APPLICATION NOTE

Liquid Chromatography

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Analysis of Aflatoxin M1 in Raw Milk by HPLC with Fluorescence Detection

Introduction

Aflatoxins B1 is considered to be the most carcinogenic/genotoxic mycotoxin known, produced by toxigenic strains of Aspergillus flavus, Aspergillus nominus, and Aspergillus parasiticus fungi after feed, crop or harvest exposure to moisture or warm temperatures. When ingested by cows, aflatoxin B1 is converted to aflatoxin M1, which, though less potent than B1, 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]

To analytically reach the EU's very low control limit for M1, HPLC with fluorescence (FL) detection is the preferred choice. Using this technique, we describe an HPLC method for monitoring aflatoxin M1 in raw milk at ppb/ppt levels, using simple immunoaffinity solid phase extraction (SPE) methodology for initial sample preparation/clean-up. Though the focus herein was on aflatoxin M1, aflatoxins B1, B2, G1 and G2 were also included as part of the standards and spikes, to confirm the chromatographic separation of M1 from other aflatoxins.

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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). A PerkinElmer Brownlee C18 3 µm, 4.6x100-mm column was used for all analyses (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, 4.6x100-mm (Part# N9303507)
SPE Column:	Afla M1™ immunoaffinity column, 3 mL (Vicam)
Mobile Phase:	17:23:60 acetonitrile/methanol/water
Analysis Time:	10 min
Flow Rate:	1.1 mL/min. (2900 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 10-µg aflatoxin M1 standard was obtained from Sigma-Aldrich and taken up in water to make a 10-µg/mL (10-ppm) stock solution. A 20-µg/mL (20-ppm) aflatoxin B1, B2, G1 and G2 standard stock solution in acetonitrile was also obtained from Sigma-Aldrich, Inc® (Allentown, PA). A 10-ppb aflatoxin working standard was then prepared by transferring 100 µL of the M1 stock solution and 50 µL of the B1/B2/G1/G2 stock solution to a 100-mL volumetric flask and diluting to 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.

Raw milk was obtained from a local health food store. The milk was tested as three separate aliquots. In a 50-mL centrifuge tube, 40 mL of each milk aliquot was spiked with 800 µL of 3.82-ppb aflatoxin calibrant, producing a 0.0765-ppb spiked sample. The spiked milk aliquots were then vortexed for one minute. The spiked milks were each diluted 10-fold by transferring 4 mL of each sample to another 50-mL tube, adding 36 mL of water and then shaking the tubes well. 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 Afla M1™ 3-mL immunoaffinity SPE columns, at a rate of 1 drop/second, without letting the column packing dry out. Allowing the column to dry out at this point may adversely affect either binding or wash efficiency. Subsequently, the columns were washed with 20 mL of water, at a rate of 1-2 drops/second, until air was seen passing through the column. Aflatoxin M1 was then eluted into a 15-mL conical-bottomed centrifuge tube by passing 2.5 mL of 60:40 acetonitrile/methanol through the SPE column, at a rate of 1 drop/second, again, waiting until air was seen passing through the column. Each eluent was then dried down to approximately 100 µL 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 the chromatogram of the Level-3 (0.382 ppb) aflatoxin calibrant, both in full view (A) and expanded view (B), to highlight M1. All five aflatoxins were isocraticly separated in under six minutes.

