APPLICATION NOTE

Liquid Chromatography

Authors:

Catharine Layton

Wilhad M. Reuter

PerkinElmer, Inc.
USA

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 B_1, B_2, G_1, and G_2. 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 B_1 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]

PerkinElmer®
For the Better

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 B_1, B_2, G_1, and G_2 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.

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

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 B_1, B_2, G_1, and G_2 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.

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 B_1, B_2, G_1, and G_2, 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.

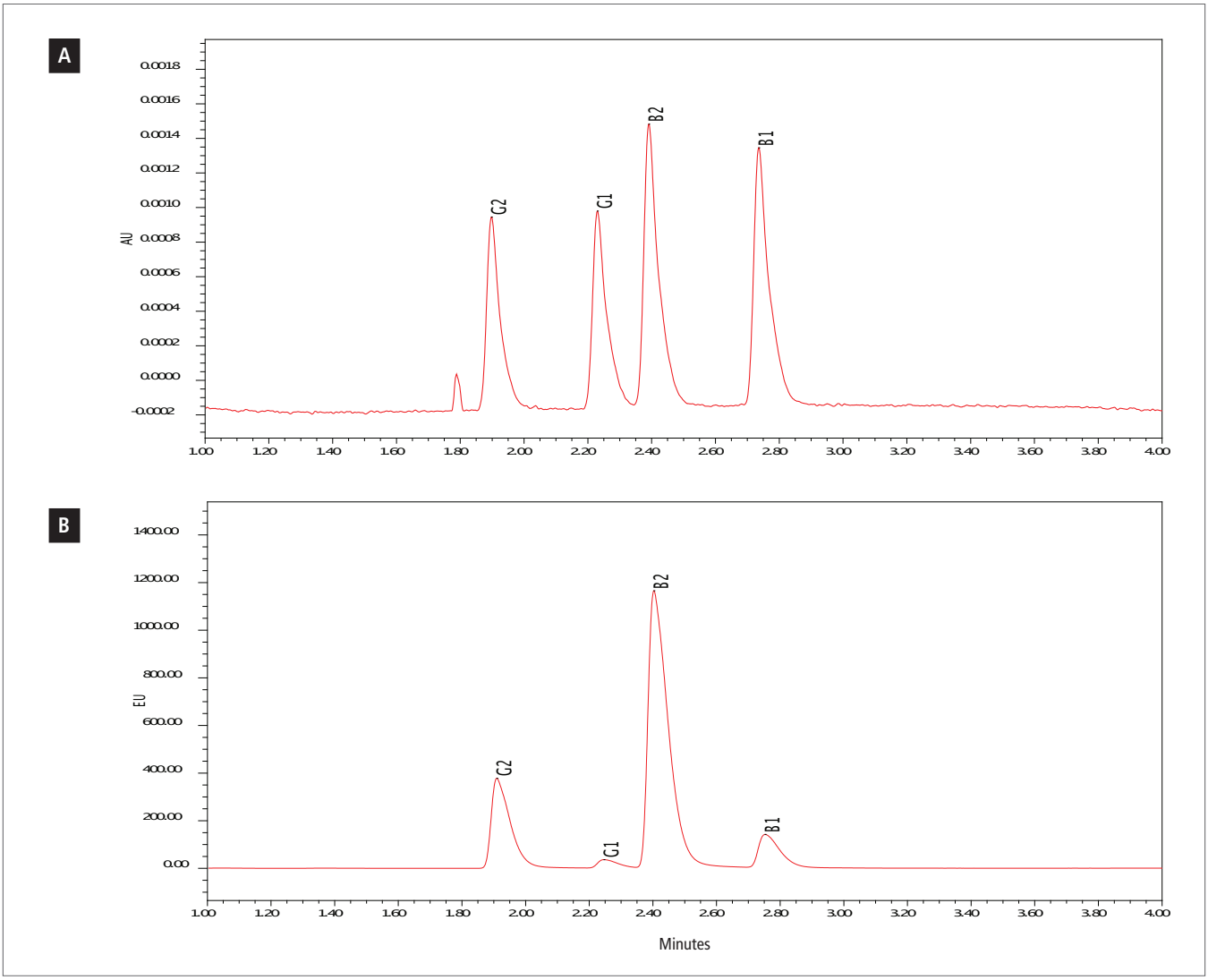

Figure 1. UHPLC chromatogram showing the 20-ppb standard solution by: (A) PDA at 360 nm; (B) FL: Ex 365 nm /Em 425 nm.

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%.

