



Liquid Chromatography

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Analysis of Aflatoxins in Pet Food by UHPLC Using PDA and Fluorescence Detection

Introduction

Commercially prepared pet foods are easy and economical ways to fulfill the nutritional requirements for pets. Dry pet food is produced with grains and cereal by-products rejected for human consumption. The contamination of

these by-products, with toxigenic fungal metabolites called mycotoxins, pose a serious health threat to pets.

Aflatoxins, some of the most carcinogenic mycotoxins known, are classified as B₁, B₂, G₁, and G₂. They are produced by toxigenic strains of *Aspergillus flavus*, *Aspergillus nominus*, and *Aspergillus parasiticus* fungi after 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 farm animals, can contaminate dairy, eggs, and meat products intended for human consumption.¹

Several aflatoxin outbreaks in commercial pet foods have been reported in the past few years. Symptoms from aflatoxin exposure include lethargy, anorexia, jaundice, and intravascular coagulation, the severity often varying based upon a pet's breed, species, age, dose, length of exposure, and nutritional status.² Even if affecting only a small percentage of commercial pet foods, problems with pet food safety impact the entire pet food industry due to recalls and loss of consumer loyalty. Such experiences have reaffirmed the need for commercial pet food manufacturers to devote extensive resources documenting product quality.²

The U.S. Food and Drug Administration (FDA) control limit for raw mycotoxins in grains is 20 ppb, while in the European Union standards are stricter, set at 10 ppb. Trace amounts of aflatoxin in some commercial pet foods are typically around 1-2 ppb.³ Post-column derivatization is commonly used to enhance the response of aflatoxin analytes at these levels using reversed phase separation and fluorescence (FL) detection.

In this application, we describe a technique for monitoring B₁, B₂, G₁, and G₂ aflatoxins at ppb to ppt levels without the need for post-column derivatization. This method uses a simple solid phase extraction (SPE) technique for sample clean-up followed by UHPLC analysis using a sub-3 μm particle column combined with fluorescence (FL) detection.

Experimental

Hardware/Software

A PerkinElmer Altus™ UPLC® system was used, including the A-30 Sampling and Solvent Delivery Module (quaternary pump), column heater, A-30 PDA (photodiode array), and FL detectors (PerkinElmer, Shelton, CT, USA). A PerkinElmer Brownlee™ SPP C18 2.7μm, 3.0 x 100-mm column was used for all separations (PerkinElmer, Shelton, CT, USA). All instrument control, analysis, and data processing was performed via Waters® Empower® 3 Chromatography Data Software (CDS).

Method Parameters

The UHPLC method parameters are shown in Table 1.

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 stock standard solution was obtained from Sigma-Aldrich® Inc. (Allentown, PA) and consisted of aflatoxins B₁, B₂, G₁, and G₂ in acetonitrile. A 20-ppb working standard was prepared by adding 10 μL of the stock standard to 10.0 mL of diluent. A 1.6-ppb working solution was prepared by adding 2 μL of aflatoxin stock standard mixture to 25.0 mL of diluent.

Table 1. UHPLC Method Parameters.

Column:	PerkinElmer Brownlee SPP C18, 2.7 μm, 3.0 x 100-mm (Part# N9308410)				
SPE Cartridge:	Supel™ Tox AflaZea, 6-mL, (Cat. No. 55314-U), Sigma-Aldrich® Inc. (Allentown, PA)				
Mobile Phase:	Solvent A: 0.1% formic acid in water Solvent B: 0.1% formic acid in 50% methanol, 50% acetonitrile Solvent program:				
		Time (min)	Flow Rate (mL/min)	%A	%B
	1	Initial	0.5	70.0	30.0
	2	4.5	0.5	40.0	60.0
	3	4.6	0.5	5.0	95.0
	4	4.8	0.5	5.0	95.0
5	5.0	0.5	70.0	30.0	
Analysis Time:	4.0 min.; wash/equilibration time = 6.0 min.				
Flow Rate:	0.5 mL/min. (~5000 psi maximum pressure)				
Oven Temp.:	35 °C				
PDA Detection:	Wavelength: 360 nm				
FL Detection:	Excitation (Ex): 365 nm, Emission (Em): 425 nm; for quantitation of B ₁ and B ₂ Excitation (Ex): 365 nm, Emission (Em): 450 nm; for quantitation of G ₁ and G ₂				
Injection Volume:	10 μL				
Sampling (Data) Rate:	10 pts./sec				
Diluent:	80:20 acetonitrile/water				

