# Occurrence of Mycotoxins in Extruded Commercial Cat Food

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ABSTRACT: The occurrence of the most important mycotoxins (deoxynivalenol, fumonisin B_1 and B_2, aflatoxins B_1, B_2, G_1, and G_2, ochratoxin A, zearalenone, T-2, and HT-2 toxins) was determined in 64 extruded cat foods purchased in Italy through ultra-performance liquid chromatography coupled with tandem mass spectrometry. Deoxynivalenol and fumonisins were the most common contaminants (quantified in 80 and 95% of the samples, respectively). Conversely, aflatoxins B_2, G_1, and G_2 were not identified in any sample. Some cat foods exceeded the regulatory limit for aflatoxin B_1 ($n = 3$) or the guidance values for zearalenone ($n = 3$), fumonisins ($n = 2$), ochratoxin A ($n = 1$), and T-2 ($n = 1$) recently established for pets in the European Union. A widespread co-occurrence of mycotoxins was observed (28, 42, and 8% of the samples contained quantifiable amounts of two, three, and four mycotoxins, respectively). This study describes criticisms regarding the mycotoxin issue in pet food and suggests an improvement of the monitoring of the pet food chain.
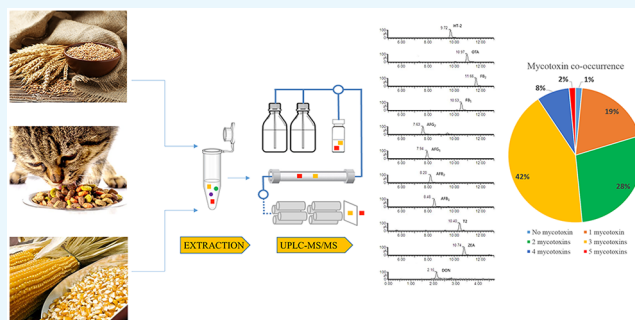
INTRODUCTION

Nowadays, feeding practice based on commercially prepared pet food is extremely widespread throughout the world since it represents an easy and economical way to meet the nutrient requirements of dogs and cats at the various stages of their life both in healthy and pathological conditions.

In consideration of the current strict human−animal bond and the importance of the feeding time perceived by pet owners as an opportunity of interaction and gratification,[1] pet food quality is considered of great importance given its recognized connection to pet health. In this regard, among recent concerns about pet food safety, mycotoxin contamination represents a well-known problem.[2] Mycotoxins are chemical compounds derived from the secondary metabolism of various genera of fungi such as *Aspergillus*, *Fusarium*, and *Penicillium* that can grow on agricultural crops. Consequently, these toxins are frequently detected in plant-based foods as well as animal feed.[3]

Although dogs and cats belong to the Carnivora order, extruded pet food typically contains relatively high amounts of cereals and cereal byproducts since the extrusion process, by favoring starch gelatinization, makes starch easily digestible.[4] Nevertheless, these particular processing conditions, even if characterized by high pressures and temperatures, do not generally guarantee the complete degradation/inactivation of mycotoxins possibly contaminating raw ingredients.[5] Consequently, extruded (or commonly named "dry") pet food results particularly at risk of contamination, as proven by several surveys recently conducted in various world regions[6−17] (Table 1).

Awareness of the worldwide occurrence of mycotoxins in food and feedstuffs, the consequent risks to human and animal health, and the detrimental effects on global trade have led in recent decades to the adoption of regulatory limits for the most important mycotoxins found in food commodities[18] and animal feed:[19] aflatoxins, fumonisins, ochratoxins (OTA), zearalenone (ZEA), and trichothecenes (in particular, deoxynivalenol (DON) and T-2 toxin (T-2)). Consequently, these particular mycotoxins are considered the most relevant from both a safety and economic perspective.

In the European Union (EU), legislation concerning mycotoxin contamination in animal feeds (mostly for livestock and only in regard to fumonisins for pets) has been promulgated in the past.[20,21]

More recently, following some scientific opinions provided by the European Food Safety Authority (EFSA),[22−24] specific "guidance values" recommended for DON, ZEA, OTA, T-2, and HT-2 also referred to the compound feed intended for dogs or cats have been introduced.[25,26]

Beyond the detrimental consequences on economic performance and reputation in the pet food industry, mycotoxin contamination poses severe health risks to pet animals although current knowledge on the toxicological effects in these species is limited.[2] Moreover, modern analytical methods based on liquid chromatography tandem mass spectrometry (LC−MS/MS), allowing simultaneous determination of different molecules, have recently highlighted the

