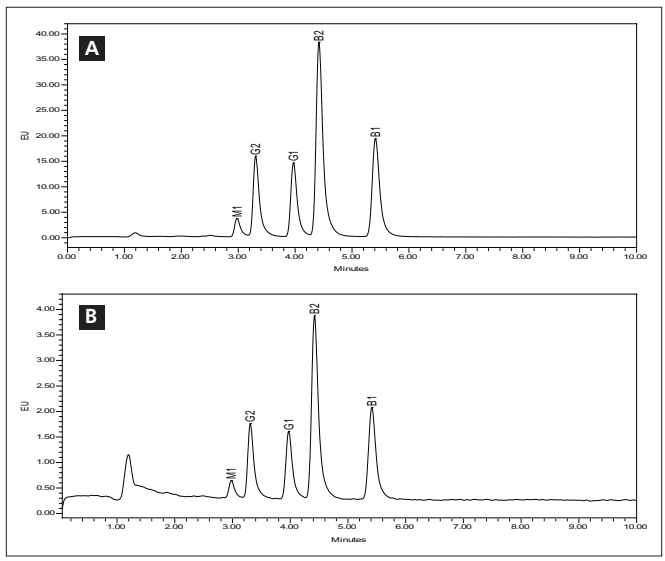

Figure 1. LC chromatograms of Level-3 (0.382 ppb) aflatoxin calibrant (A) and the Level-1 (0.0382 ppb) calibrant (B).

As shown in Figure 2, chromatographic repeatability was confirmed via ten replicate injections of the Level-3 calibrant, demonstrating exceptional reproducibility. The retention time %RSD for all analytes was ≤ 0.05%.

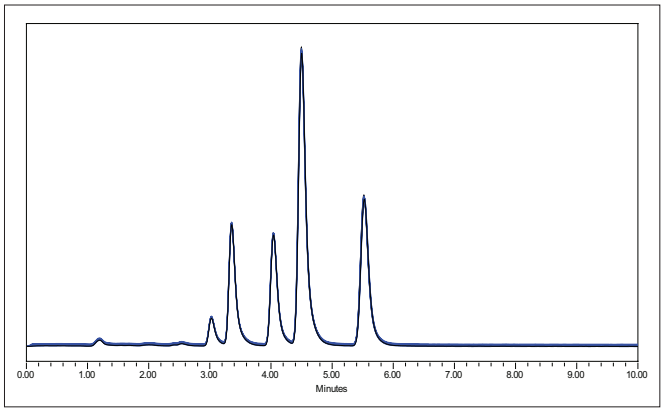

Figure 2. Overlay of 10 replicates of the Level-3 (0.382-ppb) calibrant.

To test for carryover, a mobile phase blank was injected after three replicate injections of the high Level-5 (3.82 ppb) calibrant. As shown in Figure 3, the chromatogram of this blank shows no detectable carryover.

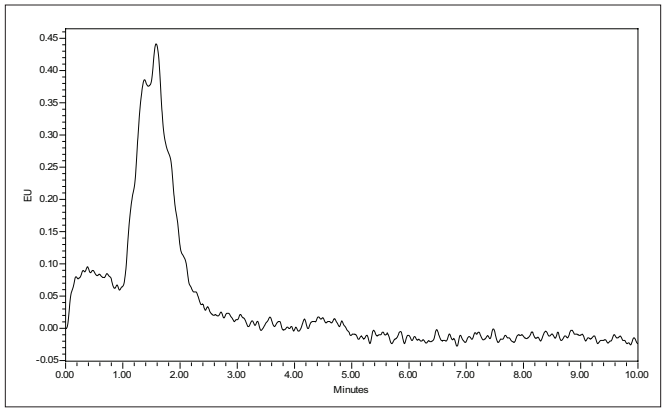

Figure 3. Chromatogram of a mobile phase blank injected after three replicate injections of Level-5 calibrant.

Figure 4 shows example calibration plots for aflatoxins M1 and B1. Calibration linearity was greater than $R^2 = 0.999$ for all analytes.

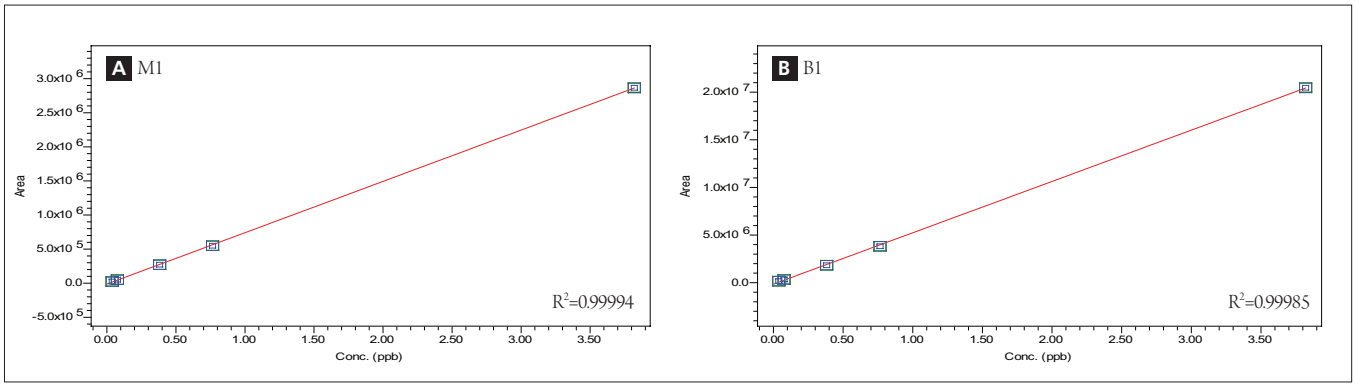

Figure 4. Linear calibration plots of aflatoxins M1 (A) and B1 (B); concentration range: 0.0382-3.32 ppb.