Commercial pet foods were obtained from a local store. To prepare pet food extracts, approximately 30 g of pet food was ground into a fine powder and 25.0 g weighed into an Erlenmeyer flask. To prepare 1.6-ppb spiked pet food, 8 μL of the 20-μg/mL aflatoxin stock standard was added to the powder. 100.0 mL of diluent was added and the flasks swirled for one hour. To prepare an unspiked pet food extract, the procedure was repeated without adding the aflatoxin standard.

For sample cleanup prior to injection, 2.0 mL of the 1.6-ppb working standard, spiked extract, and unspiked extract were each added to an individual AflaZea SPE column and quickly passed through using a vacuum pump. Prior to analysis, 200 μL of each eluent was added to 880 μL of HPLC-grade water and mixed by manual shaking. Recoveries of the aflatoxin spike in the dog and cat foods were calculated against the response of the 1.6-ppb working standard.

Results and Discussion

Figure 1 shows the chromatogram of the 20-ppb aflatoxin standard mixture containing B₁, B₂, G₁, and G₂, using PDA and fluorescence detection. The upper chromatogram, (A) was collected by PDA at 360 nm, while the lower chromatogram, (B), was collected by fluorescence at Ex 365 nm / Em 425 nm. Separation for the aflatoxins was achieved in less than 4 minutes.