Table 1. Most Recent Mycotoxin Studies on Commercial Pet Food[a]

location and year of sampling/publication	type of pet food investigated	mycotoxins detected	most relevant results	ref
Poland (2004)	57 pet foods (41 standard and 16 therapeutic samples)	ZEA (and its derivatives)	ZEA was identified in 84.2% of the samples (mean concentration 36.2 μg/kg). Max values in standard and therapeutic samples were 299.5 and 158 μg/kg, respectively.	6
Austria (2007)	29 dry dog foods and 11 wet dog foods	DON and OTA	DON was identified in all the dry samples (range between 22 and 1837 μg/kg); 27% of the samples were positive for DON (range between 95 and 170 μg/kg); OTA contaminated 10% of the dry samples (range between 7 and 40 μg/kg) and 18% of the wet samples (range between 45 and 115 μg/kg).	7
Austria (2007)	76 dry dog foods	DON, ZEA, fumonisins, OTA, and aflatoxins	83% of the samples were positive for DON (mean of 409 μg/kg, max of 1390 μg/kg); 47% of the samples were positive for ZEA (mean of 298 μg/kg); 42% of the samples were positive for fumonisins (mean of 178 μg/kg, max of 568 μg/kg); OTA was less frequently found (5% of the positive samples); aflatoxins were not detected.	8
Brazil (2010/2011)	100 dry dog foods	ZEA, fumonisins, and aflatoxins	68% of the samples were positive for fumonisins (max of $FB_1 + FB_2$, 380 μg/kg); 95% of the samples were positive for ZEA (max 442.2 μg/kg); 68% of the samples were positive for aflatoxins (max of 3.88 μg/kg).	9
Italy (2011)	41 dry dog foods (32 complete and 9 complementary)	FB_1 and FB_2	FB_1 and FB_2 were quantified in 63.4 and 56.1% of the samples, respectively. The range of $FB_1 + FB_2$ was between 150 and 8800 μg/kg. Two samples (one complete and one complementary dog food), containing 5190 and 8800 μg/kg of $FB_1 + FB_2$ respectively, exceeded the European guidance value (5000 μg/kg).	10
South Africa (2011)	60 dog foods	ZEA, fumonisins, aflatoxins, and OTA	87% of the samples were positive for aflatoxins (mainly AFB_1 and AFB_2): mean of 248 μg/kg, range between 1.2 and 353 μg/kg; most of the samples (75%) contained levels above the regulatory limits. Fumonisins were detected in 98% of the samples: mean of 1556 μg/kg, range between 5.2 and 4654 μg/kg. OTA was detected in 68% of the samples (mean of 13.7 μg/kg, range between 0.5 and 53.6 μg/kg). ZEA was detected in 96% of the samples (mean value of 354 μg/kg, range between 2.5 and 2351 μg/kg).	11
Poland (2014)	25 dry dog foods and 24 dry cat foods	DON, ZEA, fumonisins, aflatoxins, OTA, T-2, and HT-2	All the samples were positive for DON and ZEA (max of 436 and 123 μg/kg, respectively); T-2 was detected in 88% and HT-2 in 84% of the samples (max of 13.3 and 19.6 μg/kg, respectively); 29% of the samples were positive for fumonisins (max of 108 μg/kg); 45% of the samples were positive for OTA (max of 3 μg/kg); AFB_1 was identified at LOD level (0.05 μg/kg) in 8% of the samples	12
Egypt (2014)	20 pet food (5 wet dog foods, 5 wet cat foods, 5 dry dog foods, 5 dry cat foods)	ZEA, total aflatoxins, AFB_1, and OTA	15% of the samples were positive for AFB_1 (max 18.4 μg/kg); OTA was detected in most of the samples (max 6.65 μg/kg); ZEA was measured in 20% of the samples at levels between 148 and 1170 μg/kg.	13
Italy (2015)	48 dry dog foods	DON, ZEA, fumonisins, OTA, and aflatoxins	DON, fumonisins, and OTA were the most common mycotoxins (100, 88, and 81% of the positive samples, respectively); max values were 281, 1746 ($FB_1 + FB_2$), and 41.1 μg/kg, respectively. Fumonisin and OTA contamination was higher in standard than in premium dog foods. No sample contained quantifiable amounts of AFB_1 and AFG_1 (<LOQ 0.05 μg/kg). AFB_2 or AFG_2 was measured in 12% of the samples (max of 15.8 μg/kg); 25% of the samples contained ZEA at quantifiable levels (max value of 42.4 μg/kg).	14
South Africa (2017)	20 dry dog foods	ZEA, fumonisins, aflatoxins, and OTA	All the samples were positive for fumonisins (levels of FB_1 or FB_2 above 20 μg/kg; standard dog foods were more contaminated than premium ones); OTA and ZEA were detected in most of the samples at very low concentrations; aflatoxins were identified in all the samples with relatively high concentrations of AFB_1 (5 standard and 5 premium dog foods contained AFB_1 at levels above 10 μg/kg)	15
China (2017)	32 dry pet foods	DON, ZEA, FB_1, AFB_1, AFG_1, OTA and T-2, citrin, and beauvericin	Only one sample was free of contamination. All the other samples (96.9%) contained at least three mycotoxins. DON, ZEA, AFB_1, FB_1, citrin, and beauvericin displayed a relatively high occurrence (78.1, 62.5, 87.5, 93.8, 68.8, and 96.9%, respectively). AFB_1 exceeded the European regulatory limit in all the positive samples (range, 30.3–242.7 μg/kg). T-2 was found in only one sample (15.4 μg/kg) and OTA in two samples (15.1 and 17.3 μg/kg).	16
Poland (2019)	42 therapeutic foods (17 samples for cats and 25 samples for dogs)	DON, ZEA, FB_1, and nivalenol	ZEA was detected in 69% (range, 1.22–51.7 μg/kg), DON in 52% (24.87–2451 μg/kg), FB_1 in 33% (4.89–80.13 μg/kg), and nivalenol in 26% (17.43–200 μg/kg) of the samples. The highest level of mycotoxins was quantified in samples aimed to nutritionally support allergies (ZEA), renal diseases (FB_1), and obesity (DON and nivalenol).	17

[a]ZEA, zearalenone; DON, deoxynivalenol; OTA, ochratoxin A; FB_1, fumonisin B_1; FB_2, fumonisin B_2; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; HT-2, HT-2 toxin; T-2, T-2 toxin; LOQ, limit of quantification.

