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Risk assessment of the exposure to mycotoxins in dogs and cats through the consumption of commercial dry food

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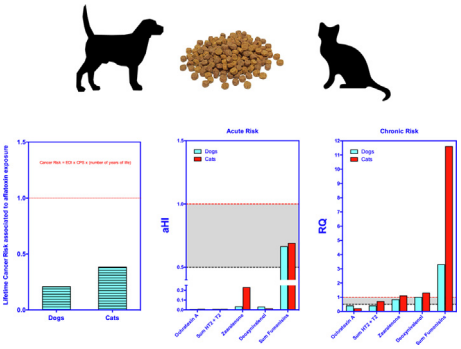
HIGHLIGHTS

- High incidence and co-occurrence of mycotoxins in dog and cat feed.
- Virtually no differences in the level of contamination between expensive and cheap feed brands.
- Low risk of acute toxicity, although some brands had worryingly high levels of fumonisins.
- Moderate chronic risk for zearalenone, deoxynivalenol, and fumonisins.
- Fumonisin exposure levels exceed up to 12 times the tolerable daily intake.

GRAPHICAL ABSTRACT



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ABSTRACT

Dry feed for dogs and cats sold in Europe are mostly formulated with cereals and cereal by-products, so the contamination of this food with mycotoxins represents a potential risk for these pets. We analyzed a representation of the best-selling feed brands in Spain. The presence of Aflatoxins (B_1, B_2, G_1, and G_2), Ochratoxin A, T-2 and HT-2 toxins, Deoxynivalenol, Zearalenone, and Fumonisins (B_1 and B_2) was quantified, using immunoaffinity columns and LC-MS/MS. In general, mycotoxins were frequently and simultaneously (6–11) detected, with AFB_1, FB_1, FB_2, Deoxynivalenol, and HT-2 detected in 100% of the samples. However, the concentrations of most of them are among the lowest reported so far. Fumonisins were the exception since we report the highest concentrations to date, particularly in cat feed. We practically found no significant differences in the level of mycotoxin contamination in relation to the presumed quality of the feed. We also calculated the daily exposure, and evaluated the acute and chronic health risk posed by these feeds. None of the brands analyzed presented acute risk for any of the mycotoxins. However, the high levels of fumonisins found in some samples could become problematic, if there are hidden forms of them. This is also evident in relation to long-term risk, since in the case of fumonisins the level of exposure exceeds the tolerable daily intake level in 3.5 and 12 times, for dogs and cats respectively. The exposure levels to zearalenone and deoxynivalenol could also be of long-term concern, especially considering the possibility that the continuous exposure to several mycotoxins simultaneously might produce potentiated toxic effects as a result of their synergistic action. Further

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research on the potential adverse health effects deriving from chronic exposure to low doses of multi-mycotoxin mixtures in pets is needed.

1. Introduction

According to data from the European Pet Food Federation, the census of pet dogs and cats in the EU reached 140 million in 2018. Thus, the economic data of the European pet care sector are overwhelming, with a turnover of € 38.5 billion (FEDIAF, 2018b). About 60% of this figure accounts for the sale of food for animals since most of these pets are fed commercial formulas instead of homemade food. In fact, the pet food market maintains an upward trend in all countries of the European Union, parallel to the increase in family spending, although there are relevant differences between countries (FEDIAF, 2018b). In the specific case of Spain, it experienced a growth of more than 3.5% last year compared to the 2017, with a market sale of 844.700 tons of dog food and 176.000 tons of cat food. Of these amounts, dry food accounted for 84.4% and 70.4% in the case of dog and cat food, respectively (MAPAMA, 2018).

Although dogs and cats have no absolute dietary requirement for carbohydrates, most pet food manufacturers take advantage of their capacity of digesting them, and employ variable amounts of cereals, such as corn, rice, wheat, barley or sorghum, as well as cereal byproducts as a cheap source of energy, proteins, fiber and other nutrients, such as thiamine and niacin (FEDIAF, 2018c; Leung et al., 2006). The proportion of these cereals in the feed formulation can be up to 70%, although it is usual to be between 30 and 50% (Kempe et al., 2004).

The fact that cereals can be affected very easily by mycotoxins is well known. Although the feed industry is a sustainable outlet for food processing industries because it allows converting byproducts into high-quality animal feed, in the particular case of cereals and mycotoxins an added risk exists, since cereal processes tend to concentrate mycotoxins into those fractions that are commonly used as ingredients of animal feed (cereal byproducts) (Kaushik, 2015; Pinotti et al., 2016).

Depending on classification, 300–400 mycotoxins are known to date (Streit et al., 2012). When ingested above certain levels these chemicals may produce acute or long-term adverse health effects. The clinical symptoms for mycotoxicosis depend on the type of mycotoxin and its concentration, the duration of exposure, and the species, gender, age and health of the host animal, and range from a simple food rejection to the development of cancers (Pinotti et al., 2016; Streit et al., 2012). However, due to a combination of frequency of occurrence and high toxicity, some are more worrying than others, and for several of them (aflatoxins, fumonisins, zearalenone, ochratoxin A, deoxynivalenol, T2 and HT2 toxins) the food safety regulatory authorities have established safety limits (MRLs) in cereals and cereal-based foods and feeds. In addition tolerable daily intakes (TDIs) have been calculated for these regulated mycotoxins for humans (EFSA, 2006, 2011a,b, 2013a,b; Marroquin-Cardona et al., 2014; WHO, 1998; Yogendrarajah et al., 2014).

However, although the European legislation on animal feed provides a framework for ensuring that feedstuffs do not present any danger to animal health or to the environment, the main focus of these limits are the farming animals and, ultimately, the human food safety. That is, the MRLs, reach human food, and also the feed for livestock, but there are not such specific MRLs for cereal-based dog and cat feed, as these are grouped as "non-food producing animals" according the European laws (EC, 2009). Therefore, pet food is regulated by the maximum mycotoxin contamination levels for all foodstuffs rather than by pet-specific legislation (Leung et al., 2006). In practical terms, this means that most food safety criteria for these pets in the EU are self-regulated by the industry, which encourages the manufacturers to follow the guides to good practice for the manufacture of safe pet foods (FEDIAF, 2018c). According to a recent study, >50% of pet owners reported giving equal priority to buying healthy food for their pets compared with themselves (Schleicher et al., 2019), and it has been also established that the most important determinant of the choice of feed by pet owners is quality, in terms of performance and health (Anders, 2013). However, there are no clear criteria and objectives that need to be met that allow classifying a pet feed as of a higher quality than another. Again, it is a matter of industries' self-regulation, which should adhere to the code of good labeling practice (FEDIAF, 2018a). However, since this is not an officially regulated matter, there is also the risk that the marketing strategies of certain manufacturers might take advantage of the desire of consumers and present products that provide an "imaginary added value", with packaging alluding to aspects of health, or labeled under the "premium" or "ultra-premium" denominations, or simply at a higher price range than typical products. In reality, these products may be of higher quality than the average, or they may simply be perceived as of higher quality, since studies in consumers' psychology indicate that price can alter our perception of a product, with the more expensive products being perceived as of better quality (Poundstone, 2011).

As far as we know, there are barely a dozen studies reporting mycotoxin contamination in feed for dogs and cats (Abd-Elhakim et al., 2016; Bissoqui et al., 2016; Blajet-Kosicka et al., 2014; Bohm et al., 2010; Frehse et al., 2015; Gazzotti et al., 2015; Maia and Pereira Bastos de Siqueira, 2002; Mulunda et al., 2013; Scudamore et al., 1997; Singh et al., 2017; Singh and Chuturgoon, 2017; Witaszak et al., 2019), and the number of those which also have carried out a health risk assessment is even smaller (Bissoqui et al., 2016; Boermans and Leung, 2007; Frehse et al., 2015; Singh et al., 2018). This is probably due to the fact that this type of feed has had a relatively minor interest, as it is not intended for productive animals. However, due to the growing interest on the part of pet owners in providing their companion animals with the most healthy and balanced diet possible, we have designed this study. We have investigated the presence of aflatoxins, ochratoxin A, zearalenone, deoxynivalenol, T2 and HT2 toxins, and fumonisins in pelleted dry food for dogs and cats containing cereals. We have included both "premium" brands sold in veterinary centers and specialty stores, at relatively high prices, and also low-price brands, sold in supermarkets and non-specialized stores. In addition, we have also made an approach to the evaluation of acute and chronic health risk posed by these feeds.

