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Comments

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1 **Use of enzyme-linked immunosorbent assay to screen for aflatoxins, ochratoxin A, and**
2 **deoxynivalenol in dry pet foods**

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Abstract

The objective of this study was to perform a market survey on dry pet foods using enzyme-linked immunosorbent assay (ELISA) to detect total aflatoxins (AFs), ochratoxin A (OTA), and deoxynivalenol (DON). Pet food products (n = 58) marketed for dogs, cats, birds, and rabbits were tested in duplicate with ELISA and results above the limit of quantitation were confirmed using liquid chromatography tandem mass spectrometry (LC-MS/MS). OTA was detected in one product (rabbit food) and AFs were detected in two products (one dog treat and one bird treat). In contrast, DON was detected in the majority (74%) of products tested. Bird and rabbit products were the most affected by DON, with levels above 0.5 µg/g in 50% and 80% of samples, respectively. One rabbit sample tested positive for both OTA and DON. Overall, the findings of this study revealed a low incidence of AFs and OTA in commercial pet food. Although DON was detected in numerous products, the levels were well below those associated with acute toxic effects.

Keywords: aflatoxins; deoxynivalenol; ELISA; mycotoxins; ochratoxin A; pet foods

28 **Introduction**

29 Pets are a growing industry in the U.S., with \$28 billion spent on pet food alone in 2016
30 (APPA 2017). Domestic pets are particularly at-risk for illness from adulterated food because a
31 single type of pet food typically comprises the majority or sole component of a pet's diet
32 (Bischoff and Rumbeiha 2012). Many ingredients commonly used in pet foods, such as corn,
33 wheat, barley, soy, and peanuts, are susceptible to contamination with mycotoxins. Due to the
34 potential for detrimental health effects resulting from mycotoxin exposure, many countries have
35 developed regulations and guidelines for mycotoxins in animal feed, including feed intended for
36 pet animals ('pet food') (for example, EC 2002; EC 2006; FDA 2000; FDA 2010).

37 Between 1996 and 2016, the U.S. Food and Drug Administration (FDA) reported 11
38 recalls in the U.S. pertaining to the presence or potential presence of mycotoxins in pet foods
39 (FDA 2017; Rumbeiha and Morrison 2011). The majority of these recalls involved dog or cat
40 food associated with aflatoxins (AFs) or aflatoxin-producing mold. In 2005-2006, over 700,000
41 pet food packages distributed to 24 states in the U.S. and 30 other countries were recalled due to
42 aflatoxin contamination (FDA 2007; Rumbeiha and Morrison 2011). The contaminated pet foods
43 were associated with the death of at least 76 dogs and caused illnesses in an undetermined
44 number of animals. In South Africa, aflatoxin-contaminated dry dog food led to an aflatoxicosis
45 outbreak and was estimated to have caused over 200 fatalities (Arnot et al. 2012).

46 Globally, several mycotoxins have been detected in retail pet foods, including AFs,
47 ochratoxin A (OTA), and DON (Abd-Elhakim et al. 2016; Arnot et al. 2012; Blajet-Kosicka et
48 al. 2014; Böhm et al. 2010; Gazzotti et al. 2015; Henke et al. 2001; Pühringer et al. 2007;
49 Razzazi et al. 2001; Sharma and Marquez 2001; Songsermsakul et al. 2007). For instance, OTA
50 was detected in dog and cat foods purchased in Austria and Poland, with the highest level of 13.1

51 ng/g in a dry dog food sample and other positive samples containing 0.21-3.2 ng/g (Razzazi et al.
52 2001). In another study, DON was detected in 97% of Austrian dry dog food samples tested by
53 ELISA and 83% of a subset of samples confirmed with high-performance liquid chromatography
54 (HPLC) (Böhm et al. 2010). Henke et al. (2001) surveyed 142 bags of wild bird seeds purchased
55 in Texas, USA, and found that 17% of samples contained AFs at levels > 100 ng/g.

56 Despite the potential for mycotoxin contamination in pet foods sold on the U.S.
57 commercial market, there is a lack of current research on this topic. There is also a paucity of
58 information regarding mycotoxin contamination of food marketed for smaller animals, such as
59 birds and rabbits, as the majority of studies have focused on dog food. Therefore, the objective of
60 this study was to perform a market survey of dry pet foods and treats marketed for dogs, cats,
61 rabbits, and birds to examine for contamination with AFs, OTA, and DON using enzyme-linked
62 immunosorbent assay (ELISA).