Table 2. Mass Spectrometry Parameters for the Selected Mycotoxins[a]

compound	precursor ion (m/z)	product ions[b] (m/z)	cone voltage (kV)	collision energy (eV)
DON	297.1	**249.20**	18	10
		231.2	18	13
^{13}C DON	312.2	**263.4**	18	12
		216.4	18	16
AFB$_1$	312.20	**285.30**	45	22
		241.30	45	36
AFB$_2$	315.05	**287.10**	45	33
		259.10	45	38
AFG$_1$	329.10	**243.30**	45	26
		283.30	45	24
AFG$_2$	331.10	**313.25**	46	33
		313.25	46	39
^{13}C AFB$_1$	330.3	**301.2**	45	22
		255.4	45	38
FB$_1$	722.20	**334.50**	52	45
		352.50	52	43
FB$_2$	706.30	**336.50**	50	38
		318.50	50	40
^{13}C FB$_1$	756.3	**356.5**	52	45
		374.6	52	40
OTA	404.15	**239.20**	25	25
		221.20	25	37
^{13}C OTA	424.1	**232.4**	52	40
		356.6	52	45
HT-2	447.25	**345.3**	36	20
		345.3	36	22
T-2	489.2	**245.1**	36	27
		387.0	36	22
^{13}C T-2	513.3	**406.2**	40	23
		260.3	40	28
ZEA	319.3	**283.20**	20	12
		185.20	20	30
^{13}C ZEA	377.3	**301.3**	17	12
		199.4	17	18

[a]DON, deoxynivalenol; AFB$_1$, aflatoxin B$_1$; AFB$_2$, aflatoxin B$_2$; AFG$_1$, aflatoxin G$_1$; AFG$_2$, aflatoxin G$_2$; FB$_1$, fumonisin B$_1$; FB$_2$, fumonisin B$_2$; HT-2, HT-2 toxin; OTA, ochratoxin A; T-2, T-2 toxin; ZEA, zearalenone. [b]Quantification ions are reported in bold.

problem of mycotoxin co-occurrence also in pet food products,[9,14,16] as described for other animal feeds.[27]

The aim of the present study was to investigate the occurrence of the most important mycotoxins that are currently covered by EU legislation in the complete dry pet food intended for cats that is commercially available in Italy.

■ RESULTS

Chemical analysis of the samples identified similar mean moisture and starch contents in the two cat food categories evaluated. In fact, excluding grain-free products, moisture contents were 46 ± 11 and 64 ± 14 g/kg, while starch contents were 259 ± 54 and 269 ± 35 g/kg (on a dry matter basis) in the premium and standard products, respectively. In cat foods not containing cereals, moisture was 50 ± 13 g/kg, and starch content was 186 ± 45 g/kg.

Positivity for and concentrations of the different mycotoxins in the two cat food categories are reported in Tables 3 and 4, respectively.

ZEA was identified in 75% of the samples. Higher concentrations of this mycotoxin than those of the corresponding LOQ (5 μg/kg) were found in 42% of the samples. Standard cat foods were less contaminated than premium ones (8.89 vs 20.8 μg/kg, respectively; $P < 0.05$). Furthermore, in this last category, three dietetic samples (one for obesity management and two for renal diseases), containing 112, 101, and 104 μg of ZEA/kg, exceeded the reference guidance value recently established for cats used for reproduction and for kittens (100 μg/kg) (Figure 1). On the other hand, these three last samples respected the limit referred to compound feed intended for adult cats other than for reproduction (200 μg/kg).[26]

DON was quantified in 62% of the premium and 100% of the standard cat foods (at concentrations ≥ the corresponding LOQ, 3 μg/kg), with higher values in this last price category (77.7 vs 209 μg/kg in premium and standard samples, respectively; $P < 0.05$). All of the positive samples resulted in compliance with the corresponding European guidance value recommended for cat food (5000 μg/kg).[26]

Concerning aflatoxins, analyses revealed a general low occurrence among the cat foods evaluated. In particular, no sample contained AFB$_2$, AFG$_1$, and AFG$_2$ at levels above the corresponding LOD (1 μg/kg) (Table 3). AFB$_1$ was identified in trace amounts in 16% of the samples (values > the corresponding LOD, 1 μg/kg, but < the corresponding LOQ, 3 μg/kg) and quantified in eight samples (one premium and seven standard cat foods) at concentrations between 3 and 18.4 μg/kg. In particular, three of these last positive samples (one premium sample for renal diseases and two standard samples for adult cats), containing values of 17.4, 18.4, and 16.5 μg of AFB$_1$/kg, exceeded the regulatory maximum content established for compound feeds for animals other than for livestock (10 μg/kg)[20] (Figure 1).

