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 niger, 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. 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.

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. 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 2.7 µm, 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.

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.
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 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.

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).

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>LOQ via PDA (ppb)</th>
<th>LOD via PDA (ppb)</th>
<th>LOQ via FL (ppb)</th>
<th>LOD via FL (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₂</td>
<td>3.39</td>
<td>1.02</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td>G₁</td>
<td>3.43</td>
<td>1.03</td>
<td>2.18</td>
<td>0.65</td>
</tr>
<tr>
<td>B₁</td>
<td>2.68</td>
<td>0.80</td>
<td>0.53</td>
<td>0.16</td>
</tr>
<tr>
<td>B₂</td>
<td>2.43</td>
<td>0.73</td>
<td>0.07</td>
<td>0.02</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>B₁ (%)</th>
<th>B₂ (%)</th>
<th>G₁ (%)</th>
<th>G₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat food</td>
<td>82.3</td>
<td>84.7</td>
<td>120.7</td>
<td>70.2</td>
</tr>
<tr>
<td>Dog food</td>
<td>93.5</td>
<td>94.7</td>
<td>100.4</td>
<td>79.2</td>
</tr>
</tbody>
</table>

**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**