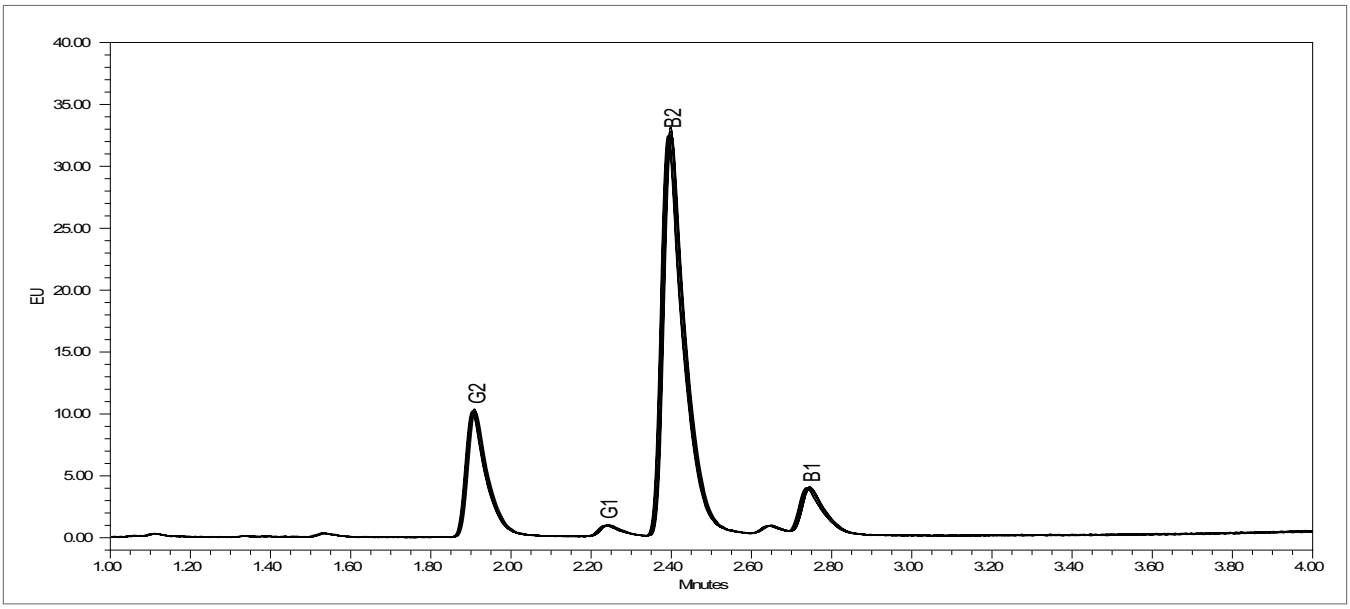

Figure 2. Overlay of 10 replicates of the 1.6-ppb aflatoxin working standard solution by fluorescence.

Linearity was determined for aflatoxins B_1, B_2, G_1, and G_2 by both PDA and FL detection at ppb levels. Representative calibration plots for B_1 and G_2 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 B_1, B_2, G_1, and G_2 are quantifiable down to approximately 3 ppb by PDA. Using fluorescence detection, aflatoxins B_1, B_2 and G_2 are quantifiable down to less than 600 ppt. For aflatoxin G_1, the LOQ by fluorescence was approximately 2 ppb.

Table 2. LOQ and LOD of aflatoxins B_1, B_2, G_1, and G_2 .

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 B_1, B_2, G_1, and G_2 at 1.6 ppb detected by fluorescence. Recoveries of the 1.6-ppb spike ranged from 70-120% (Table 3).

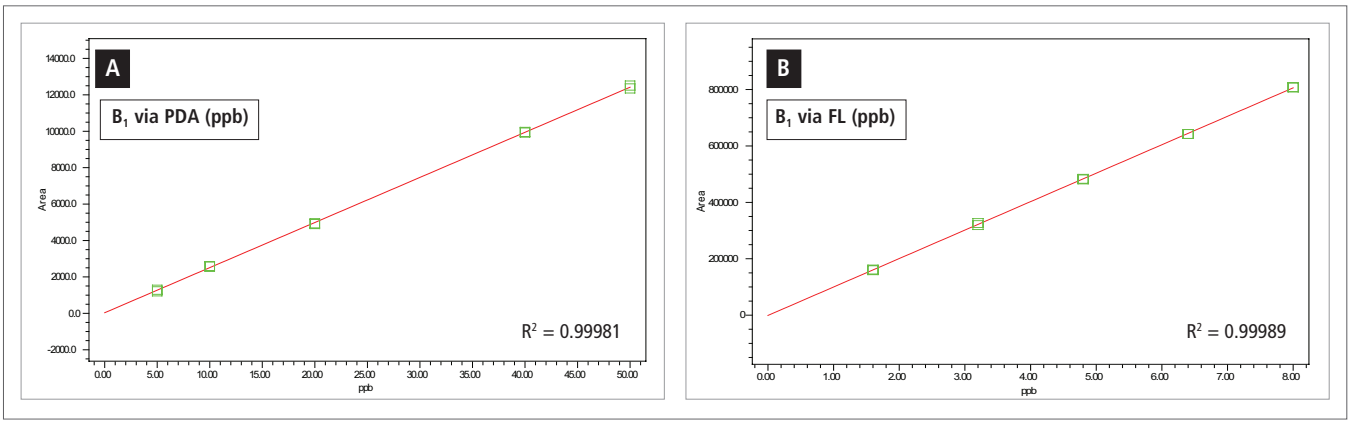

Figure 3. Linearity plots of B_1 at concentrations between 5-50 ppb by PDA at 360 nm (A) and between 1.6-8 ppb by fluorescence (B); Ex and Em wavelengths as specified by the method.