Fumonisins were the most common mycotoxins. With only one exception (represented by a grain-free product), all of the samples presented at least one of the two types of fumonisins evaluated (FB$_1$ or FB$_2$) at concentrations above the corresponding LOD (1 μg/kg). In particular, FB$_1$ and/or FB$_2$ were quantified in 95% of the samples (their concentration was ≥ the LOQ, 3 μg/kg), with no differences between standard and premium samples. The average levels of total fumonisins (FB$_1$ + FB$_2$) were relatively high although a wide range of contaminations was found (overall mean concentration of 1297 μg/kg and median concentration of 376 μg/kg). Furthermore, two dietetic products formulated for obesity management (containing 7494 and 7933 μg of total fumonisins/kg, respectively) exceeded the corresponding guidance value for pets (5000 μg/kg)[26] (Figure 1).

OTA was identified in seven standard samples among which only two (both intended for adult cats) contained quantifiable amounts of this mycotoxin (at concentrations of 5.1 and 14 μg/kg, respectively). According to the current European guidance value for compound feeds for dogs and cats (10 μg/kg),[26] one sample was illegal (Figure 1).

Finally, T-2 and HT-2 were detected in trace amounts (when the concentration was between the corresponding LOD (5 μg/kg for both toxins) and LOQ (10 μg/kg for T-2 and 20 μg/kg for HT-2)) in 28 and 11% of the total samples, respectively. Only T-2 was quantified in five samples (two standard and three premium), with mean and maximum concentrations of 35.6 and 69.6 μg/kg, respectively. This last value, found in a dietetic product intended for cats affected by

Table 3. Positivity for Mycotoxins of Commercial Dry Cat Food[a]

	number of positive samples					
	LOD[b] < mycotoxin < LOQ[c]			mycotoxin ≥ LOQ[c]		
mycotoxin	standard (n = 30)	premium (n = 34)	total (n = 64)	standard (n = 30)	premium (n = 34)	total (n = 64)
ZEA	13 (43%)	8 (24%)	21 (33%)	12 (40%)	15 (44%)	27 (42%)
DON	0	7 (21%)	7 (11%)	30 (100%)	21 (62%)	51 (80%)
AFB_1	5 (17%)	5 (15%)	10 (16%)	7 (23%)	1 (3%)	8 (13%)
AFB_2	0	0	0	0	0	0
AFG_1	0	0	0	0	0	0
AFG_2	0	0	0	0	0	0
FB_1	1 (3%)	1 (3%)	2 (3%)	29 (97%)	32 (94%)	61 (95%)
FB_2	1 (3%)	2 (6%)	3 (5%)	29 (97%)	31 (91%)	60 (94%)
fumonisins[d]	1 (3%)	1 (3%)	2 (3%)	29 (97%)	31 (91%)	60 (94%)
OTA	5 (17%)	0	5 (8%)	2 (7%)	0	2 (3%)
T-2	7 (23%)	6 (18%)	13 (20%)	2 (7%)	3 (9%)	5 (8%)
HT-2	3 (10%)	4 (12%)	7 (11%)	0	0	0
T-2/HT-2[e]	1 (3%)	1 (3%)	2 (3%)	0	0	0

[a]ZEA, zearalenone; DON, deoxynivalenol; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; FB_1, fumonisin B_1; FB_2, fumonisin B_2; OTA, ochratoxin A; T-2, T-2 toxin; HT-2, HT-2 toxin. [b]LOD: limit of detection (FB_1, FB_2, AFB_1, AFB_2, AFG_1, AFG_2 and DON: 1 μg/kg; ZEA and OTA: 2 μg/kg; T-2 and HT-2: 5 μg/kg). [c]LOQ: limit of quantification (FB_1, FB_2, AFB_1, AFB_2, AFG_1, AFG_2 and DON: 3 μg/kg; ZEA and OTA: 5 μg/kg; T-2: 10 μg/kg; HT-2: 20 μg/kg). [d]Fumonisins: positivity for both fumonisins (FB_1 and FB_2). [e]T-2/HT-2: positivity for both toxins (T-2 and HT-2).

Table 4. Concentrations of Mycotoxins (μg/Kg) in Commercial Dry Cat Food[a]

	standard cat foods (n = 30)				premium cat foods (n = 34)				European guidance values (μg/kg)[26]
mycotoxin	mean ± SD[b]	median[c]	min[d]	max[e]	mean ± SD[b]	median[c]	min[d]	max[e]	
ZEA	8.89[f] ± 8.36	5.0	LOD	34.1	20.8[f] ± 31.3	5.0	LOD	112	100[g] 200[h]
DON	209[f] ± 351	69.2	3.0	1588	77.7[f] ± 117	28.1	LOD	423	5000[i]
FB_1	774 ± 853	441	LOQ	3277	648 ± 929	138	LOD	3464	
FB_2	438 ± 558	209	LOQ	2257	724 ± 1180	112	LOD	4837	
FB_1 + FB_2	1212 ± 1326	630	LOQ + LOQ	4258	1372 ± 2072	269	LOD + LOD	7933	5000[j]

