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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

Introduction

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

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

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

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

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

Materials and Methods

Sample collection and preparation

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

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

ELISA verification for dry pet food screening

Pet foods were tested for AFs and OTA using the following ELISA kits from Helica Biosystems, Inc. (Santa Ana, CA, USA): AFs low matrix kit for grains and cereal, silage, nuts, spices, animal feed and OTA low matrix kit for coffee, cocoa, cocoa butter and spices. Because these kits had not previously been verified by the manufacturer for use with dry pet foods, they were subjected to spike-recovery testing prior to use in the market survey to ensure there were no matrix interferences that would yield false results. The spike-recovery tests were carried out with three dry pet food samples: dog food, bird food, and rabbit food. Dry dog food was used to represent both dog and cat foods, as these foods typically contain similar types of ingredients. The three samples were tested by spiking 0.50 ± 0.05 g of ground samples with the target mycotoxin at concentrations along the range of the standard curve supplied with each mycotoxin kit. Mycotoxin stock solutions were acquired from Sigma-Aldrich (St. Louis, MO, USA) for aflatoxin B_1 (AFB_1) and OTA. Each of the three pet food samples were spiked with 0.0, 1.0, 5.0, and 20.0 ng/g of AFB_1 and, separately, with 0.0, 2.5, 10.0, 40.0 ng/g of OTA. Spiked samples were left uncovered and dried overnight at room temperature in a SterilGARD® II Class II biological safety cabinet (The Baker Company, Sanford, ME, USA) with the window sash completely closed. Mycotoxin extraction and ELISA assays were performed the next day, as described below. The kits were considered acceptable with mycotoxin recovery rates of 80-120%. The Veratox® DON 5/5 ELISA kits used in this study were previously validated for use

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

Mycotoxin extraction and ELISA screening

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

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

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

LC-MS/MS confirmation of quantifiable DON samples

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

Statistical analysis

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

Results and Discussion

ELISA verification results

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

ELISA screening for mycotoxins in dry pet food

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

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

Similar to the OTA results, the incidence of AFs was low, at 3.4%, with detection in just two samples (Table 2). The main suspect ingredients in the two samples were peanut butter (dog treat) and peanuts (bird treat). Both ingredients were the first of only four or five ingredients listed on the label. While other pet food samples containing peanuts or peanut butter were below the detection limit for AFs (0.02 ng/g), these components were further down on the ingredient list. The estimated levels of aflatoxin for the two products in this study were 1.5 ng/g (bird treat) and 12.3 ng/g (dog treat). These levels are below the FDA action level of 20 ng/g for aflatoxin in animal feed ingredients intended for the products tested in this study (FDA 2000) and below the European Commission maximum allowed levels of AFB_1 in feed materials (20 ng/g). The dog treat was above the European Commission maximum level for AFB_1 in complete feedingstuffs (10 ng/g) applicable to the products tested in this study; however, the assay used in the current study measured total AFs and did not differentiate AFB_1 from other aflatoxins (EC 2002).

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

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

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

Confirmation of quantifiable DON with LC-MS/MS analysis

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

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

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

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

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

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

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Conflicts of Interest

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

Ethical Standards

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

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Figure caption

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

Table 1 Recoveries of AFB_1 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

[a]Based 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 µg/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**

[a]One sample of rabbit food contained detectable levels of both OTA and DON