63 **Materials and Methods**

64 *Sample collection and preparation*

65 A total of 58 dry pet foods containing grain- and peanut-based ingredients were
66 purchased from six retail stores in Orange County, CA, USA. These samples consisted of food
67 and treats marketed for dogs, cats, birds, and rabbits (see Online Resource 1 for further details).
68 The products selected for this study represented 36 pet food brands and 28 manufacturers or
69 distributors. Following collection, the samples were cataloged, assigned a sample ID, and stored
70 at room temperature. For sample preparation, each pet food package was thoroughly mixed by
71 hand-agitation of the external packaging for one minute, then aseptically sampled. A portion of
72 each sample (100 ± 5 g) was finely ground using an Oster[®] Blender base and sterilized Blend-N-
73 Go Smoothie Kit (Jarden Corporation, Rye, NY, USA) until 75% or more of the sample passed

74 through a 0.841 mm sieve (Helica 2014a; Helica 2014b; Neogen 2008). Ground and
75 homogenized samples were stored in sterile 50 mL conical centrifuge tubes (Corning, Inc.,
76 Corning, NY, USA) at 4°C until analysis.

77 *ELISA verification for dry pet food screening*

78 Pet foods were tested for AFs and OTA using the following ELISA kits from Helica
79 Biosystems, Inc. (Santa Ana, CA, USA): AFs low matrix kit for grains and cereal, silage, nuts,
80 spices, animal feed and OTA low matrix kit for coffee, cocoa, cocoa butter and spices. Because
81 these kits had not previously been verified by the manufacturer for use with dry pet foods, they
82 were subjected to spike-recovery testing prior to use in the market survey to ensure there were no
83 matrix interferences that would yield false results. The spike-recovery tests were carried out with
84 three dry pet food samples: dog food, bird food, and rabbit food. Dry dog food was used to
85 represent both dog and cat foods, as these foods typically contain similar types of ingredients.
86 The three samples were tested by spiking 0.50 ± 0.05 g of ground samples with the target
87 mycotoxin at concentrations along the range of the standard curve supplied with each mycotoxin
88 kit. Mycotoxin stock solutions were acquired from Sigma-Aldrich (St. Louis, MO, USA) for
89 aflatoxin B₁ (AFB₁) and OTA. Each of the three pet food samples were spiked with 0.0, 1.0, 5.0,
90 and 20.0 ng/g of AFB₁ and, separately, with 0.0, 2.5, 10.0, 40.0 ng/g of OTA. Spiked samples
91 were left uncovered and dried overnight at room temperature in a SterilGARD[®] II Class II
92 biological safety cabinet (The Baker Company, Sanford, ME, USA) with the window sash
93 completely closed. Mycotoxin extraction and ELISA assays were performed the next day, as
94 described below. The kits were considered acceptable with mycotoxin recovery rates of 80-
95 120%. The Veratox[®] DON 5/5 ELISA kits used in this study were previously validated for use

96 with pet foods by Neogen Corporation (Lansing, MI, USA) and were not subjected to spike-
97 recovery testing in this study (Neogen 2013).

98 *Mycotoxin extraction and ELISA screening*

99 Mycotoxin extraction was performed on all ground pet food samples per the
100 manufacturers' specifications (Helica 2014a; Helica 2014b; Neogen 2008) with modifications to
101 the starting sample amount and volume of extraction solvent called for by the Helica kits.
102 Extractions for AFs and OTA were conducted using 0.5-1.0 g of ground sample mixed at a 1:5
103 ratio (w/v) with ACS grade acetonitrile (Fisher Scientific, Hampton, NH, USA) diluted to 80%
104 with deionized water, while DON extractions were carried out with 10.0 g of ground sample
105 mixed at a 1:10 ratio with deionized water. AFs and OTA samples were vortexed for 10 min at
106 high speed, then centrifuged at 1370 x g for 10 min. The supernatant extracts were diluted
107 according to the manufacturer's specifications with the kit-supplied wash buffer for AFs and
108 70% methanol (RICCA Chemical Company, Arlington, TX, USA) for OTA (Helica 2014a;
109 Helica 2014b). DON samples were vortexed at moderate speed for 5 min and left undisturbed at
110 room temperature for 3 min. The supernatant extract was passed through filter syringes filled
111 with 1 g of chopped glass wool (Neogen Mycotoxin Extraction Kit). The pH of each sample
112 extract was determined and adjusted to a pH range of 6.0 – 8.0 according to the manufacturer's
113 instructions using ACS grade 1.0 mol/L sodium hydroxide solution (Sigma-Aldrich) (Neogen
114 2008; Neogen 2013).