[a]ZEA, zearalenone; DON, deoxynivalenol; FB_1, fumonisin B_1; FB_2, fumonisin B_2; LOD, limit of detection (DON, FB_1, and FB_2: 1 μg/kg; ZEA: 2 μg/kg); LOQ, limit of quantification (DON, FB_1 and FB_2: 3 μg/kg; ZEA: 5 μg/kg). The values for aflatoxins are not reported since AFB_2, AFG_1 and AFG_2 were not detected and AFB_1, OTA, T-2 and HT-2 levels were lower than their corresponding limit of quantification in 87%, 97%, 92% and 100% of the samples, respectively. [b]Arithmetic mean ± standard deviation. [c]Median of all samples. [d]Minimum value. [e]Maximum quantified value. [f]Means within a row differ (P < 0.05). [g]Guidance value relative to a compound feed for puppies, kittens and dogs and cats for reproduction. [h]Guidance value relative to a compound feed for adult dogs and cats other than for reproduction. [i]Guidance value relative to a compound feed for cats. [j]Guidance value relative to a compound feed for pets.
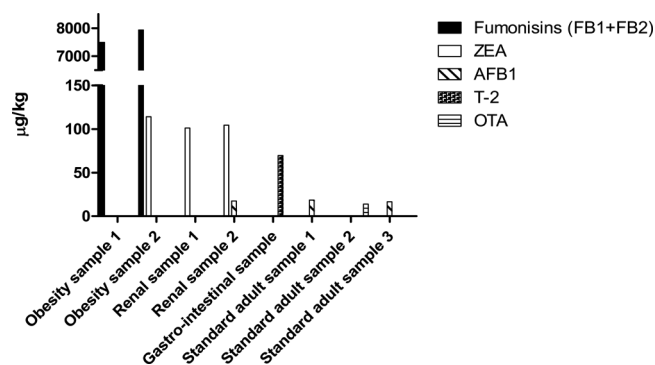

Figure 1. Samples of commercial dry cat food that did not comply with the current EU legislation concerning mycotoxin contamination.

gastrointestinal diseases, was higher than the current guidance value established for T-2 + HT-2 regarding compound feeds for cats (corresponding to 50 μg/kg)[26] (Figure 1).

In regard to grain-free products, one sample did not contain detectable levels of any mycotoxin, while in the other four samples, only fumonisins were quantified (at concentrations ≥ the corresponding LOQ, 3 μg/kg) in a range between 11.1 and 125 μg of FB_1 + FB_2/kg.

The present study showed that 80% of the cat foods evaluated (51 of 64 samples) contained quantifiable concentrations of at least two types of mycotoxins. In particular, 28% of the cat foods (11 standard and 7 premium samples) were contaminated by two different mycotoxins (among which 89% contained DON + fumonisins), 42% (14 standard and 13 premium samples) by three (among which 82% contained ZEA + DON + fumonisins), 8% (3 standard and 2 premium samples) by four, and 2% (1 standard sample) by five mycotoxins (ZEA + DON + fumonisins + OTA + AFB_1) (Table 5). The simultaneous quantification of at least DON and fumonisins (FB_1 and/or FB_2) was the most frequent: 77% of the samples evaluated (29 standard and 20 premium) revealed measurable concentrations of these two *Fusarium* mycotoxins.

In regard to the correlation study between starch content (on a dry matter basis) and mycotoxin concentration, only

Table 5. Mycotoxins Co-occurrence in Commercial Dry Cat Food

number of mycotoxins	standard cat foods ($n = 30$)	premium cat foods ($n = 34$)
1	1	11
2	11	7
3	14	13
4	3	2
5	1	0

total fumonisins, ZEA, and DON were considered because of their widespread occurrence in over half of the samples. Nevertheless, the Pearson coefficient test failed to show a linear relationship for all three mycotoxins evaluated ($r = 0.058$, $r = 0.002$, and $r = 0.195$ in regard to the correlation between starch content and total fumonisins, ZEA, and DON, respectively).

■ DISCUSSION

The present monitoring compared, for the first time, the occurrence of the most important mycotoxins in cat food with the guidance values for pets that were recently introduced by EU legislation.[26] Based on our results, a relatively high number of samples (five premium dietetic samples and three standard samples for adult cats) exceeded the European regulatory limits established for some mycotoxins (Figure 1). Astonishingly, two dietetic samples even exceeded the guidance values for two mycotoxins simultaneously. Nevertheless, it must be emphasized that cat food sampling preceded the adoption of the current European Recommendation concerning DON, ZEA, and OTA contamination in pet food.[26]

The lack of compliance with European rules appears particularly critical since this outcome mainly concerned dietary products aimed at nutritional support for cats affected by common pathological conditions such as obesity and renal and gastrointestinal disorders. In fact, such animals might present a suboptimal immunological status, and prolonged consumption of mycotoxin-contaminated pet food could be detrimental to their health.[2] Dietetic pet food is poorly investigated in this regard as most of the published studies on mycotoxin contamination have been carried out on pet food formulated for healthy dogs and cats (Table 1).

The results from the present monitoring partially disagreed with those highlighted by our previous similar study on Italian dry dog food.[14] In the previous work, interesting evidence such as a widespread multicontamination in most of the samples and differences in the concentration of some mycotoxins (fumonisins and OTA) based on the price category was obtained.[14] Conversely, the present investigation on cat food showed that a high price does not necessarily guarantee a mycotoxin-free product.

The mean starch content in standard and premium categories was similar given the high variability of this complex carbohydrate among the cat foods evaluated. Furthermore, the correlation study between the starch content and the concentration of the most prevalent mycotoxins (total fumonisins, ZEA, and DON) failed to demonstrate a linear relationship. On the other hand, the five grain-free samples evaluated, containing starch from legumes and potatoes, showed an overall low contamination. In most of the other samples, cereals and cereal byproducts represented the first or second ingredient (or ingredient category) listed on the label

(corn in primis when the type of cereals was specified). Anyway, information on the exact amount of the different ingredients was not provided. Consequently, it is only possible to speculate on the relationship between mycotoxin contamination and the relatively large quantity of cereals used to produce dry pet food.