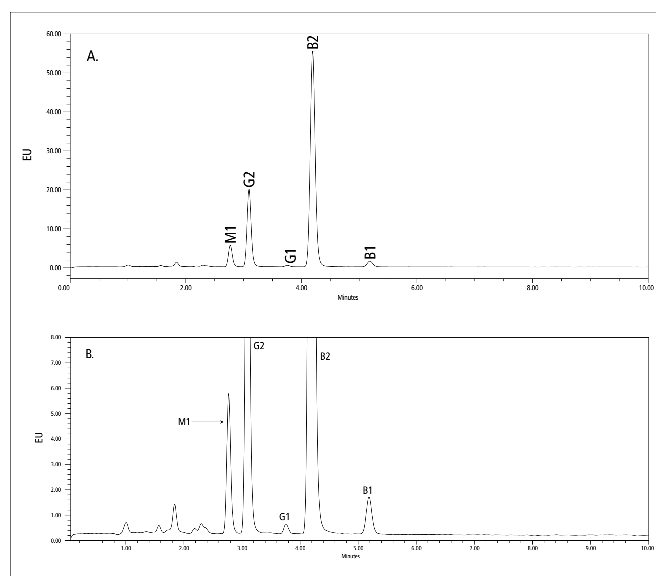

Figure 1. LC chromatogram of Level-3 (0.382 ppb) aflatoxin calibrant; full (A) and expanded (B) views

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 M1 was 0.05%.

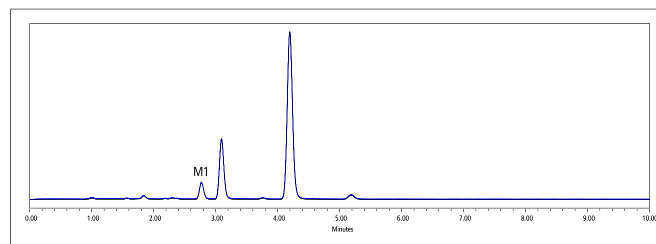

Figure 2. Overlay of 10 replicates of the Level-3 (0.382-ppb) calibrant.

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

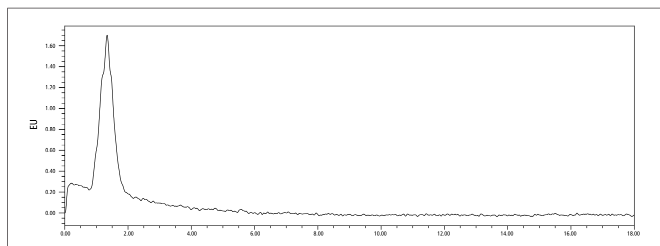

Figure 3. Chromatogram of a mobile phase blank injected after three replicate injections of Level-5 calibrant.

Figure 4 shows the calibration plot for aflatoxin M1. Calibration linearity (R^2) was greater than 0.9999.

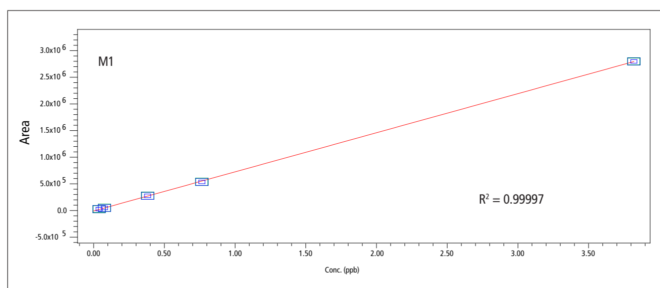

Figure 4. Linear calibration plot of aflatoxin M1; concentration range: 0.0382-3.32 ppb.

The limit of quantitation (LOQ) for aflatoxin M1, based upon a S/N of ≥10/1, was calculated to be 0.011 ppb, making M1 easily quantifiable down to the 0.05 ppb level established by the EU as the maximum tolerable level in milk.

Figures 5 shows the full and expanded chromatographic views of a 0.0765-ppb spiked raw milk sample. The run time had to be extended to 15 minutes to account for the large unidentified matrix peak that consistently eluted at 11 minutes.

It should be noted that, upon calculating the LOQ for M1 based upon this spiked sample, the LOQ was 0.014 ppb, also considerably below the EU control limit.

The chromatographic overlay in Figure 6 highlights the repeatability for three replicate injections of a 0.0765-ppb spiked raw milk sample.