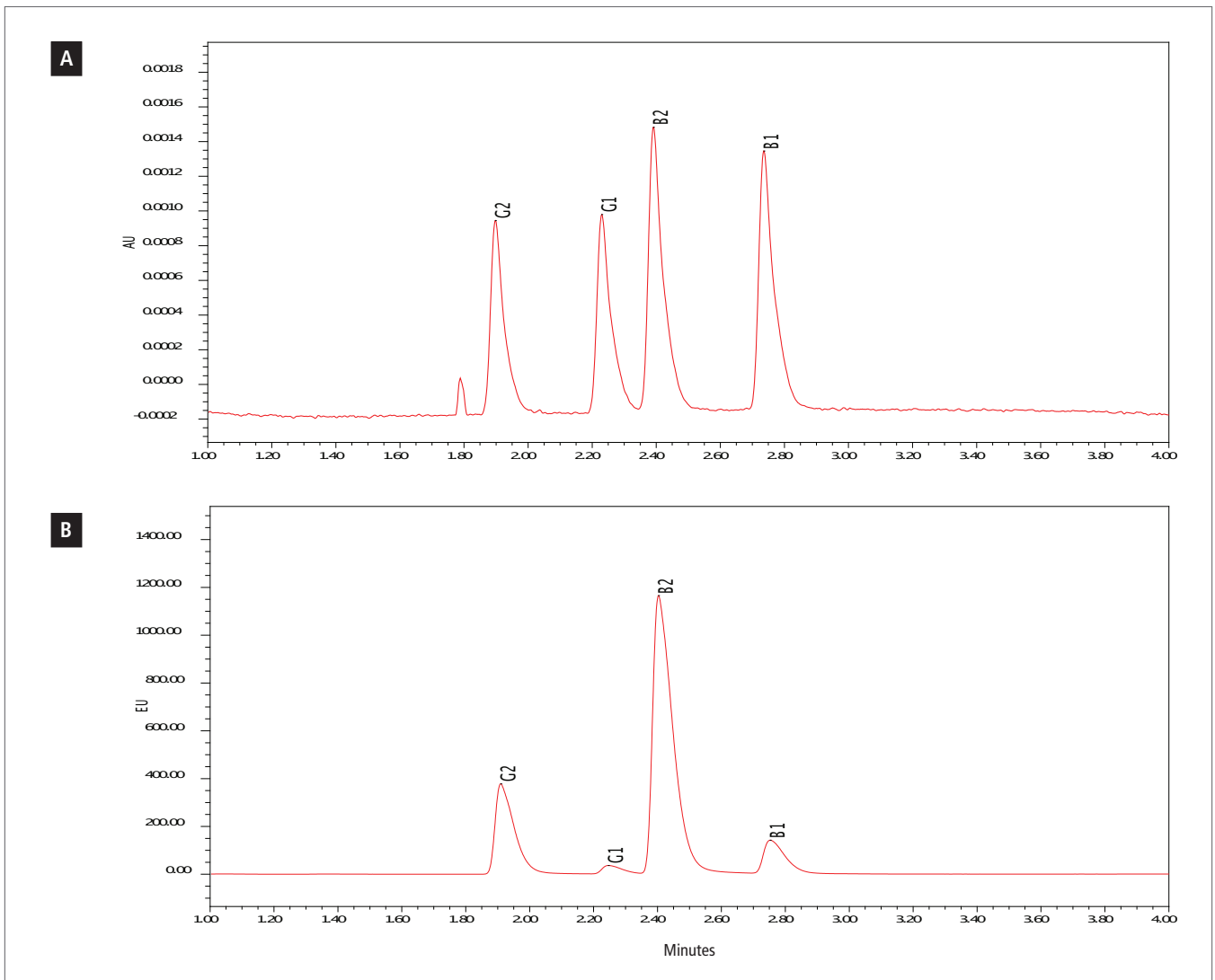


Figure 1. UHPLC chromatogram showing the 20-ppb standard solution by: (A) PDA at 360 nm; (B) FL: Ex 365 nm /Em 425 nm.

As shown in Figure 2, chromatographic repeatability was confirmed via 10 injections of the 1.6-ppb standard by fluorescence detection, demonstrating exceptional reproducibility. The retention time %RSD for all peaks was less than 0.2%.

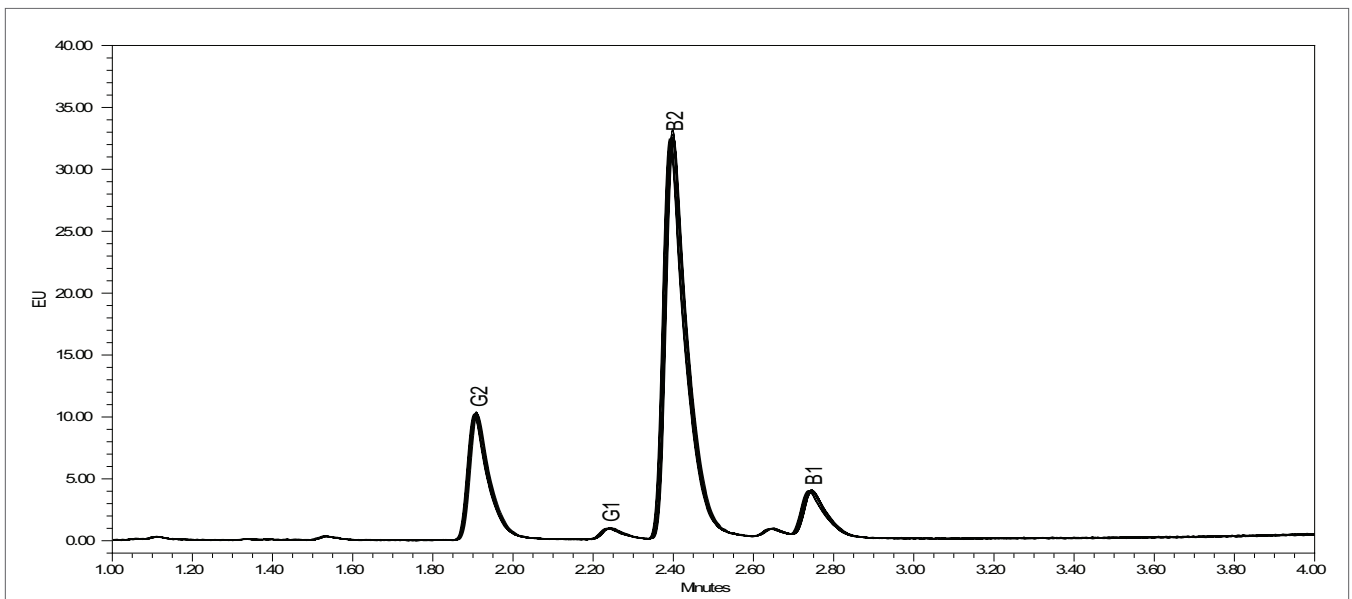


Figure 2. Overlay of 10 replicates of the 1.6-ppb aflatoxin working standard solution by fluorescence.