Mold growth and mycotoxin production strongly depend on several factors along the cereal supply chain, such as weather and storage conditions (i.e., temperature and humidity), microbial and insect damages, and mechanical injuries.[28,29] This situation partially explains the difficulties in the prediction and control of the problem as well as the heterogeneous levels of contamination emerging from the comparison of the results described by the different studies conducted on pet food published in recent years (Table 1).

In the present study, in accordance with our previous investigation on dry dog food[14] and other similar investigations,[8,12,16,17] a widespread occurrence of the most important *Fusarium* mycotoxins (DON, fumonisins, and, to a lesser extent, ZEA) was observed.

These toxic molecules are commonly found in cereals (corn, in primis) and in compound feeds (typically containing different grains).[30] Furthermore, DON, ZEA, and in particular fumonisins are particularly heat-stable, and only temperatures above 150 °C have demonstrated a significant reduction of their toxicity.[31] Consequently, these toxins are a matter of great concern for the pet food industry (particularly fumonisins) since extrusion typically provides lower temperatures.

According to previous investigations, the situation concerning aflatoxin occurrence in pet food is widely heterogeneous (Table 1). Surprisingly, our study revealed a non-negligible presence of AFB$_1$ in the cat foods evaluated, with three samples exceeding the European regulatory limit established for animal feeds including pet food.[20]

Several outbreaks of canine aflatoxicosis are reported in the literature.[32−34] Conversely, to our knowledge, no case involving cats has been described. Anyway, aflatoxins represent a common cause of pet food recalls.[35] Given the absence of critical control points for aflatoxins along the manufacturing process (due to their stability to the extrusion process), routine screening for these contaminants in cereal ingredients (especially corn and corn byproducts) is necessary.[36] Furthermore, even if lower levels of AFB$_1$ than 20 μg/kg have previously shown to be insufficient for causing noticeable clinical signs in companion animals, the chronic exposure should not be ignored.[2] Surely, the situation concerning AFB$_1$ as indicated in the present study represents an important warning sign and suggests for rigorous screening tests on incoming raw ingredients.

OTA contamination was very limited even if one of the two positive standard samples contained a level above the current European guidance value recently established for companion animals.[26] OTA represents a nephrotoxic compound usually detected not only in several agricultural commodities such as corn, wheat, and barley but also in animal-derived products (in particular, meat and milk) given its high fat solubility and the consequent accumulation in animal tissues, particularly in swines.[18] For this reason, OTA could be detected both in dry and wet pet food since the latter product typically contains large amounts of animal-derived ingredients such as muscles and offal.[37] Different levels of contamination have been reported by several studies carried out both in Europe and

in African countries (Table 1). Interestingly, a recent retrospective study by Meucci et al.[38] evaluating the blood concentration of OTA in healthy and nephropathic dogs showed a higher incidence of OTA positivity in this last group. In this regard, other authors had previously pointed out this mycotoxin etiology in canine renal failure syndrome.[39] Certainly, these results contribute to supporting the need for rigorous monitoring by the pet food industry regarding OTA.

According to toxicological studies, T-2 represents the most toxic compound among trichothecenes. Given the rapid in vivo conversion of T-2 to HT-2, in vivo toxicity of T-2 is recognized to include that of HT-2.[31] According to a recent EFSA opinion, cats are extremely sensitive to this mycotoxin category,[40] and probably for this reason, some years ago, the European legislation was implemented with a guidance value for T-2 + HT-2 only referred to this last species.[25] Recently, some studies have reported a wide occurrence of T-2 and HT-2 in cereals and animal feed.[28,29] In regard to pet food, only a few studies have evaluated the occurrence of these mycotoxins, and they reported conflicting results.[12,16] In the present monitoring, probably because of the corresponding relatively high LODs and LOQs, few positive samples were identified among which a dietetic product did not comply with the current legislation.[26]

The present study confirmed the noteworthy problem of the co-occurrence of different mycotoxins, previously reported by other surveys on pet food (Table 1) as well as on feed raw materials and feedingstuffs.[28,29] Certainly, the detection of one single mycotoxin in a feed sample represents the exception, and it is well-known that both humans and animals are usually exposed to several mycotoxins (mainly at low levels) at the same time.[27] Nevertheless, worldwide regulations and past toxicological studies usually refer to individual mycotoxins. Recently, an increasing number of investigations evaluating the toxicological effects of different mycotoxin combinations have been published, both in vivo[41] and in vitro conditions,[27] even if the experimental doses were usually higher than those found in "natural" contaminations and often exceeding the international regulatory limits.[42] In the present study, one of the most common co-occurrence was ZEA + DON + fumonisins, with these mycotoxins being the most frequent in the samples evaluated. In this regard, several Fusarium strains producing ZEA also produce trichothecenes such as DON, and in general, a frequent co-occurrence of ZEA with other Fusarium toxins has been described in cereals, especially in corn.[43] Furthermore, a synergy among these Fusarium toxins has been recognized[42] even though additive, synergistic, and antagonistic effects have been described under in vitro conditions, often depending on the dose of the toxins.[27]

Pets are traditionally fed with the same type of diet for long periods of their life. Therefore, the scientific community should be aware of the potential chronic exposure of dogs and cats to relatively low levels of different mycotoxins and the consequential detrimental risks to their health.