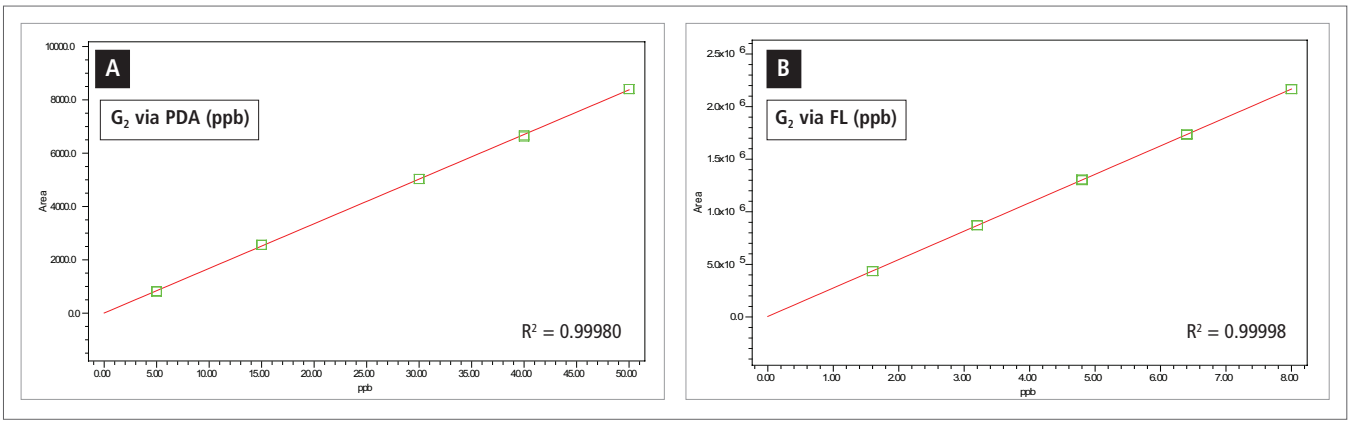

Figure 4. Linearity plots of G_2 at concentrations between 5-50 ppb by PDA at 360 nm (A) and between 1.6-8 ppb by fluorescence (B); Ex and Em wavelengths as specified by the method.