2. Materials and methods

2.1. Sampling

A total of sixty packages of pelleted dry food for cats (0.8–2 kg), and sixty-two packages of pelleted dry food for dogs (1.25–4 kg) were purchased from different specialized stores, retail outlets and supermarkets located in Gran Canaria (Canary Islands, Spain). The expiration date was taken into account, and no sample that

expired before 4 months was acquired. The sampling protocol was designed taking into account two premises: a) only brands that declared a minimum content of 6% of cereals or cereal byproducts in the label were included, and b) the brands selected needed to cover the entire price range present in the market. For this second premise, information previously provided by wholesalers and distributors in a telephone interview was employed (Supplementary Tables 1 and 2). Given that the smaller the package size, the cost per kilo is higher, the prices of several package sizes of each brand were taken, and the average in €/kg for each sample was calculated. Thus, dog food brands from 0.7 to 9.1 €/kg, and cat food brands from 0.8 to 13.5 €/kg were selected. No samples of bulk dog/cat feed were included in this study. Although the samples were acquired in Gran Canaria island, none of the brands selected are locally produced, but national and international distribution brands were chosen. All these brands sampled are sold throughout Spain and more than 85% of them throughout the EU. Until sample preparation the samples were stored in a dark and dry environment at room temperature, without removing them from the commercial packaging.

2.2. Chemicals and reagents

Mycotoxin standards, including 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), ochratoxin A (OTA), T-2 toxin (T2), HT-2 toxin (HT2), deoxynivalenol (DON), and zearalenone (ZEA), as well as MS-PREP® monoclonal antibody multi-mycotoxin immunoaffinity columns for simultaneous extraction of deoxynivalenol, zearalenone, T2 and HT2 (DZT columns), or aflatoxins, ochratoxin A and fumonisins (AOF columns) were supplied by Trilogy (Washington, USA). The synthetic mycotoxin compound zearalanone was employed as internal standard (IS) and was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Solvents (acetonitrile and methanol), formic acid, and ammonium formate were of LC/MS grade (Optima™, Fisher Scientific, Pittsburgh, USA). Ultrapure (UP) water was produced in the laboratory using a Milli-Q Gradient A10 apparatus (Millipore, Molsheim, France). Sterile phosphate buffered saline (PBS), pH 7.4 was purchased from VWR International (Pennsylvania, USA). 1 μm glass fiber prefilters and 0.20 μm polyester syringe filters were purchased from Macherey-Nagel (Düren, Germany).

For the preparation of standard solutions of mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, OTA, T2, HT2, DON, and ZEA) dissolutions at 0.1 mg/ml of each one in methanol were prepared, which were stored at −20 °C in sealed vials until use. According to the results of a previous pilot study and the expected ranges of concentration in real samples, two intermediate mycotoxin mixes in methanol were prepared, which were stored at 4 °C (up to three months): a) Mix 1 containing FB_1, FB_2, DON, and ZEA at 20 μg/ml each, and b) Mix 2 containing AFB_1, AFB_2, AFG_1, AFG_2, OTA, T2, and HT2 at 1 μg/ml each. These mixes were employed to prepare daily calibration curves (4000, 2000, 1000, 400, 100, 50, 10, 5, 1 and 0.5 ng/ml for Mix 1; and 100, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01 and 0.005 ng/ml for Mix 2), by the appropriate dilutions with mobile phase.

2.3. Mycotoxin extraction from pelleted dry food samples

Twenty-five grams of grinded dog/cat dry food were placed in 250-ml glass beakers tubes and mixed with 5 g of NaCl and 100 ml of the extractant solution (methanol/water: 70/30 v/v). The samples thus prepared were thoroughly mixed for 3 min using a Yellow Line DI-25 basic homogenizer (IKA-Werke, Staufen, Germany), and the homogenate was passed through cellulose laboratory paper filter cones, and the filtrate was collected in 50-ml polypropylene conical tubes. The tubes were centrifuged at 4200 rpm, 10 °C, 10 min. The supernatant was subjected to dilution with PBS in two different proportions, using conical polypropylene tubes: a) Twenty ml of the supernatant were diluted with 30 ml of PBS and employed for the determination of aflatoxins, OTA, and fumonisins; and b) other 5 ml of the supernatant were diluted with 60 ml of PBS and employed for the determination of DON, ZEA, T2 and HT2. These diluted extracts were centrifuged at 4200 rpm, 10 °C, 10 min and filtered through a 1/0.2 μm glass fiber-polyester tandem syringe filter set. Forty ml of each dilution were sequentially passed through a tandem of AOF and DZT immunoaffinity columns at a maximum flow rate of 2 ml/min. The columns were washed with ultrapure water (10 ml) and allowed to dry under vacuum for 15 min. The elution of the retained mycotoxins was performed using 1 ml of methanol followed by 1 ml of ultrapure water. The combined eluate was filtered using a 0.2 μm polyester syringe filter and placed in an amber glass chromatography vial. Finally, 20 μl of the IS (zearalenone, 50 ng/ml) were added to each vial and were subjected to chromatographic analysis without further purification steps.

To verify the effectiveness of the columns, at least one sample of the Certified Reference Materials TR-A100 (for AOF columns) and TR-Z100 (for DZT columns) was extracted for each box of immunoaffinity columns (50 units). For this, exactly the same method described above was employed.

2.4. Analytical method

The mycotoxin LC-MS/MS analyses were performed with an Agilent 1290 UHPLC tandem coupled to an Agilent 6460 mass spectrometer. The chromatographic separations were performed using a Poroshell 120 EC-C18 2.1 × 100 mm, 2.7 μm with a Poroshell Guard pre-column of the same characteristics and a length of 2.1 × 5 mm (Agilent Technologies Inc., Palo Alto, CA, USA). The mobile phase A consisted on 0.1% formic acid in water, and the mobile phase B consisted on 0.1% formic acid in methanol. A binary gradient using mobile phases A and B was programmed as follows: 0 → 8.0 min, 10% B; 8.0 → 14.1 min, 50% B; 14.1 → 15.00 min, 90% B; 15.0 → 17.0 min, 10% B. The flow rate was set at 0.3 ml/min. The injection volume was 3 μl. The column oven temperature was set at 50 °C. Total run time was 17 min. Transitions and conditions for the pure mycotoxins were optimized using the Agilent MassHunter Optimizer software with flow injection of the diluted stock solutions as previously reported (Luzardo et al., 2016). For all the mycotoxins, the $[M+H]^+$ species produced the most abundant precursor ion, except for ZEA, which achieved the highest sensitivity in negative mode with $[M-H]^-$ as the precursor. The MS/MS ionization was performed with a Jet Stream electrospray ionization (ESI). Two MS/MS transitions were optimized for each analyte for identification and quantification purposes. Nitrogen was used as nebulizer and collision gas. The operating conditions for the analyses in positive and negative ESI were the following: gas temperature 140 °C; nebulizer gas flow 16 l/min; Nebulizer pressure 25 psi; Sheath gas temperature 350 °C; Sheath gas flow 11 l/min; Capillary voltages 4000 V (positive); 3000 V (negative); Cycle time 400 ms; Dwell time 50 ms.

The analytical method was assessed for selectivity, linearity, precision, and repetitiveness. Selectivity was checked by injecting 5 μl of mycotoxin standard solution three times before injecting extracted samples and comparing the peak retention time, and the ion ratio of the transitions of each mycotoxin. Standard curves were generated by linear regression of peak areas against concentrations. Precision was established by determining the levels of the targeted mycotoxins in fortified dog or cat dry food samples by triplicate. For these experiments we selected the lowest residue level brands that we detected in a preliminary pilot study, with 5

samples of each food type. All the correlation coefficients (r^2) were higher than 0.985, and the RSD lower than 13% for all the analytes. As the limit of detection (LOD), we employed the lowest non-zero calibrator that demonstrated that all detection and identification criteria were met. As the limit of quantification (LOQ), we employed the lowest non-zero calibrator that additionally fulfilled the bias and precision criteria. For these calculations, a minimum of three samples per run of the lowest calibrator was analyzed over three runs, but additional samples/replicates were needed for some mycotoxins (i.e. aflatoxins or OTA) to meet the minimum of nine data points (Scientific Working Group for Forensic, 2013; Wille et al., 2017). The LOQs of the targeted mycotoxins were: AFs = 0.025 ng/g; OTA = 0.1 ng/g; FBs = 2.5 ng/g; T2 = 0.2 ng/g; HT2 = 0.1 ng/g; DON = 5 ng/g; and ZEA = 0.04 ng/g.