The results from the present study showed that mycotoxin contamination represents a critical point for pet food safety. Certainly, given the high stability of mycotoxins through the cooking process used to produce dry pet food,[5] scrupulous monitoring of incoming ingredients undoubtedly represents the most effective strategy to prevent mycotoxin contamination.

■ MATERIALS AND METHODS

Sampling. In order to evaluate a representative selection of the different types of cat food available on the Italian market, 64 complete commercial dry products of different brands were collected from stores in the province of Bologna (Italy) from June to September 2015. Specifically, they included 30 standard cat foods (5 for kittens, 5 for senior, and 20 for adult cats) and 34 premium cat foods (5 for kittens, 5 for senior cats, 5 grain-free, and 19 dietetic products including 6 for obesity management, 7 for renal diseases, and 6 for gastrointestinal disorders). Standard samples consisted of products ranging in price from 0.80 to 4.00 €/kg that were sold by discount and mass-market retailers. Conversely, premium samples consisted of more costly diets ranging from 4.00 to 15.00 €/kg that were purchased in specialized pet stores. The size of the packages ranged from 250 g to 2 kg. In grain-free samples, the main sources of starch declared on the label were legumes (lentils, peas, chickpeas, and beans) and potatoes. The main cereals and cereal by-products listed on the package of the other cat foods were corn, wheat, rice, corn gluten, wheat gluten, corn starch, barley, spelt, and yellow millet. In particular, corn and corn byproducts (corn starch and corn gluten) represented the most common vegetable ingredients placed in the first positions of the ingredient list declared on cat food labels.

All of the cat foods were stored in a cool, dry place inside their original hermetically sealed package until chemical analysis. With the aim of obtaining a representative sample of each cat food, approximately half of the content of each product was randomly taken from the original package (from four different sites), finely ground, stored at −20 °C, and analyzed within its shelf life.

Nutrient Analyses. The cat food samples were chemically analyzed to determine the moisture and starch content according to the official methods of the Association of Official Analytical Chemists (method 950.46 for moisture and method 996.11 for starch).[44]

Determination of Mycotoxin Concentration. The most important mycotoxins currently regulated in the EU regarding pet food have been evaluated in cat food samples.

Chemicals and Reagents. Aflatoxin B_1 (AFB$_1$), aflatoxin B_2 (AFB$_2$), aflatoxin G_1 (AFG$_1$), aflatoxin G_2 (AFG$_2$), fumonisin B_1 (FB$_1$), fumonisin B_2 (FB$_2$), deoxynivalenol (DON), zearalenone (ZEA), ochratoxin A (OTA), T-2 toxin (T-2), and HT-2 toxin (HT-2) standards were purchased from Sigma-Aldrich (Steinheim, Germany).

U-[$^{13}C_{17}$]-aflatoxin B1 (^{13}C AFB$_1$), U-[$^{13}C_{34}$]-fumonisin B1 (^{13}C FB$_1$), U-[$^{13}C_{15}$]-deoxynivalenol (^{13}C DON), U-[$^{13}C_{18}$]-zearalenone (^{13}C ZEA), U-[$^{13}C_{20}$]-ochratoxin A (^{13}C OTA), and U-[$^{13}C_{24}$]-T-2 toxin (^{13}C T-2) were obtained from Romer Lab Inc. − Biopure (Tulln, Austria).

Methanol, formic acid, and ammonium acetate, used as mobile phases, were of LC−MS grade and were purchased from Riedel-de Haën (Seelze, Germany). Acetonitrile and acetic acid, used in the extraction procedures, were purchased from Merck (Darmstadt, Germany).

Reverse osmosis and ultrapure water, respectively used as an extraction solvent and the mobile phase, were produced using a human-powered apparatus from Human Co. (Seoul, South Korea).

Sample Preparation. The method set up by Zhang et al.[45] was slightly modified and used in this work. A ground sample

(500 mg) was weighed in a beaker, fortified with labeled standards, and extracted with 5 mL of acetonitrile/water (50:50). The sample was shaken for 15 min, and 500 μL of the supernatant was collected and transferred in an Amicon ultra centrifugal filter (0.5 mL, 3 K; Millipore, Carrigtwohill, Ireland). After a centrifugation step, 14,000 rpm for 30 min at 20 °C, the sample, once filtered, was analyzed using ultra-performance liquid chromatography in combination with tandem mass spectrometry (UPLC−MS/MS). The samples with a mycotoxin concentration greater than 1,000 μg/kg were diluted to obtain a correct analyte concentration in the curve range.

UPLC−MS/MS Equipment and Conditions. Analyses were conducted with a UPLC−MS/MS system, composed of a Waters Acquity UPLC binary pump, equipped with a Waters Acquity BEH C_{18} reversed-phase column (50 × 2.1 mm, 1.7 μm) coupled to a VanGuard guard column with identical packaging (Waters, Milford, MA, USA).

Two different mobile phases were optimized, one for DON and the other for all of the other analytes.