115 ELISA was performed on all sample extracts per the manufacturers' specifications using
116 the kits mentioned above for AFs, OTA, and DON. The extract from each sample was tested in
117 duplicate microplate wells using ELISA. Absorbance was measured at 450 nm for the AFs and
118 OTA ELISAs and at 650 nm for the DON ELISAs using a SpectraMax M2e multi-mode

119 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and SoftMax[®] Pro, version 6.4
120 software with 4-parameter logistic (4PL) curve fit. Pet food samples were considered to contain
121 detectable levels of AFs, OTA, and/or DON if the average results exceeded the minimum
122 detection limit of the respective assay. The detection limits of the AFs and OTA kits were
123 determined during spike-recovery testing to be 0.02 ng/g and 0.05 ng/g, respectively. According
124 to the manufacturer, the detection limit of the DON kit was 0.1 µg/g and the limit of quantitation
125 (LOQ) was 0.5 µg/g (Neogen 2008).

126 *LC-MS/MS confirmation of quantifiable DON samples*

127 Samples found to be above the LOQ for DON (0.5 µg/g) were submitted to Eurofins
128 Scientific (Garden Grove, CA, USA) for confirmation with liquid chromatography coupled with
129 tandem mass spectrometry (LC-MS/MS). Samples (25 g) were extracted and purified according
130 to the MultiSep[®] 226 protocol (Romer Labs, (Getzersdorf, Austria). Chromatographic separation
131 for DON was performed with a Shimadzu Nexera x2 (Kyoto, Japan) with a SCIEX Triple Quad
132 5500 (Framingham, MA, USA). A Thermo Scientific[™] Acclaim[™] 120Å, C18, 2.2 µm column
133 (Waltham, MA, USA) was used with an injection volume of 5.0 µL. Mobile phase A consisted
134 of 0.1% acetic acid in 10% methanol/90% water and mobile phase B consisted of 0.1% acetic
135 acid and 5 mmol/L ammonium acetate in 45% methanol/45% acetonitrile/10% water. A flow rate
136 of 0.400 mL/min at 35°C was used with a gradient elution of 10% B for 3 min, 10-70% B from 3
137 to 7 min, increased to 90% B in 0.1 min, and then held at 90% B for 2 min, followed by re-
138 equilibration for 3 min at 10% B. MS/MS was performed with an electrospray ionization (ESI)
139 source in multiple reaction monitoring (MRM) mode. Ion source parameters for ESI+ were:
140 spray voltage of 4500 V, curtain gas at 30 PSI (207 kPa), gas source 1 at 60 PSI, gas source 2 at
141 80 PSI, and collision gas at 12 PSI, with a temperature of 550°C.

142 *Statistical analysis*

143 The results of the 16 samples quantified with DON ELISA were compared to the results
144 of the same samples tested with LC-MS/MS using a Wilcoxon signed-rank test, with a pre-
145 determined level of significance of $p < 0.05$, two-tailed. Statistical analysis was carried out using
146 IBM SPSS Statistics 23 (Armonk, NY, USA).

147 **Results and Discussion**

148 *ELISA verification results*

149 Mean recoveries for mycotoxins in the spiked samples were within the predetermined
150 acceptable range of 80-120% for pet food samples tested with the AFs and OTA ELISA kits
151 (Table 1). The mean recoveries were 87.4-110.4% for the AFs kit and 96.6-118.5% for the OTA
152 kit. Recovery levels were similar across the three different matrices, with a coefficient of
153 variation (CV) under 20.0% for all spike levels. Evaporation of the extract solvent can
154 concentrate the samples, resulting in >100% recovery (Scudamore et al. 1997). Recoveries may
155 also be affected due to cross-reacting antibodies, matrix interference, and/or the presence of
156 masked mycotoxins (Berthiller et al. 2013; De Saeger et al. 2016; Rahmani et al. 2009; Tangni et
157 al. 2011). The recoveries obtained in this study were consistent with the typical recoveries
158 achieved in previous studies using methods such as ELISA, thin layer chromatography (TLC),
159 and HPLC (Maia and de Siqueira 2002; Scudamore et al. 1997; Urusov et al. 2015).