2.5. Exposure assessment and risk characterization

In order to estimate the probability and severity of potential adverse health effects of the presence of targeted mycotoxins in dog and cat pelleted dry food, a quantitative exposure assessment was performed for both species, and exposure values were compared with the acute or chronic toxicity reference values. It is important to note that toxicity references, both acute and chronic, for dogs and cats are very scarce, or directly non-existent. Some specific susceptibilities to certain mycotoxins have been reported, both for dogs and cats. Thus, cats seem to be particularly susceptible to T2 and HT2 toxins, due to their inability to excrete them and their metabolites via glucuronide conjugation (EFSA, 2011a), and dogs have been reported to be more to susceptible to AFs due to their low glutathione-S-transferase activity, which plays an important role in detoxification of this mycotoxin (Singh and Chuturgoon, 2017). Despite these differences, in the absence of appropriate reference values, we have decided to use the same reference points as those derived for humans to give an indication on the possible risk, assuming that, although some exceptions exist, for most mycotoxins the toxicokinetics in dogs and cats are not substantially different to that of humans (EFSA, 2011a, 2013b, 2017a,b, 2018b). However, the results should be interpreted with caution.

2.5.1. Short-term or acute exposure

These calculations were made in order to assess the acute (short-term) health risk for those dogs or cats consuming the most contaminated feed brands of the whole series. The estimated short-term intake (ESTI) was calculated according to the following formula:

$$\mathrm{ESTI} = \mathrm{HRM} \times K \tag{1}$$

where HRM represents the highest residue level of the mycotoxin found in the series and K is the recommended amount of feed per kilo and day of that feed, according to the manufacturer's recommendation.

The acute hazard index, which represents ratio between the exposure to a single dose of a toxic substance and the acute reference dose of toxicity for that pollutant, was calculated for each mycotoxin and species according to the following formula:

$$aHI = \frac{ESTI}{LOAEL} \tag{2}$$

In this case, the lowest observed adverse effect dose (LOAEL) described in the literature for mammals was considered as the acute reference dose of toxicity (aRfD) for FB_1, FB_2, OTA, T2, HT2, DON, and ZEA. Because aflatoxins are proven genotoxic/mutagenic compounds, no aRfD or LOAEL have been defined for them. For the calculation of aHI of aflatoxins, either individually considered or as a sum, we have employed the lowest oral acute toxic dose that has been published to date for dogs and cats (Newberne and Butler, 1969).

2.5.2. Long-term or chronic exposure

The long-term estimated daily intakes (EDIs) of mycotoxins through the consumption of each brand were obtained by the combination of mycotoxin analysis of each brand with the amounts of consumption of each feed, according to the manufacturer's recommendation, expressed in grams of feed/kg body weight (b.w.). We only took into account differences in species (dogs vs. cats), but not sex or age. Therefore, the estimated daily intake for each mycotoxin in each species was expressed in ng/kg b.w./day and was calculated according to the following formula:

$$EDI = M_e(C_n \times K_n) \tag{3}$$

where M_e represents the median value of all the series; C is the content of a mycotoxin (ng/g); K is the recommended amount of feed (g/kg b.w./day), according to the manufacturer's recommendation; and the subindex n represents each brand.

To evaluate the long-term risk of exposure to non-carcinogenic mycotoxins through the consumption of dog or cat dry food, we calculated the risk quotient as follows:

$$RQ = \frac{EDI}{TDI} \tag{4}$$

where tolerable daily intake values (TDI) have been expressed in the same units that EDI (ng/kg b.w./day) and are derived from the TDIs, PTDIs or PTWIs of each mycotoxin as described by EFSA (EFSA, 2006, 2011a,b, 2013a,b, 2014).

In the case of aflatoxins, as these have been classified as group 1 carcinogens (IARC, 2002), no TDI can be established, and the Margin of Exposure (MoE) approach has been employed, as recommended for substances in food that are genotoxic and carcinogenic (Benford et al., 2010; EFSA, 2013a). The MoE is calculated as a ratio between as a reference point on the dose-response curve (e.g. a benchmark dose lower confidences limit derived (BMDL), calculated from a carcinogenicity study in laboratory animals) and the estimated daily intake.

$$MoE = \frac{BMDL_{10}}{EDI} \tag{5}$$

According to EFSA, the most sensitive animal model for aflatoxins is the rat, and a $BMDL_{10}$ of 170 ng/kg b.w./day has been defined as the confidence limit for an extra cancer risk of 10% (EFSA, 2018a). Following the commonly adopted criteria, EDI would be considered to be of concern if the value of the MoE is less than 10,000. This value includes a factor of 100 for differences among species (since carcinogenicity studies are made in rats), a factor of 10 because $BMDL_{10}$ is linked to a measurable tumor incidence of 10%, and another additional factor of 10 for considering intra-species differences in DNA repair capacity and cell cycle control. It should be noted that the MoE is dimensionless, and does not quantify the risk, but indicates the level of concern. This means that when the calculated MoE is less than 10,000 additional characterization of the carcinogenic risk should be made. In our case, this characterization has been carried out as the increased cancer risk from a lifetime oral exposure to a carcinogenic agent, and can be calculated according to the Eq. (6) (USEPA, 2005):

$$\text{Cancer Risk} = \mathrm{EDI} \times \mathrm{CPS} \times (\text{number of years of life}) \tag{6}$$

where EDI is the individual's lifetime estimated daily intake of the carcinogenic mycotoxin; CPS is the carcinogenic potency slope for ingestion, as defined by the international organisms. For this study, although the life expectancy of dogs and cats varies greatly from breed to breed, we have employed an average of 12 years. We

assume that if the value of risk is equal to or less than 1, then it can be considered that there is no increased risk of cancer attributable to the carcinogenic mycotoxin, and if it is higher than 1, an increased risk would exist.

2.6. Statistical analysis

All the statistical analyses were done using GraphPad Prism v6.0 (GraphPad Sofware, CA, USA). The distribution of the variables included in this study was evaluated through Kolmogorov-Smirnov test. The concentration of the mycotoxins included in this study did not follow a normal distribution; therefore, the results, apart than as mean ± SD, are expressed in terms of the median and interquartile range (percentiles 25th to 75th). Differences of contaminants among groups were tested with the non-parametric Mann-Whitney *U* test test. A *P* value of less than 0.05 (two-tailed) was considered to be statistically significant.

3. Results and discussion

3.1. Occurrence of mycotoxins in pelleted dry food for dogs and cats

Tables 1 and 2 show the descriptive analysis of mycotoxins in pelleted dry food for dogs and cats, respectively. There are several issues that attract attention to the results we have obtained. The first of these is the high detection frequency for almost all mycotoxins in feed brands, both for dogs and cats. Thus, AFB_1, HT2 toxin, DON, and FB_1 and FB_2 were detected in 100% of the samples analyzed. Also striking is the fact that none of the mycotoxins regulated in the EU were totally absent from the series of samples analyzed, and that even the very rare AFG_2 was detected in 2 samples of dog food. In addition, as can be seen in Fig. 1, the number of mycotoxins detected simultaneously was high, with a minimum number of 6 different residues per sample, and even in 4 samples the 11 mycotoxins analyzed were found. The most frequent combination of mycotoxins in feed, both for dogs and cats, was that of AFB_1 + HT2 + ZEA + DON + FB_1 + FB_2. In the oldest available studies, the authors report detection frequencies much lower than ours, in the range of 2 to 28% (Maia and Pereira Bastos de Siqueira, 2002; Martins et al., 2003; Scudamore et al., 1997). However, this is probably because the LOQs reported by the authors are much higher than those of our method (around an order of magnitude higher). In fact, our LOQs are among the lowest reported in studies that analyze mycotoxins in pet feeds, and consequently, this is probably the reason why the detection frequencies that we report in this paper are the highest so far. In fact, the different available studies generally report higher detection frequencies the lower the LOQ of the methods employed (Abd-Elhakim et al., 2016; Bissoqui et al., 2016; Bohm et al., 2010; Frehse et al., 2015; Mulunda et al., 2013; Singh et al., 2017; Singh and Chuturgoon, 2017; Witaszak et al., 2019), the highest being those found in Poland in 2014 (Blajet-Kosicka et al., 2014), which reports very low LOQs, similar to ours. However, it is noteworthy that these authors, despite being able to quantify levels as low as 0.02 ng/g only report 8% of samples positive to total aflatoxins, compared to 100% that we detected in our study. In addition, of course, it also contributes to the detection frequencies we report, the fact that we only selected food brands that declare on the label that they use at least 6% cereals. Although it would have been interesting to study whether there is any type of association with the type of cereal used or with the concentration of the same, this has not been possible due to the heterogeneity of the labeling, which prevents being sure of which ingredients were employed exactly, and the relative percentages of each of them.