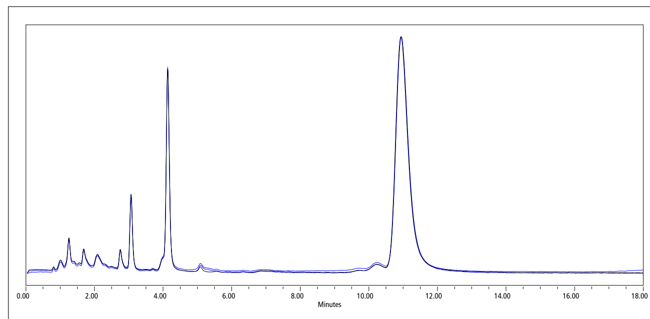

Figure 6. Chromatographic overlays of three replicate injections of a 0.0765-ppb spiked raw milk sample.

Recoveries for the three aliquots of spiked raw milk are shown in Table 2. All individual values are an average of three replicates. Overall, the recoveries very good considering the rather difficult matrix for sample extraction and very low analyte concentrations. The somewhat higher recovery for Raw Milk 2 may very well be attributable to the limited exactness in the graduation lines of the 15-mL centrifuge tubes upon bringing the partially dried SPE eluent back to 2 mL with 70:30 water/methanol.

Table 2. Aflatoxin M1 recovery results for the three aliquots of 0.0765-ppb spiked raw milk (n=3 for each aliquot).

Sample	M1 (%)
Raw Milk 1	97.8
Raw Milk 2	112.5
Raw Milk 3	101.4
Avg. for 3 aliquots	103.9

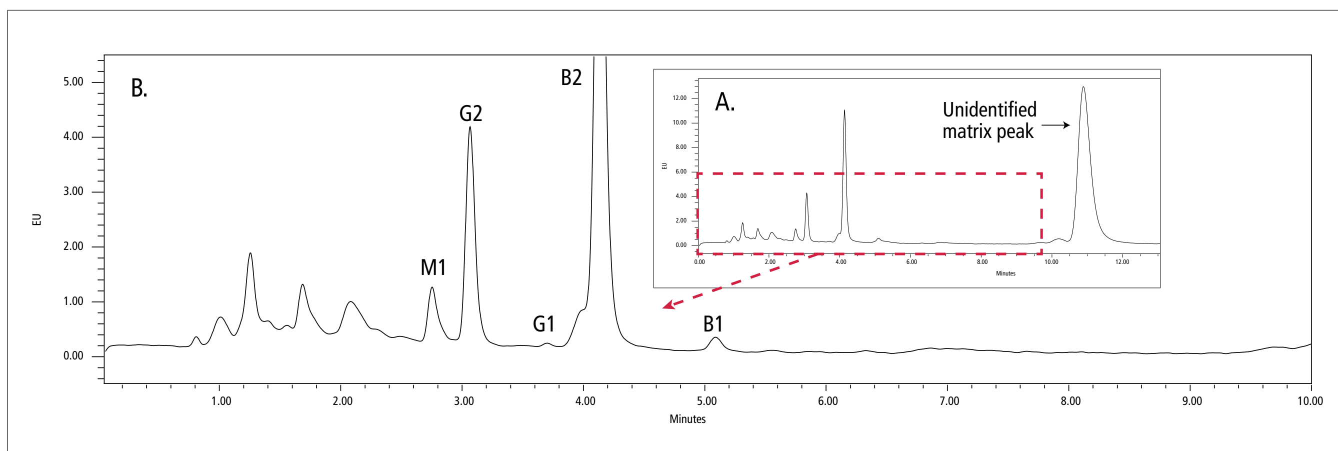

Figure 5. Chromatogram of 0.0765-ppb spiked raw milk. A) full view; B) expanded view.

Conclusion

This work demonstrated the effective chromatographic separation and quantitation of aflatoxin M1 using a PerkinElmer Altus HPLC system with an A-10 FL detector and immunoaffinity SPE sample cleanup. The results exhibited exceptional linearity over the tested concentration range. Quantitation is achievable down to < 0.02 ppb, with very good recoveries. The sub-0.02 ppb LOQ allows for the routine analysis of aflatoxin M1, well below the 0.05 ppb acceptable limit in milk, as established by the EU.

Note: For a focus on aflatoxins M1, B1, B2, G1 and G2 in 1% and whole milk, please refer to the application note entitled *Analysis of Aflatoxins in Milk by HPLC using Kobra Cell and Fluorescence Detection.*

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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