For all the analytes, water containing 0.1% formic acid and methanol containing 0.1% formic acid were employed as mobile phases under programmed conditions at a flow rate of 0.42 mL/min. Analyses were carried out over 16 min using a previous method developed by Jackson et al.[46]

Some changes about LC and MS conditions for the DON analysis were necessary to improve the sensitivity of the determination. For DON, the mobile phase consisted of 5 mM ammonium acetate (A) and methanol (B), and the flow rate was 0.3 mL/min. The following gradient program, time (%A−%B), was applied: 0 min (99−1), 1 min (95−5), 2 min (25−75), 2.1 min (1−99), 4 min (99−1), 6 min (99−1). For both methods, the column heater temperature was set at 40 °C, and the volume injection was 10 μL.

The mass spectrometer was a Quattro Premier XE, a triple quadrupole instrument equipped with an ESCI multimode ionization source (Waters, Milford, MA, USA).

The mass spectrometer was operated in the positive electrospray ionization mode (ESI+) using multiple reaction monitoring (MRM). The capillary voltage was set at 3.5 kV for all analytes and 3.0 kV for DON; the MRM transitions, cone voltages, and collision energies are shown in Table 2.

Data acquisition and processing was performed using Mass Lynx 4.1 Software (Waters Corporation, Milford, USA).

Method Validation. The proposed method was validated in-house according to the European Commission Decision 2002/657/EC[47] and Commission Regulation 2006/401/EC.[48] The following parameters were evaluated: specificity, linearity, trueness, precision, limits of quantification (LOQs), and limits of detection (LODs).

One grain-free sample exhibiting no measurable concentrations of the analytes of interest was used for the validation of the analytical method.

Fortified samples were obtained adding different volumes of mycotoxin standard solutions to blank samples before the extraction steps. Spiked samples were left at least for 2 h at room temperature to allow for the evaporation of the solvents and the equilibration between the analytes and matrix.

The matrix-matched calibration curve ($R^2 > 0.98$) occurred over the range of 0 to 1000 μg/kg using seven calibration standards (0, 5, 20, 50, 100, 200, and 1000 μg/kg).

The recovery and precision of the methods were evaluated at three quality control levels (20, 50, and 100 μg/kg).

The injection of four replicates of three quality control levels demonstrated satisfying precision, with maximum relative standard deviations to the mean of 12.2% as well as good trueness values expressed as the relative bias between the mean value measured and the spiked concentration, ranging from −12.1 to 4.5%. The trueness and precision (in terms of repeatability) values obtained fulfill the performance criteria established by the abovementioned regulations.[47,48] Specificity was assessed excluding the presence of potential interferences around mycotoxin retention times in the chromatograms of non-contaminated samples. Moreover, the blank sample used for the method validation was used to evaluate the matrix effects, as ion suppression or enhancement; in particular, it was injected while standard solutions (1 μg/mL) were directly infused with a flow of 10 μg/min through a T-connection device into the LC eluate.[49] The ion currents were stable, and no interference was recorded at the specific retention times of the considered mycotoxins.

Limits of quantification of the method, defined as the concentrations providing a chromatographic signal with a signal-to-noise ratio equal to 10, were 3 μg/kg for FB_1, FB_2, AFB_1, AFB_2, AFG_1, AFG_2, and DON, 5 μg/kg for ZEA and OTA, 10 μg/kg for T-2, and 20 μg/kg for HT-2. Limits of detection of the method, defined as the concentrations providing a chromatographic signal with a signal-to-noise ratio equal to 3 for the qualification transition, were 1 μg/kg for FB_1, FB_2, AFB_1, AFB_2, AFG_1, AFG_2, and DON, 2 μg/kg for ZEA and OTA, and 5 μg/kg for T-2 and HT-2.

Standard curves and quality controls were run at the beginning and end of each analytical run day.

Statistical Analyses. The concentrations of the different mycotoxins in the two price categories of cat foods were statistically analyzed by using Student's *t* test. For samples in which they were not detected or quantified, a specific mycotoxin was assigned according to the corresponding LOD or LOQ. The level of significance was set at $P < 0.05$. Furthermore, the correlations between the starch content (expressed on a dry matter basis) and the concentration of the different mycotoxins were analyzed using the Pearson correlation test. All of the statistical computations were performed with Statistica 10.0 (Stat Soft Italia, Italy).

■ APPENDIX

Guidelines of the European Commission

Guidance values recommended by the European Commission:[26]

1. For total fumonisins ($FB_1 + FB_2$): 5000 μg/kg relative to compound feeds for pets.
2. For ZEA: 100 μg/kg relative to compound feeds for puppies, kittens, and dogs and cats for reproduction.
3. For $T_2 + HT_2$: 50 μg/kg relative to compound feeds for cats.
4. For OTA: 10 μg/kg relative to compound feeds for dogs and cats.

Maximum content established by the European Commission for AFB_1:[20] 10 μg/kg for complete feeds for animal species other than for livestock.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; DON, deoxynivalenol; EFSA, European Food Safety Authority; EU, European Union; FB_1, fumonisin B_1; FB_2, fumonisin B_2; HT-2, HT-2 toxin; LC−MS/MS, liquid chromatography coupled to mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; OTA, ochratoxin A; T-2, T-2 toxin; UPLC-MS/MS, ultra-performance liquid chromatography coupled to tandem mass spectrometry; ZEA, zearalenone

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