As listed in Table 2, the limits of quantitation (LOQ) were established for all analytes, based upon a S/N of >10/1. As shown, aflatoxin M1 was easily quantifiable down to the 0.05 ppb level established by the EU as the maximum tolerable level in milk.

Table 2. Retention times (RT) and LOQs for aflatoxins M1, G2, G1, B2 and B1; as extrapolated based upon the Level-1 (0.0382 ppb) standard.

Aflatoxin	RT (min)	LOQ (ppb)
M1	2.98	0.018
G2	3.31	0.005
G1	3.97	0.005
B2	4.42	0.002
B1	5.41	0.005

Figures 5 and 6 show the chromatograms of the 0.0765-ppb spiked 1% milk and the 0.0765-ppb spiked whole milk, respectively. It should be noted that, upon calculating the LOQs for M1 based upon these spiked samples, the values were 0.018 ppb for 1% milk and 0.025 ppb for whole milk, still ≤ half the EU control limit.

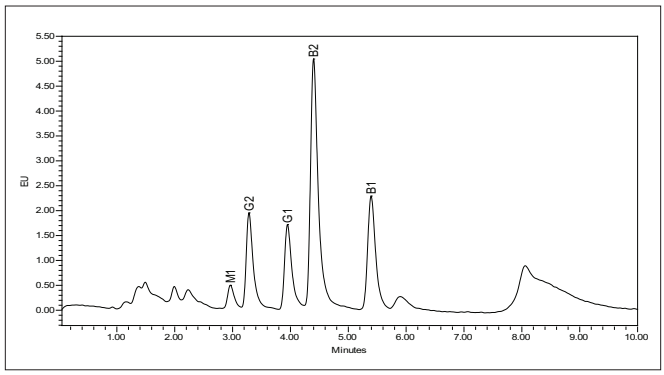

Figure 5. Chromatogram of the 0.0765-ppb spiked 1% milk.

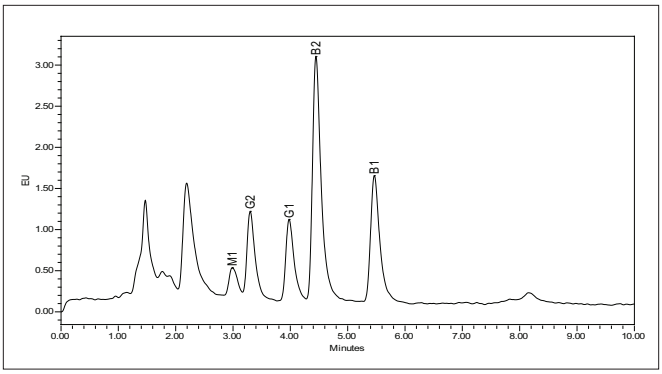

Figure 6. Chromatogram of the 0.0765-ppb spiked whole milk.

Recoveries for the spiked milk samples, run as duplicate sets, are shown in Table 3. All the recovery values were very consistent across the duplicate sets and the recoveries for the 0.0765-ppb spiked 1% milk were exceptional. The recoveries for spiked whole milk were also good considering the more difficult matrix for sample extraction and very low analyte concentrations.

Table 3. Recovery results for duplicate sets of 0.0765-ppb spiked milk samples (n=3 for each sample).

Sample	B1 (%)	B2 (%)	G1 (%)	G2 (%)	M1 (%)
1% Milk #1	91.4	94.1	95.3	70.2	85.6
1% Milk #2	91.0	97.7	94.6	70.2	92.8
Avg. for 1% Milk	91.2	95.9	95.0	70.2	89.2
Whole Milk #1	87.7	76.5	70.1	68	92.4
Whole Milk #2	81.3	75.2	68.0	65.5	86.8
Avg. for Whole Milk	81.5	75.9	69.1	66.8	89.6

Conclusion

This work demonstrated the effective separation and quantitation of B1, B2, G1, G2 and M1 aflatoxins using SPE sample cleanup and using a PerkinElmer Altus HPLC system with an A-10 FL detector and a Kobra cell for online post-column derivatization. The results exhibited exceptional linearity for each aflatoxin over the tested concentration range. Quantitation was afforded down to below 0.02 ppb with consistently good recoveries. The sub-0.02 ppb LOQs allow for routine analysis of all five aflatoxins down to well below the 0.05 ppb acceptable limit for M1 in milk, as established by the EU.

References

1. FDA Compliance Policy Guide, under Inspections, Compliance, Enforcement, and Criminal Investigations; CPG Section 527.400 Whole Milk, Lowfat Milk, Skim Milk - Aflatoxin M1.
2. Commission Regulation (EU) No 165/2010. Amending Regulation (EC) No 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins. Official Journal of the European Union, Feb 26, 2010, pp L 50/8 – L 50/12.

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