160 *ELISA screening for mycotoxins in dry pet food*

161 Of the 58 pet food samples tested in this study, 45 had detectable levels of AFs, OTA, or
162 DON, with both DON and OTA being detected in one sample (Table 2). OTA had the lowest
163 incidence rate (1.7%), with detection in one sample of rabbit food containing soy and wheat-

164 based ingredients as three of the first four ingredients listed. However, other rabbit food products
165 containing predominantly soy and wheat-based ingredients did not test positive for OTA,
166 indicating that this may be an isolated incident. The estimated level of OTA in the rabbit food
167 product was 2.6 ng/g, based on ELISA testing. While the FDA has not established limits for
168 OTA in animal feed, the European Commission has a guidance value of 25 ng/g for OTA in
169 cereals and cereal products used in feed (EC 2006).

170 Similar to the OTA results, the incidence of AFs was low, at 3.4%, with detection in just
171 two samples (Table 2). The main suspect ingredients in the two samples were peanut butter (dog
172 treat) and peanuts (bird treat). Both ingredients were the first of only four or five ingredients
173 listed on the label. While other pet food samples containing peanuts or peanut butter were below
174 the detection limit for AFs (0.02 ng/g), these components were further down on the ingredient
175 list. The estimated levels of aflatoxin for the two products in this study were 1.5 ng/g (bird treat)
176 and 12.3 ng/g (dog treat). These levels are below the FDA action level of 20 ng/g for aflatoxin in
177 animal feed ingredients intended for the products tested in this study (FDA 2000) and below the
178 European Commission maximum allowed levels of AFB₁ in feed materials (20 ng/g). The dog
179 treat was above the European Commission maximum level for AFB₁ in complete feedingstuffs
180 (10 ng/g) applicable to the products tested in this study; however, the assay used in the current
181 study measured total AFs and did not differentiate AFB₁ from other aflatoxins (EC 2002).

182 As compared to the AFs and OTA results, the incidence rate for DON in pet food was
183 much higher, at 74.1%, with bird and rabbit products being the most affected (Table 2). The high
184 incidence of detectable DON is similar to that found by previous studies conducted in Austria,
185 Italy, and Poland, which reported the presence of DON in 83-100% of the dry dog and/or cat
186 food samples tested (Blajet-Kosicka et al. 2014; Böhm et al. 2010; Gazzotti et al. 2015). Of the

187 43 samples in this study with detectable DON levels ($\geq 0.1 \mu\text{g/g}$), 27 products were below the
188 LOQ ($0.5 \mu\text{g/g}$) and 16 products were above the LOQ, with the highest ELISA result at $1.6 \mu\text{g/g}$
189 for a rabbit treat (Online Resource 1). The 16 products with quantifiable levels were further
190 tested with LC-MS/MS for confirmation, as discussed in the following section. The FDA
191 advisory level for DON is $5 \mu\text{g/g}$ in grains and grain by-products for animals other than cattle
192 and chickens, with a recommendation that DON-contaminated ingredients make up less than 20-
193 40% of the diet, depending on the animal (FDA 2010). Similarly, the European Commission has
194 a guidance value of $5 \mu\text{g/g}$ for DON in complete feedingstuffs (EC 2006).

195 The only sample in the current study with detectable levels of multiple mycotoxins was a
196 rabbit food product containing both OTA and DON (Table 2). OTA was estimated to be present
197 at a level of 2.6 ng/g and DON was quantified at $< 1.0 \mu\text{g/g}$. The co-contamination of
198 mycotoxins in crops has been previously reported and more research on the additive and
199 synergistic effects is needed (Kovalsky et al. 2016; Smith et al. 2016).

200 *Confirmation of quantifiable DON with LC-MS/MS analysis*

201 The 16 pet food samples with DON levels above the LOQ ($0.5 \mu\text{g/g}$) for the ELISA kit
202 were confirmed using LC-MS/MS (Fig. 1). The average quantifiable DON level for the 16
203 samples tested by ELISA was $0.9 \mu\text{g/g}$ with a range of $0.6 - 1.6 \mu\text{g/g}$, and the average LC-
204 MS/MS result was $0.6 \mu\text{g/g}$ with a range of $0.1 - 1.0 \mu\text{g/g}$. When the levels determined for these
205 16 samples were compared statistically, the results of ELISA were significantly higher than the
206 results of LC-MS/MS, based on a Wilcoxon signed-rank test ($p < 0.05$, two-tailed). This may
207 have been due to cross-reactivity of the ELISA antibodies with related compounds (Tangni et al.
208 2010). The results of LC-MS/MS and ELISA were correlated, with an R-squared value of 0.82

209 (Fig. 1), suggesting that ELISA may be used as a screening method for DON in dry pet foods.
210 However, testing with LC-MS/MS is recommended in order to verify quantitative results.