Although they are frequently detected, the concentrations of most mycotoxins do not seem too high, at least from the point of view of legal recommendations, except in the case of fumonisins. In Fig. 2 we have represented the concentrations found, expressed in terms of the percentage of the maximum recommended values by the EU authorities for animal feed (MRV). The MRVs employed are those for compound feed for livestock since there are no such recommendations for dogs and cats (EC, 2006). It can be seen that there are some samples that would exceed these MRVs for ZEA and FB, particularly in the case of cat feeds, in which up to 30% of the analyzed samples exceeded the recommended maximum limit for fumonisins. In general, the food for cats appears to be more contaminated by mycotoxins than those of dogs (Table 3), and the levels of all mycotoxins, except OTA and DON, are significantly higher in cat than in dog feeds. In the specific case of fumonisins, levels are approximately 4 times higher in feed for this species (median values of 1189,97 ng/g and 324,71 ng/g, for cat and dog feed, respectively). Comparing our results with those reported by other authors, we found that our levels are generally low, except for fumonisins. So, regarding aflatoxins, although detectable in 100% of the samples, the concentrations we found (median values = 0.06 ng/g and 0.19 ng/g in dog and cat feed, respectively) are among the lowest reported so far (Abd-Elhakim et al., 2016; Blajet-Kosicka et al., 2014; Bohm et al., 2010; Gazzotti et al., 2015; Maia and Pereira Bastos de Siqueira, 2002; Scudamore et al., 1997; Singh et al., 2017; Singh and Chuturgoon, 2017) (Tables 1-3). These results contrast with the extremely high values found in South Africa in 2013 (Mulunda et al., 2013), where a med-

Table 1
Comparative analysis of mycotoxin contamination of supermarket and premium brand pelleted dog food in Spain. Results are expressed in ng/g feed.

	Supermarket brands (n = 34)				Premium brands (n = 26)				
Mycotoxin	Mean ± SD	Median	P25-P75	Frequency	Mean ± SD	Median	Range	Frequency	*p*
Aflatoxin B_1	0.33 ± 0.99	0.04	0.01–0.19	100%	0.11 ± 0.09	0.06	0.03–0.19	84.6%	n.s.
Aflatoxin B_2	0.14 ± 0.50	0.00	0.00–0.08	29.4%	0.02 ± 0.03	0.00	0.00–0.03	23.1%	n.s.
Aflatoxin G_1	0.06 ± 0.17	0.01	0.00–0.03	58.8%	0.02 ± 0.02	0.00	0.00–0.04	46.2%	n.s.
Aflatoxin G_2	0.03 ± 0.11	0.00	0.00–0.00	5.9%	–	–	–	0%	–
Sum Aflatoxins	0.56 ± 1.77	0.05	0.02–0.28	100%	0.14 ± 0.11	0.10	0.05–0.26	92.3%	n.s.
Ochratoxin A	0.71 ± 0.42	0.78	0.28–1.05	88.2%	0.07 ± 0.11	0.02	0.00–0.18	53.8%	0.0014
HT2	1.06 ± 0.53	1.06	0.66–1.52	100%	0.80 ± 0.31	0.84	0.53–0.91	100%	n.s.
T2	1.34 ± 0.95	1.23	0.65–2.04	94.1%	1.17 ± 0.52	1.07	0.76–1.70	100%	n.s.
Sum HT2 + T2	2.41 ± 1.44	2.15	1.33–3.51	100%	1.96 ± 0.95	1.71	1.39–2.67	100%	n.s.
Zearalenone	9.53 ± 7.58	8.42	5.46–12.36	88.2%	11.33 ± 14.90	7.46	4.18–12.32	100%	n.s.
Deoxynivalenol	75.23 ± 50.15	54.71	37.77–121.71	100%	82.81 ± 138.3	36.05	13.96–60.73	100%	n.s.
Fumonisin B_1	263.88 ± 351.65	174.91	8.99–377.9	100%	204.90 ± 204.42	150.51	39.14–323.31	100%	n.s.
Fumonisin B_2	390.51 ± 858.04	138.33	10.81–273.34	100%	256.12 ± 392.88	181.2	32.11–304.6	100%	n.s.
Sum Fumonisins	654.3 ± 1182.32	313.2	21.49–651.2	100%	461.0 ± 580.5	336.2	71.25–665.7	100%	n.s.

Table 2
Comparative analysis of mycotoxin contamination of supermarket and premium brand pelleted cat food in Spain. Results are expressed in ng/g feed.

	Supermarket brands (n = 32)				Premium brands (n = 30)				
Mycotoxin	Mean ± SD	Median	P25-P75	Frequency	Mean ± SD	Median	Range	Frequency	*p*
Aflatoxin B_1	0.14 ± 0.12	0.08	0.04–0.24	100%	0.17 ± 0.13	0.16	0.11–0.21	100%	n.s.
Aflatoxin B_2	0.05 ± 0.06	0.04	0.00–0.09	56.2%	0.06 ± 0.07	0.06	0.00–0.10	62.5.1%	n.s.
Aflatoxin G_1	0.04 ± 0.05	0.03	0.00–0.04	68.8%	0.04 ± 0.03	0.03	0.02–0.05	86.7%	n.s.
Aflatoxin G_2	–	–	–	0.0%	–	–	–	0%	–
Sum Aflatoxins	0.22 ± 0.67	0.15	0.05–0.36	100%	0.28 ± 0.21	0.22	0.13–0.37	100%	n.s.
Ochratoxin A	0.57 ± 0.66	0.33	0.16–0.79	87.5%	0.19 ± 0.13	0.17	0.13–0.29	80.0%	n.s.
HT2	1.19 ± 0.49	1.31	0.66–1.51	100%	1.72 ± 1.08	1.55	0.79–2.61	100%	n.s.
T2	1.92 ± 1.33	1.76	0.60–3.23	93.4%	3.72 ± 2.54	3.97	0.66–5.74	93.3%	0.0329
Sum HT2 + T2	3.11 ± 1.77	2.98	1.24–4.73	100%	5.44 ± 3.58	5.71	1.45–7.98	100%	0.053
Zearalenone	21.72 ± 38.30	12.77	0.00–23.08	62.5%	32.67 ± 30.40	15.24	11.53–44.78	93.3%	n.s.
Deoxynivalenol	89.43 ± 58.46	71.73	40.53–135.92	100%	113.11 ± 93.66	98.12	31.86–192.12	100%	n.s.
Fumonisin B_1	417.92 ± 443.63	283.71	34.04–750.79	100%	699.72 ± 602.33	692.3	94.12–937.3	100%	n.s.
Fumonisin B_2	609.61 ± 801.84	214.35	28.77–1058.24	100%	1201.22 ± 958.68	1213.12	164.12–1943.22	100%	0.0317
Sum Fumonisins	1028.31 ± 1200.43	498.05	58.58–1775.23	100%	1901.43 ± 1547.86	1906.12	244.22–2721.34	100%	n.s.