Linearity was determined for aflatoxins B₁, B₂, G₁, and G₂ by both PDA and FL detection at ppb levels. Representative calibration plots for B₁ and G₂ are shown in Figure 3 and Figure 4.

As listed in Table 2, LOQ and LOD levels were established for each of the aflatoxins based upon a *s/n* of >10/1 for LOQ and > 3/1 for LOD. Aflatoxins B₁, B₂, G₁, and G₂ are quantifiable down to approximately 3 ppb by PDA. Using fluorescence detection, aflatoxins B₁, B₂ and G₂ are quantifiable down to less than 600 ppt. For aflatoxin G₁, the LOQ by fluorescence was approximately 2 ppb.

Table 2. LOQ and LOD of aflatoxins B₁, B₂, G₁, and G₂.

Aflatoxin	LOQ via PDA (ppb)	LOD via PDA (ppb)	LOQ via FL (ppb)	LOD via FL (ppb)
G ₂	3.39	1.02	0.11	0.03
G ₁	3.43	1.03	2.18	0.65
B ₁	2.68	0.80	0.53	0.16
B ₂	2.43	0.73	0.07	0.02

The overlaid chromatograms in Figure 5 and Figure 6 show pet food spiked with analytes B₁, B₂, G₁, and G₂ at 1.6 ppb detected by fluorescence. Recoveries of the 1.6-ppb spike ranged from 70-120% (Table 3).

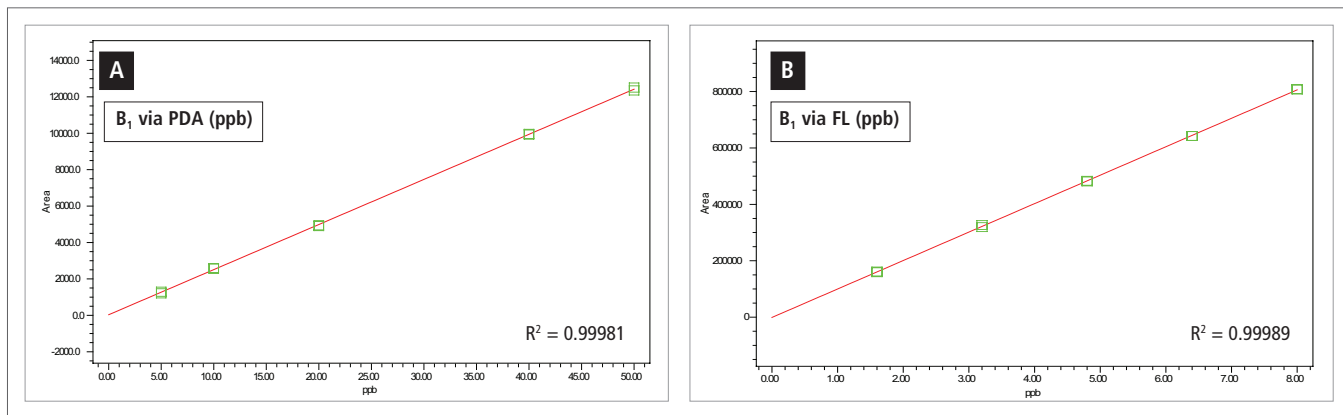


Figure 3. Linearity plots of B₁ at concentrations between 5-50 ppb by PDA at 360 nm (A) and between 1.6-8 ppb by fluorescence (B); Ex and Em wavelengths as specified by the method.