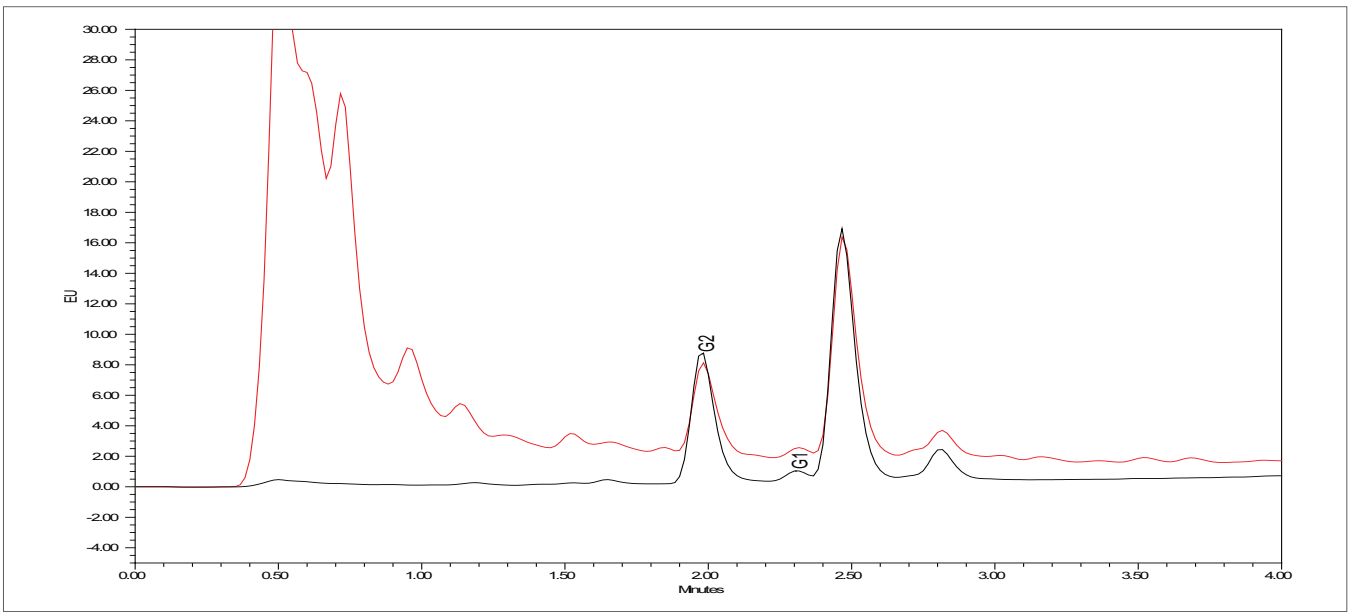

Figure 5. Overlaid chromatograms of the 1.6-ppb aflatoxin standard (black) and 1.6-ppb spiked dog food (red) by fluorescence at Ex 365 nm, Em 450 nm; used for quantitation of G_1 and G_2.

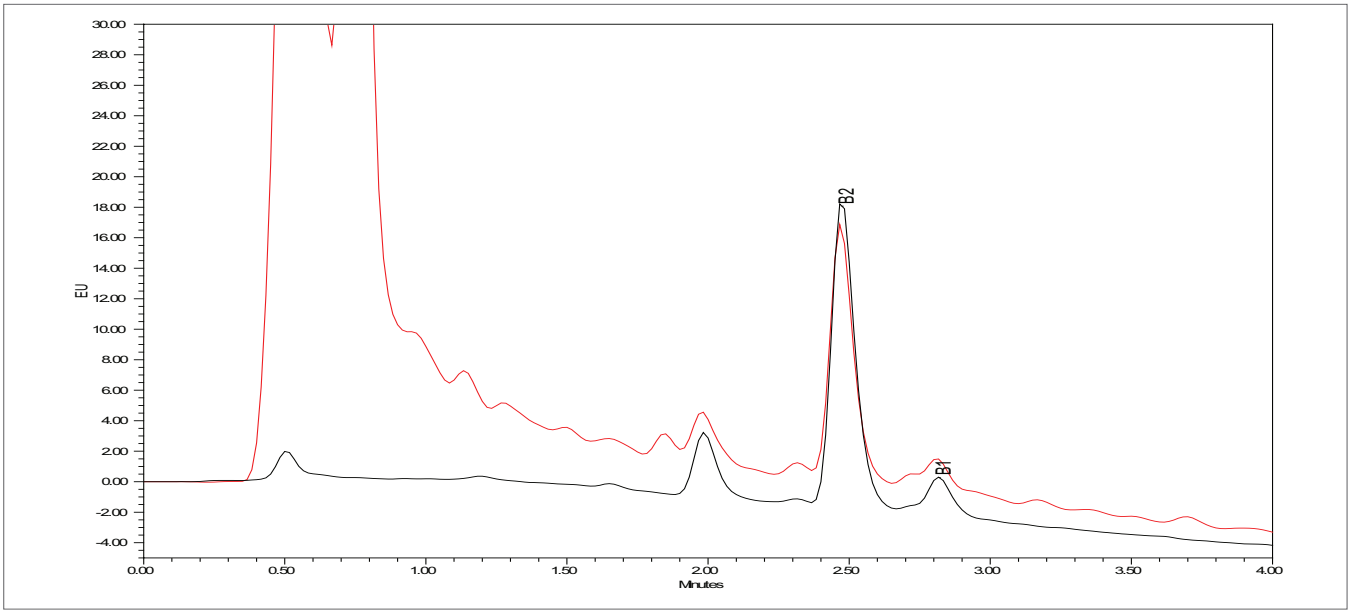

Figure 6. Overlaid chromatograms of the 1.6-ppb aflatoxin standard (black) and 1.6-ppb spiked cat food (red) by fluorescence at Ex 365 nm, Em 425 nm; used for quantitation of B_1 and B_2.

Recoveries of the spiked 1.6-ppb B_1, B_2, G_1, and G_2 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 G_1, 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 B_1, B_2, G_1, and G_2 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 B_1, B_2, G_1, and G_2 at ppb levels without the need for derivatization.

References

1. Hudler, George W., Magical Mushrooms, Mischievous Molds: The Remarkable Story of the Fungus Kingdom and Its Impact on Human Affairs, 1998.
2. Simone Aquino and Benedito Corrêa, "*Aflatoxins in Pet Foods: A Risk to Special Consumers*", *Aflatoxins - Detection, Measurements and Control,* 2011.
3. http://www.poisonedpets.com/top-us-pet-food-brands-test-positive-for-aflatoxin-melamine-and-cyanuric-acid/.

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

012274_01 PKI