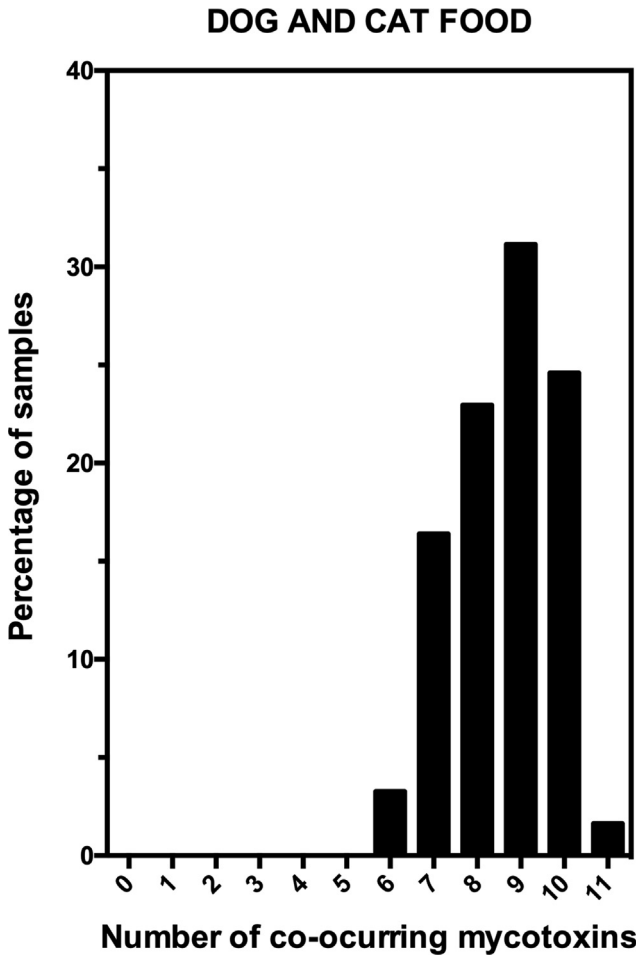

Fig. 1. Percentage of samples co-contaminated with different mycotoxins.

ian of 248.3 ng/g was reported. However, in this case, the authors studied the feed samples in the context of an aflatoxicosis outbreak previously described, which produced signs of acute intoxication and mortality of at least 220 dogs (Arnot et al., 2012). We also report very low concentrations of OTA, similar to those reported in Poland (Blajet-Kosicka et al., 2014) and South Africa (Singh et al., 2017), and very far from the relatively high values that have been published in Italy (Gazzotti et al., 2015), as well as in the same outbreak of intoxication in dogs previously mentioned, in which high levels of other mycotoxins were also detected (Mulunda et al., 2013). However, to date, very few studies have reported OTA levels that surpass the recommended threshold of 50 ng/g (EC, 2006). Something similar happened with the rest of the mycotoxins studied, with our concentrations being among the lowest reported (Bissoqui et al., 2016; Blajet-Kosicka et al., 2014; Bohm et al., 2010; Frehse et al., 2015; Gazzotti et al., 2015; Singh et al., 2017; Singh and Chuturgoon, 2017), except in the case of fumonisins, as mentioned above. In this case, the exact opposite occurs, and only the concentrations of fumonisins found in the mycotoxicosis outbreak in South Africa (Mulunda et al., 2013) exceeded those we have found in this series, particularly in cat feed. That is, we not only detect them in 100% of dog and cat feed, but the concentrations are much higher than those reported by other research groups. Finally, with respect to the concentrations of T2 and HT2, as far as we know only Blajet-Kosicka et al. (2014) have studied them in feed for dogs and cats and the median value they report is practically identical to that of this study (3 ng/g vs. 2.25 ng/g, respectively).

Finally, another result of our study that attracts attention is the lack of differences in the level of mycotoxin contamination according to the supposed quality of the feed. In the absence of another more reliable criterion, we have divided the series of feed brands for each species into two groups according to the median price per kilo (€ 2.86/kg for dog feed and € 4.14/kg for feed for cats). Thus, we are assuming that the highest priced feed are those brands of higher quality. However, neither the detection frequency, nor the co-occurrence of mycotoxins, or the concentrations were significantly different in most cases (Tables 1 and 2). In fact, only OTA levels were significantly higher in economic feed for dogs than in the most expensive ones (Table 1). Moreover, the rest of the significant differences we found – T2 and HT2 toxins, and FB1 in cat feed (Table 2) – gave the opposite result than expected and were higher in the higher-priced feed. The result of FB1 was especially striking, as it presented a median value 6 times higher in the more expensive brands than that of economic feed. This is not a very surprising result since other authors had reported that there was not much difference in the level of mycotoxin contamination between what they called "premium feed" and "supermarket feed" (Frehse et al., 2015; Gazzotti et al., 2015; Singh et al., 2017; Singh and Chuturgoon, 2017). However, to date, no study had reported that the levels of certain mycotoxins were significantly higher in the feed of higher presumed quality.

3.2. Dietary intake and risk assessment

Based in the previous results, we have calculated also the daily exposure of pet dogs and cats and made an approach to the evalu-

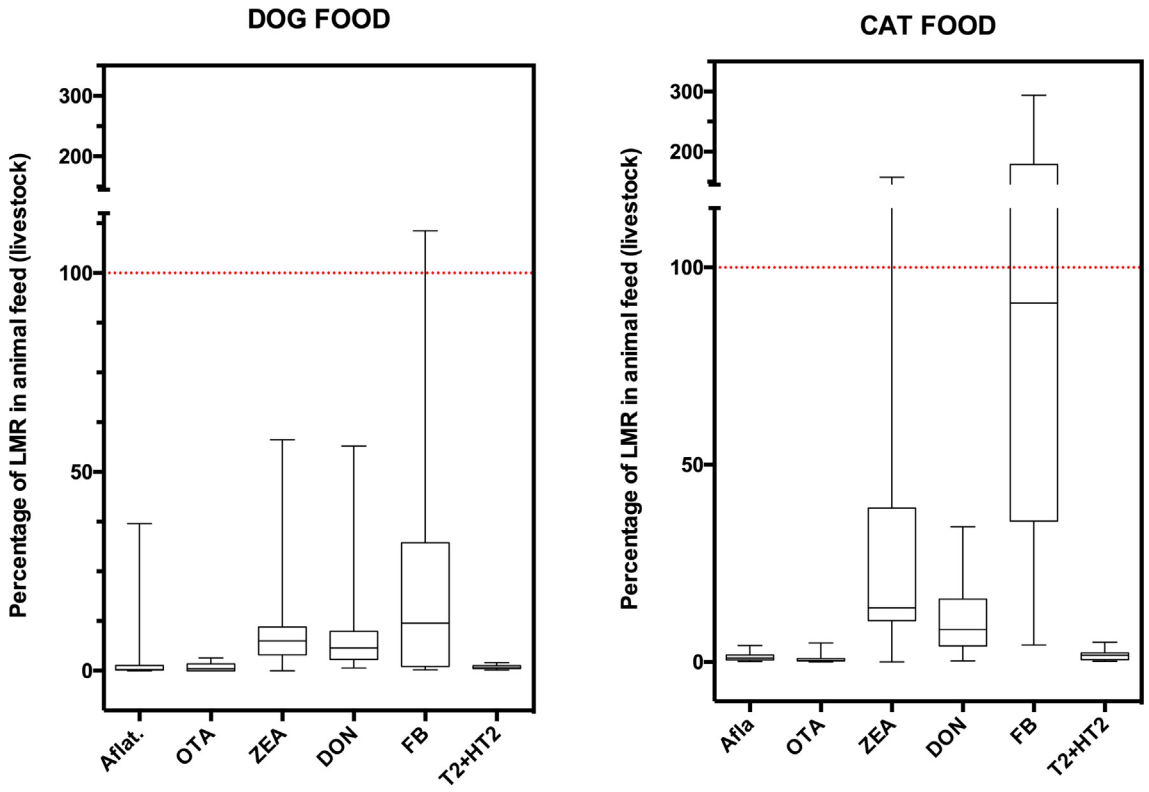

Fig. 2. Box and whiskers graphs showing the distribution of the mycotoxin contents expressed as a percentage of its corresponding maximum recommended values (MRV) (EC, 2006) in dog (left panel) and cat (right panel) feed samples. The lines show the medians, the boxes cover the 25th to 75th percentiles, and the minimal and maximal values are shown by the ends of the bars.

Table 3
Comparative analysis of mycotoxin contamination between pelleted dog and cat food in Spain. Results are expressed in ng/g feed.