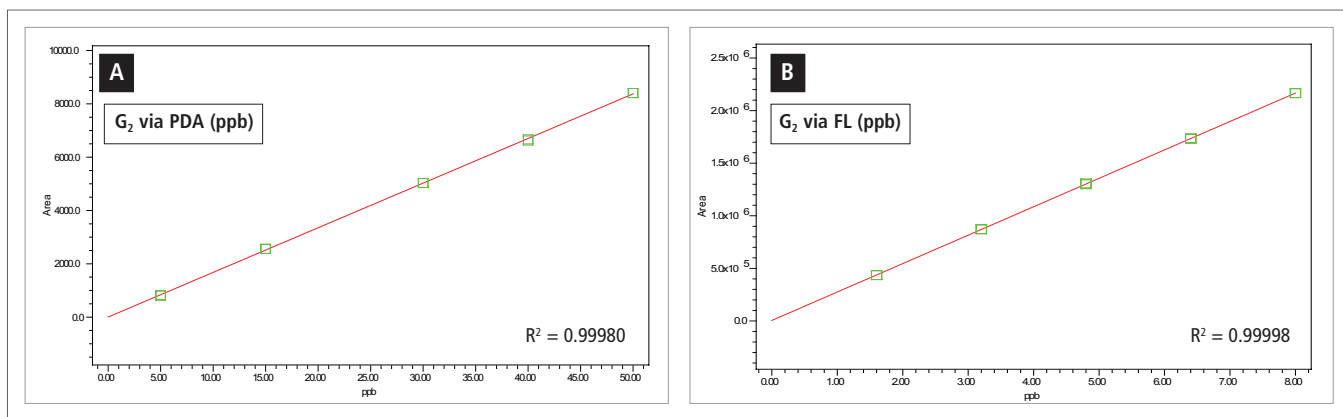


Figure 4. Linearity plots of G₂ at concentrations between 5-50 ppb by PDA at 360 nm (A) and between 1.6-8 ppb by fluorescence (B); Ex and Em wavelengths as specified by the method.

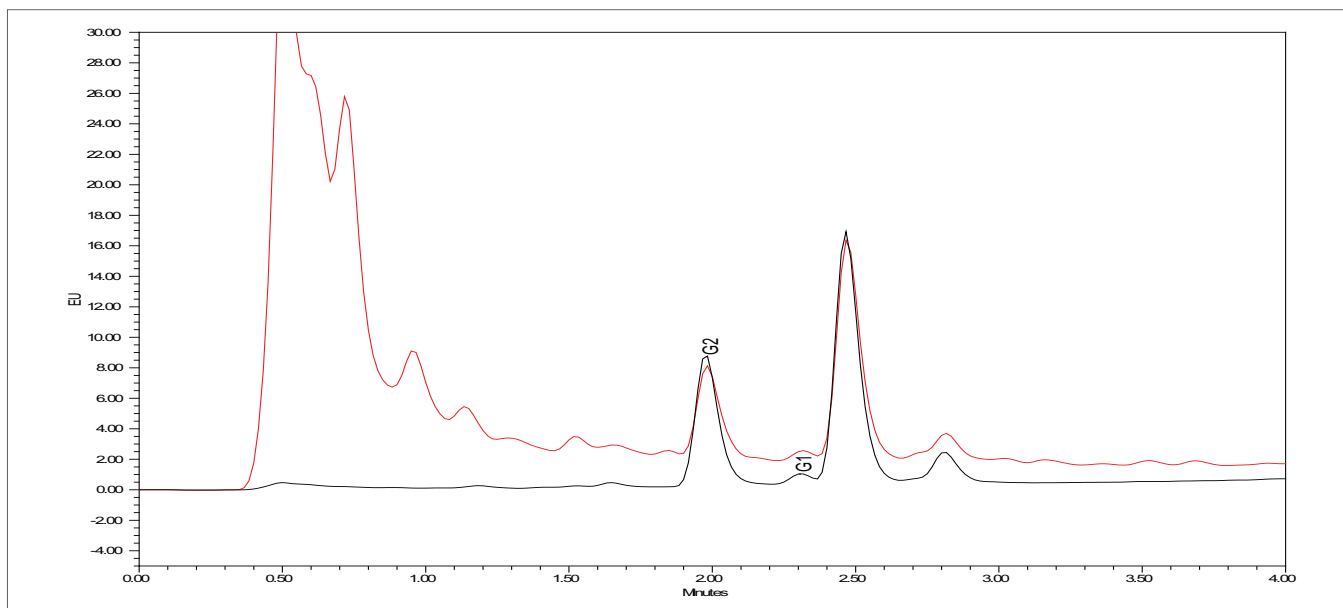


Figure 5. Overlaid chromatograms of the 1.6-ppb aflatoxin standard (black) and 1.6-ppb spiked dog food (red) by fluorescence at Ex 365 nm, Em 450 nm; used for quantitation of G₁ and G₂.