211 Among the 16 samples with quantifiable DON levels, products marketed for rabbits
212 (50%) and birds (25%) were the most affected, followed by dogs (19%) and cats (6%). When
213 considering potential sources of DON in the products, wheat-based ingredients were found in the
214 greatest number of products, followed by ingredients containing corn, soy, and oats (Online
215 Resource 1). The presence of DON in foods containing a high proportion of grain ingredients is
216 consistent with the results of the 2016 mycotoxin survey of animal feed commodities, where
217 DON was found in 85% of finished feed samples, 73% of corn samples and 70% of cereal grain
218 samples (wheat, barley, and oats) tested within North America (Biomin 2017). The levels of
219 DON detected in this study are similar to the findings of Austrian studies on dry dog food, which
220 reported average levels of 0.41-0.48 $\mu\text{g/g}$ (Böhm et al. 2010; Songsermsakul et al. 2007).

221 The levels of DON detected in this study were well below those that cause acute toxicity
222 symptoms (i.e., vomiting) in dogs and cats, which have been reported to be 8 $\mu\text{g/g}$ and 10 $\mu\text{g/g}$,
223 respectively (Hughes et al. 1999). However, prolonged exposure to lower levels of DON may
224 contribute to chronic toxic effects, such as loss of appetite, weight loss, and nutritional
225 deficiencies (Pestka 2007). Although these effects have not been widely studied in cats or dogs,
226 studies on other animals have found partial feed refusal in pigs exposed to levels as low as 1-2
227 $\mu\text{g/g}$ and significant body weight reduction in mice fed 5 $\mu\text{g/g}$ DON over a period of 2 years.

228 Overall, the results of this study indicate a low incidence of AFs and OTA in dry pet
229 foods sold on the U.S. commercial market. Although DON was frequently detected in these
230 foods, the results were well below regulatory limits and would not be expected to cause acute
231 health effects. However, there remains a risk of chronic low-level exposure to mycotoxins in pet

232 food due to the focused consumption pattern of these animals. In this study, ELISA served as a
233 rapid and reliable screening method for the detection of mycotoxins in dry pet foods.
234 Furthermore, ELISA and LC-MS/MS methods for quantifying DON in dry pet foods were
235 correlated. Due to the potential health risks of chronic exposure to mycotoxins, regular screening
236 of pet foods is recommended and pets should be carefully monitored for symptoms of exposure.

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246 **Conflicts of Interest**

247 T.P. Huynh is employed by Helica Biosystems, Inc.; T.A. Okuma was formerly employed by
248 Helica Biosystems.

249 **Ethical Standards**

250 These experiments comply with the current laws of the United States of America.

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363 **Figure caption**

364 **Figure 1** Comparison of DON quantitation results based on testing with ELISA and LC-MS/MS

Table 1 Recoveries of AFB₁ and OTA using ELISA with spiked pet food samples. The detection limits were determined to be 0.02 ng/g for AFs and 0.05 ng/g for OTA.

Mycotoxin	Spike Level (ng/g)	Mean Recovery ^a (ng/g)	Standard Deviation of Recovery ^a	Mean % Recovery
AFB ₁	20.00	21.93	0.91	109.6
	5.00	4.37	0.09	87.4
	1.00	1.10	0.11	110.4
	0.00	N/A	N/A	N/A
OTA	40.00	39.13	6.46	97.8
	10.00	9.66	0.73	96.6
	2.50	2.96	0.58	118.5
	0.00	N/A	N/A	N/A

^aBased on combined results for three spiked pet foods (dog, rabbit, and bird) tested with ELISA in duplicate.

Table 2 Summary of mycotoxin detections resulting from ELISA testing of dry pet food samples ($n = 58$), based on detection limits of 0.02 ng/g for AFs, 0.05 ng/g for OTA, and 0.1 $\mu\text{g/g}$ for DON.

Pet food type	Number of samples collected	Number of samples with detectable mycotoxins		
		OTA	AFs	DON
Dog food	9	0	0	6
Dog treats	10	0	1	6
Cat food	9	0	0	8
Cat treats	10	0	0	5
Bird food	6	0	0	5
Bird treats	4	0	1	3
Rabbit food	7	1 ^a	0	7
Rabbit treats	3	0	0	3
Overall	58	1	2	43

^aOne sample of rabbit food contained detectable levels of both OTA and DON