	Dog food (n = 60)				Cat food (n = 62)				
Mycotoxin	Mean ± SD	Median	P25-P75	Frequency	Mean ± SD	Median	Range	Frequency	*p*
Aflatoxin B_1	0.23 ± 0.74	0.05	0.02–0.18	93.3%	0.16 ± 0.12	0.12	0.07–0.21	100%	0.0453
Aflatoxin B_2	0.09 ± 0.38	0.00	0.00–0.06	26.6%	0.06 ± 0.06	0.05	0.00–0.09	58.1%	0.0255
Aflatoxin G_1	0.04 ± 0.37	0.01	0.00–0.03	53.3%	0.04 ± 0.04	0.03	0.01–0.05	77.4%	0.0141
Aflatoxin G_2	0.02 ± 0.08	0.00	0.00–0.00	3.3%	–	–	–	0%	–
Sum Aflatoxins	0.38 ± 1.33	0.06	0.03–0.26	96.6%	0.25 ± 0.20	0.19	0.09–0.37	100%	0.0173
Ochratoxin A	0.44 ± 0.45	0.26	0.00–0.83	73.3%	0.39 ± 0.51	0.20	0.16–0.42	83.9%	n.s.
HT2	0.95 ± 0.46	0.84	0.62–1.25	100%	1.44 ± 0.86	1.41	0.79–1.72	96.6%	0.0136
T2	126 ± 0.78	1.12	0.73–1.87	96.6%	2.79 ± 2.18	2.98	0.66–3.97	96.6%	0.0086
Sum HT2 + T2	2.21 ± 1.21	1.88	1.36–3.12	100%	4.24 ± 2.99	4.37	1.45–5.74	100%	0.0071
Zearalenone	10.31 ± 11.15	7.97	4.53–11.70	86.7%	27.02 ± 34.58	13.71	10.45–39.01	77.4%	0.0095
Deoxynivalenol	78.52 ± 96.51	51.54	26.58–88.82	100%	100.92 ± 77.12	74.03	36.43–143.72	100%	n.s.
Fumonisin B_1	238.32 ± 293.91	162.73	11.49–344.21	100%	554.43 ± 536.78	514.73	51.08–901.62	100%	0.0089
Fumonisin B_2	332.21 ± 688.92	153.91	13.34–286.91	100%	895.83 ± 917.04	737.91	69.85–1443.12	100%	0.0068
Sum Fumonisins	570.55 ± 959.12	324.71	24.59–665.71	100%	1450.32 ± 1426.12	1189.97	121.93–2483.34	100%	0.0056

ation of acute and chronic health risk posed by these feeds. In the following lines we detail the result of this analysis for each mycotoxin.

3.2.1. Aflatoxins

It is well known that aflatoxins may be very acutely toxic for animals, and as we said above the dog seems to be a particularly sensitive species since they metabolize this mycotoxin inefficiently (Singh and Chuturgoon, 2017). In fact, several reports of poisoning outbreaks can be found in the scientific literature, with the involvement and even death of hundreds of dogs (Arnot et al., 2012; Bruchim et al., 2012; Newman et al., 2007; Stenske et al., 2006; Wouters et al., 2013). However, for aflatoxicosis to occur, considerably high levels in feed must be given (>1–2 mg/kg), and as we have pointed out, the levels we report in this study are not too high. Even so, we have carried out the characterization of the potential risk of acute poisoning, and as expected the maximum aHI we obtained is much lower than 1 (and even <0.0005), indicating that these feedstuffs do not have the potential for acute toxicity (Table 4).

However, as it also occurs with other mycotoxins, the cases of extreme contamination leading to acute toxicosis are rare, and the greatest concern in food safety of aflatoxins is continued dietary exposure (EFSA, 2013a), since even the continued exposure to low doses of certain mycotoxins might lead to severe adverse health effects, such as cancer. The mycotoxins most clearly associated with this disease are aflatoxins, which in fact have been classified as group 1 carcinogens, by their known role in the

Table 4
Estimations of aflatoxin intake of dogs and cats through the consumption of pelleted food sold in Spain. Results are expressed in ng/kg b.w./day.

Mycotoxin	Acute toxicity reference dose [a]	$BMDL_{10}$ [b]	DOGS				CATS				P [g]
			Estimated short-term intake (ESTI) [c]	Acute Hazard Index [d]	Estimated Daily Intake (EDI) [e]	MoE [f]	Estimated short-term intake (ESTI) [c]	Acute Hazard Index [d]	Estimated Daily Intake (EDI) [e]	MoE [f]	
Aflatoxin B_1	500,000	170	116,4	0,0002328	1,18	143,5	9,76	0,00001952	1,74	97,7	n.s.
Aflatoxin B_2			58,76	0,00011752	0	–	3,86	0,00000772	0,81	209,9	n.s.
Aflatoxin G_1			20,31	0,00004062	0,32	523,1	2,17	0,00000434	0,43	395,3	n.s.
Aflatoxin G_2			13,18	0,00002636	0	–	0	0	0	–	n.s.
Sum Aflatoxins			208,7	0,0004174	1,60	105,9	14,86	0,00002972	2,95	57,6	0.043

[a] Because aflatoxins are genotoxic/mutagenic compounds, no aRfD or LOAEL has been defined for them. We have employed the lowest oral acute toxic dose that has been published to date for dogs and cats (Newberne and Butler, 1969).
[b] The benchmark dose level (BMDL) is a dose or concentration that produces a predetermined change in the response rate of an adverse effect. This predetermined change in response is called the benchmark response (BMR), and in the case of aflatoxins it usually refers to liver cancer. The default BMR for the calculation of the risk of exposure to aflatoxins is 10% (meaning the dose of aflatoxins that would cause a 10% increase in the incidence of liver cancer relative to the response of control group).
[c] The estimated Short-term Intake (ESTI) may be used to estimate exposure based on the highest reported 97.5th percentile intake during a single day by a given consumer.
[d] The acute hazard indez (aHI) indicates the potential non-cancer health impacts resulting from a single exposure to toxic substances. Is the ratio between the ESTI and the acute toxicity reference dose, and a value >1 would indicate that a significant adverse event might occur. This number is dimensionless.
[e] The EDI—estimated daily intake is calculated taking into account the food-consumption data (grams/kg b.w./day) and the residue level in the commodity, usually employing the median values (ng/g).
[f] The MoE—Margin of Exposure—is the ratio of no-observed-adverse-effect level (NOAEL) obtained from animal toxicology studies to the predicted exposure level or dose in a given species. For genotoxic and carcinogenic compounds, since NOAEL values cannot be identified the $BMDL_{10}$ is. In general, for a genotoxic/mutagenic compound a MoE $\geq$ 10.000 is considered to be protective, and figures below 10.000 should be considered with caution. MoE is dimensionless.
[g] the statistical comparison was performed between the EDIs of dogs and cat, this is, referred to chronic exposure.

development of cancer in humans, mainly liver cancer (IARC, 2002). In dogs, a recent study established an association between feed contamination with low levels of AFB_1, AFG_1, and AFG_2 and the development of breast cancer (Frehse et al., 2015). For this reason, as recommended by the EFSA, we calculated the MoE associated with the current level of intake of aflatoxins through the consumption of pelleted dry food for both, dogs and cats (Table 4), and we found that the calculated values are much lower than the threshold considered to be protective (MoE > 10,000). In fact, a MoE value as low as 58 was found for the sum of aflatoxins in cats, with exposure to aflatoxins in this species being significantly higher than that of dogs (Table 4). Since the value of MoE indicated that there could exist risk, we employed the cancer potency factor established for aflatoxins for humans (non-exposed to the hepatitis B virus), which is 0.01 cancers per year per 10^5 population per nanogram of $\sum$AFs per kg b.w. per day (JECFA, 1998) to further characterize this risk in dogs and cats. Thus, when we applied Eq. (6) for cancer risk calculation, we obtained values of 0.21 for dogs and 0.39 for cats (Fig. 3). Therefore, our estimates indicate that a very low risk of aflatoxin-induced cancer is associated with the lifetime consumption of dry food in dogs and cats. This is probably due to the short life span of these species, since a similar level of dietary exposure in humans would give a fairly significant risk.

3.2.2. Ochratoxin A

The disease caused by OTA exposure is known as ochratoxicosis, and the primary target is the kidney. Pigs, dogs and poultry seem to be particularly sensitive to the nephrotoxicity (EFSA, 2004). The presence of OTA in animal feed contributes significantly to health disorders, that range from acute renal failure and death (very rare) to decreased production in livestock (Denli and Perez, 2010). As far as we know, there are no reports of ochratoxicosis outbreaks in dogs, and only the symptoms of intoxication after experimental exposure have been described, although the doses required for this were very high (0.2–3 mg/kg b.w.) (Szczech et al., 1973). These doses are far from the maximum levels of exposure that we have calculated in this study, which are at least 3500 times lower (Table 5). Therefore, the risk of acute poisoning that we have calculated is negligible (aHI = 0.004 in dogs and 0.007 in cats, Fig. 4).