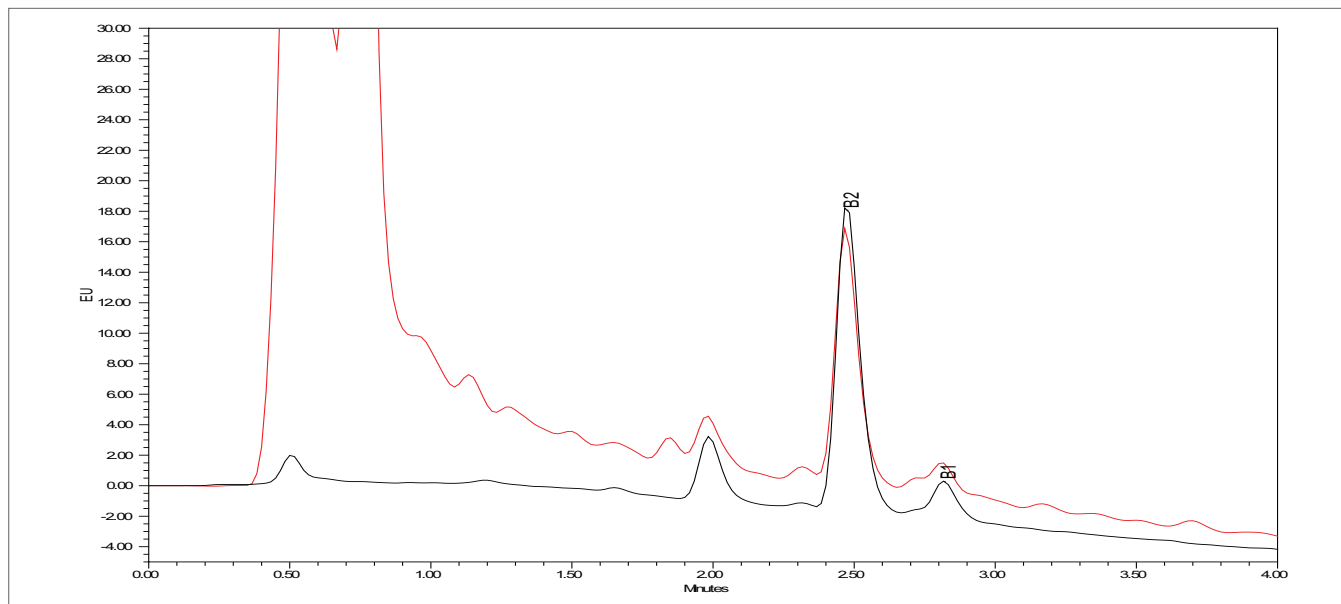


Figure 6. Overlaid chromatograms of the 1.6-ppb aflatoxin standard (black) and 1.6-ppb spiked cat food (red) by fluorescence at Ex 365 nm, Em 425 nm; used for quantitation of B₁ and B₂.

Recoveries of the spiked 1.6-ppb B₁, B₂, G₁, and G₂ analytes from the pet food ranged from 70-120% (Table 3). Although the 1.6-ppb spiked aflatoxin level was slightly below the calculated LOQ for G₁, recovery was acceptable at 100-121%.

Though not shown, no quantifiable aflatoxins were observed in the unspiked dog food and cat food.

Table 3. Recovery results for spiked dog food and cat food at 1.6 ppb by fluorescence detection (n=2).

Sample	B ₁ (%)	B ₂ (%)	G ₁ (%)	G ₂ (%)
Cat food	82.3	84.7	120.7	70.2
Dog food	93.5	94.7	100.4	79.2

Conclusion

This work demonstrated the effective chromatographic separation and quantitation of B₁, B₂, G₁, and G₂ aflatoxins using a PerkinElmer Altus UPLC® system with A-30 PDA and FL detectors. The results exhibited exceptional linearity for each aflatoxin over the tested concentration ranges.

Though none of the analyzed pet foods showed any detectable amount of aflatoxin, the spike recovery analysis demonstrated the ability of the A-30 FL detector to detect aflatoxins B₁, B₂, G₁, and G₂ at ppb levels without the need for derivatization.

References

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- Simone Aquino and Benedito Corrêa, "Aflatoxins in Pet Foods: A Risk to Special Consumers", *Aflatoxins - Detection, Measurements and Control*, 2011.
- <http://www.poisonedpets.com/top-us-pet-food-brands-test-positive-for-aflatoxin-melamine-and-cyanuric-acid/>.