In some previous studies, the possible relationship between the long-term exposure to OTA in dog and cat food and long-term adverse effects at the renal level has been evaluated by some

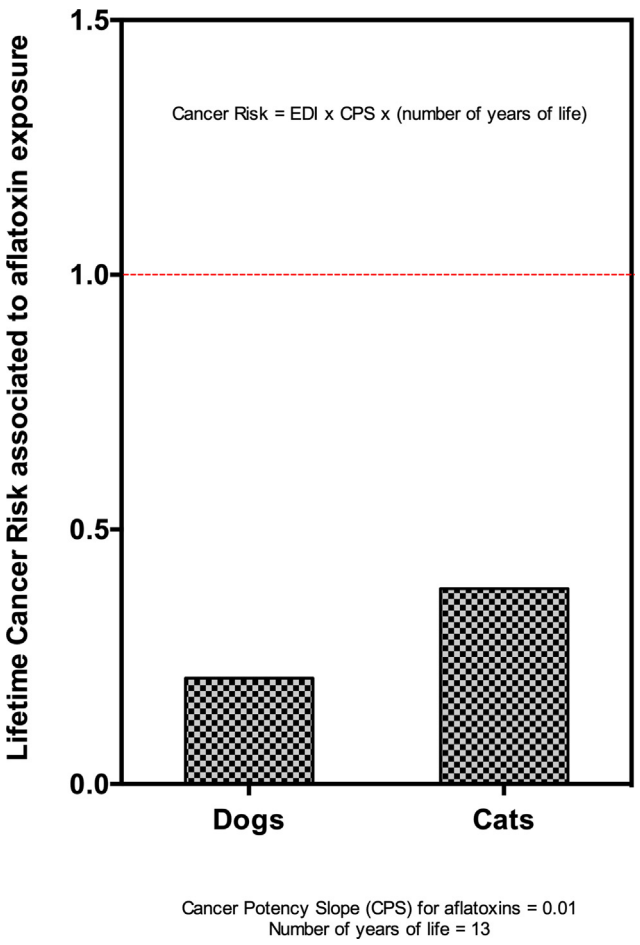

Fig. 3. Lifetime cancer risk derived from the exposure to aflatoxins through the consumption of cereal-based pelleted dry food for dogs and cats.

Table 5
Estimations of the mycotoxin intake of dogs and cats through the consumption of pelleted food sold in Spain. Results are expressed in ng/kg b.w./day.

			DOGS		CATS		
Mycotoxin	LOAEL[a]	PMTDI[b]	ESTI[c]	EDI[d]	ESTI[c]	EDI[d]	*P*[e]
Ochratoxin A	8000	15	31,37	7,01	53,15	3,36	n.s.
HT2	29,000	100	55,34	17,49	60,62	20,8	n.s.
T2			110,31	20,91	119,40	44,51	n.s.
Sum HT2 + T2			165,64	38,4	180,02	65,31	0.0402
Zearalenone	56,000	200	818,13	229	5711,32	266,7	n.s.
Deoxynivalenol	400,000	1000	11698,23	955,6	4854,12	1321	n.s.
Fumonisin B_1	200,000	2000	33878,82	3107	35038,11	9203	0.0363
Fumonisin B_2			98682,12	3032	102930,23	12,241	0.0168
Sum Fumonisins			132560,11	6636	137968,45	23,179	0.0190

[a] As reference value for acute toxicity we have considered de Low Observed Adverse Effect Level (LOAEL)
[b] PMTDI (provisional maximum tolerable daily intake) is the threshold set for contaminants with no cumulative properties. Its value represents permissible human as a result of the natural occurrence of the substance in food and in drinking-water.
[c] The estimated Short-term Intake (ESTI) may be used to estimate exposure based on the highest reported 97.5th percentile intake during a single day by a given consumer.
[d] The EDI—estimated daily intake—is calculated taking into account the food-consumption data (grams/kg b.w./day) and the residue level in the commodity, usually employing the median values of a wide series of measurements (ng/g).
[e] The statistical comparison was performed between the EDIs of dogs and cat, this is, referred to chronic exposure.

authors (Meucci et al., 2017; Razzazi-Fazeli et al., 2001). Although, as reported in this study, other authors indicated that OTA is frequently found in the feed for cats and dogs (Abd-Elhakim et al., 2016; Blajet-Kosicka et al., 2014; Gazzotti et al., 2015; Razzazi-Fazeli et al., 2001; Singh et al., 2017; Singh and Chuturgoon, 2017), and that some authors were also able to detect this mycotoxin in the kidneys or serum of animals (Meucci et al., 2017; Razzazi-Fazeli et al., 2001), no relation between pathological findings and OTA levels could be assessed. In our study, taking as a reference the value of TDI proposed for OTA (EFSA, 2004), the risks associated with long-term exposure to concentrations of this mycotoxin in feed for dogs and cats do not seem to be of concern, since the calculated risk ratio is lower than 1 (RQ = 0.4 and 0.2, for dogs and cats respectively, Fig. 4B), even in the high percentile of consumption (data not shown).

3.2.3. Trichothecenes

Trichothecenes are a broad family of chemically related mycotoxins, mainly produced by *Fusarium* species. In this study we have included the 3 trichothecenes that are regulated by the EFSA (T2, HT2 and DON), since they frequently appear as contaminants in animal feed (EFSA, 2011a, 2017b). In fact, our results confirm this, as they appear in almost 100% of the samples analyzed. This coincides with that reported in the few studies that have quantified the DON (Blajet-Kosicka et al., 2014; Bohm et al., 2010; Gazzotti et al., 2015; Martins et al., 2003), and in the only one that has done so

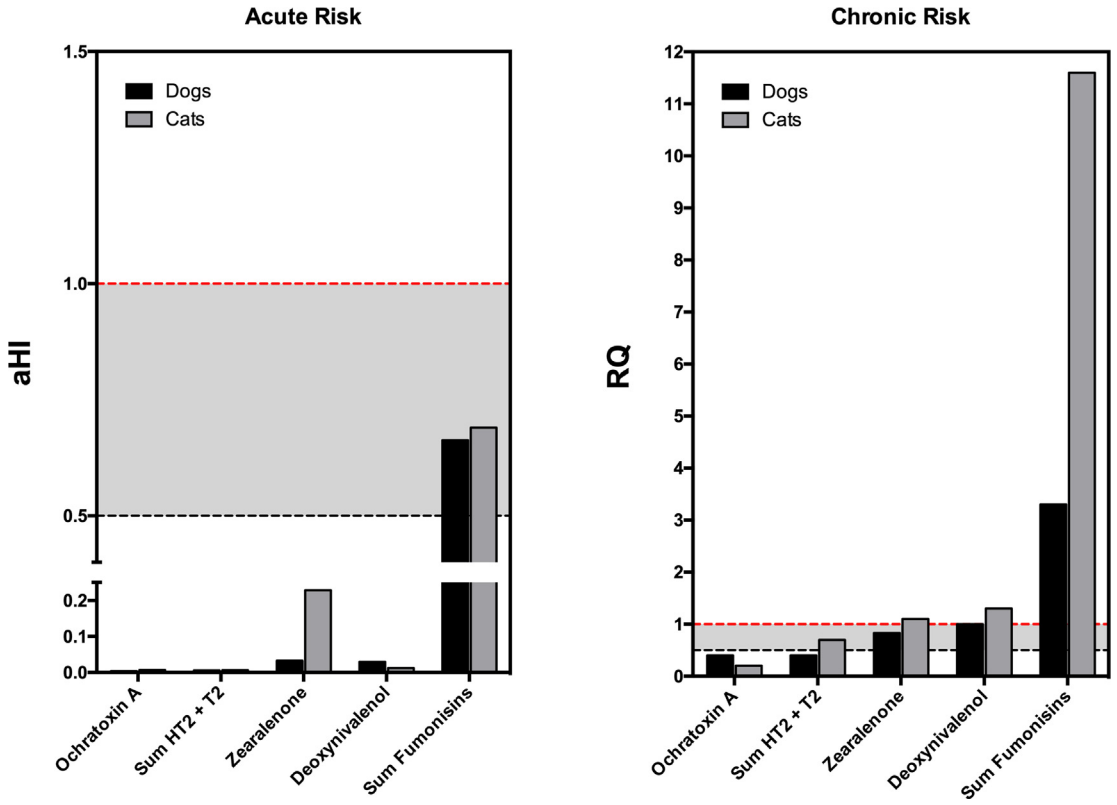

Fig. 4. Hazard ratios of the contaminants for acutely toxic effects (A) and potential toxic effects after long-term exposure (B) in dogs and cats via consumption of pelleted dry feed. The red line indicates the threshold for toxic effect (aHI or RQ = 1).

with T2 and HT2 (Blajet-Kosicka et al., 2014). All these toxins are toxic at the subcellular, cellular and organic levels, but cases of acute poisoning are rare, since their bad taste produces food aversion and rejection, at relatively low concentrations (>5 ppm) (Osweiler, 2019). Even ignoring this fact, which would be a protective factor for acute intoxication, according to the calculations we have made with the levels of consumption of trichothecenes, even in the most contaminated sample, the risk of acute poisoning in dogs and cats is negligible (aHIs < 0.03, Fig. 4A). This was expected since these levels of exposure (Table 5) are very far from the NOAELs/LOAELs for acute effects in dogs and cats, when these have been established (EFSA, 2017b).

In regard to chronic exposure, the levels we calculated from the median values in feed cannot be considered too low, especially with regard to DON (Fig. 4B). However, our calculations (Table 5) are lower than those recently calculated by the EFSA (ref 2017). The CONTAM Panel of EFSA has not reported a TDI for dogs and cats, but in their reports they indicate that dogs and especially cats might be especially sensitive to the adverse effects of some trichothecenes (EFSA, 2011a). For this reason, we have employed the generic TDI calculated for humans, and our calculations would indicate that there is a moderate risk of long-term adverse effects (Fig. 4B) since at least in the case of cat feed the RQ calculated for the DON is 1.3 in the 50th percentile of consumption. In the high percentile of consumption, all the trichothecenes in both species would surpass the no-risk threshold (RQ = 1, data not shown).

3.2.4. Zearalenone

Like trichothecenes, the ZEA is produced by *Fusarium* species, and dogs are considered a particularly sensitive species to this estrogenic mycotoxin (EFSA, 2017a). Based on experimental studies in this species, the EFSA CONTAM Panel has established a LOAEL of 25 μg/kg b.w. (adult bitches), a dose that would produce lesions in the myometrium and endometrium. No data could be identified concerning the effects of ZEN in cats (EFSA, 2017a). Considering the LOAEL for dogs and the concentrations of the most contaminated feeds, and although the levels in cat feeds are up to 10 times higher than those in dogs, our calculations indicate that the risk of acute poisoning would be very low for both species (aHIs = 0.02 and 0.22 for dogs and cats respectively, Fig. 4A).

However, the risks associated with long-term exposure, calculated considering the median value of feed (Table 5) and the PMTDI value established for ZEA, do not seem so low. Thus, in the 50th percentile of consumption, we calculated RQ values >1 in both species (Fig. 4B), which indicates that a certain degree of risk might exist. It should be borne in mind that the ZEA is a recognized endocrine disruptor, and that it has recently pointed to the possibility that it can act at low doses as a promoter of breast cancer and other hormone-dependent tumors, either alone or in conjunction with other disruptors endocrine (Kowalska et al., 2018; Yip et al., 2017). Other long-term effects, such as eryptosis (erythrocyte rupture due to oxidative stress) or reproductive alterations, have also been related to exposure to low doses of ZEA (Gajecka et al., 2015) as well. Therefore, we cannot rule out that the levels of ZEA found in our study, might cause long-term adverse effects in animals (particularly in females), especially considering that the presence in feed of other endocrine disruptors with estrogenic potential has been reported, and that these could act synergistically with ZEA (Ruiz-Suarez et al., 2015).

3.2.5. Fumonisins

Finally, the exposure to the mycotoxins that we detected at higher concentrations, fumonisins, which are also produced by *Fusarium* species, is particularly worrying. The presence of these mycotoxins in animal feed is associated with toxic effects such as interference with cell membrane metabolism, inhibition of sphingolipid metabolism and lung, liver and immunological lesions, depending on the exposure scenario. In our series, some pet feeds showed quite high levels of the sum of fumonisins, which led the dogs and cats that consumed these brands to be exposed to concentrations as high as 138 μg/kg b.w. (Table 5). These amounts are below the LOAEL established for these mycotoxins (200 μg/kg), although this data has been established for other species of monogastric animals since no reference points for dogs or cats have been identified (EFSA, 2018b). Although the calculated aHI remains below 1 (Fig. 4A), it is also important to note that the EFSA CONTAM Panel recommends multiplying the exposure data by a factor of 1.6 to take into account the hidden (unquantified) forms of fumonisins. This is because Fumonisins B_3, B_4 or other hidden fumonisins of the B series, are not usually quantified, and it is considered that these have the same mode of action and a similar toxicological profile and potency (EFSA, 2018b). If we considered this multiplying factor, some food brands would contain such high levels of fumonisins that they might cause adverse effects after a single meal.

The average exposure levels (using the median values) are much lower than the extreme values found in some brands, but they can still be considered worryingly high, particularly in the case of cats, which are exposed 4 times more than dogs to this group of mycotoxins (Table 5). For animals, a TDI value has not been established, but in its report on the risks of fumonisins in food, the EFSA CONTAM Panel mentions as a reference the value of 2 μg/kg b.w. for the sum of fumonisins established for humans, which is based on a higher incidence of megalocyte hepatocytes found in a chronic study with mice (EFSA, 2018b). Taking this reference, our results indicate that dogs and cats may be far exceeding this tolerability threshold (Fig. 4B), independently whether they are fed with premium or low-cost feed. It is very difficult to assess the real toxicological significance of this apparently high exposure, since there are no data on adverse effects of chronic exposure to fumonisins in cats and dogs (EFSA, 2018b). However, in pigs and other monogastric species at these exposure levels alterations in the metabolism of sphingolipids measurable in serum and urine and alteration of some biochemical parameters have been reported after only 8 weeks of exposure (ALP, ALT and AST activities, cholesterol, GGT, GOT) (EFSA, 2018b). Therefore, it cannot be ruled out that something similar could also occur in dogs and cats after exposure to these levels for months or even years.

In any case, the results of this study should be taken into consideration, especially because the possibility exists that the presence of several mycotoxins can simultaneously produce potent toxic effects as a result of their synergistic action (Alassane-Kpembi et al., 2017; Streit et al., 2012; Yip et al., 2017), and in addition aggregated exposure, i.e. from molds present in the indoor environment, could exist. We consider that further research on the potential adverse health effects deriving from chronic exposure to low doses of the multi-mycotoxin mixture that pet species are subject to today is needed, especially considering that most of these animals are feed a mono-diet of commercial brands, usually the same brand for months or years. Especially noteworthy is the fact that we did not find differences in the level of mycotoxin contamination between presumed feed qualities, which indicates that it is also necessary to emphasize the control of production processes, and probably also that established clear criteria for the classification of their commercial quality are needed. The lack of data for the evaluation of the risk of pets, and non-productive animals, in general, is striking, so we consider that this is a field of research that should be given more attention in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134592.

References

Abd-Elhakim, Y.M., El Sharkawy, N.I., Moustafa, G.G., 2016. An investigation of selected chemical contaminants in commercial pet foods in Egypt. J. Vet. Diagn. Invest. 28, 70–75.

Alassane-Kpembi, I., Schatzmayr, G., Taranu, I., Marin, D., Puel, O., Oswald, I.P., 2017. Mycotoxins co-contamination: methodological aspects and biological relevance of combined toxicity studies. Crit. Rev. Food Sci. Nutr. 57, 3489–3507.

Anders, M., 2013. The Determinants of Pet Owners' Feed Choice. Wageningen University, Germany.

Arnot, L.F., Duncan, N.M., Coetzer, H., Botha, C.J., 2012. An outbreak of canine aflatoxicosis in Gauteng Province, South Africa. J. S. Afr. Vet. Assoc. 83, 2.

Benford, D., Leblanc, J.C., Setzer, R.W., 2010. Application of the margin of exposure (MoE) approach to substances in food that are genotoxic and carcinogenic: example: aflatoxin B1 (AFB1). Food Chem. Toxicol. 48 (Suppl. 1), S34–S41.

Bissoqui, L.Y., Frehse, M.S., Freire, R.L., Ono, M.A., Bordini, J.G., Hirozawa, M.T., de Oliveira, A.J., Ono, E.Y., 2016. Exposure assessment of dogs to mycotoxins through consumption of dry feed. J. Sci. Food Agric. 96, 4135–4142.

Blajet-Kosicka, A., Kosicki, R., Twaruzek, M., Grajewski, J., 2014. Determination of moulds and mycotoxins in dry dog and cat food using liquid chromatography with mass spectrometry and fluorescence detection. Food Addit. Contam. Part B Surveill. 7, 302–308.

Boermans, H.J., Leung, M.C., 2007. Mycotoxins and the pet food industry: toxicological evidence and risk assessment. Int. J. Food Microbiol. 119, 95–102.

Bohm, J., Koinig, L., Razzazi-Fazeli, E., Blajet-Kosicka, A., Twaruzek, M., Grajewski, J., Lang, C., 2010. Survey and risk assessment of the mycotoxins deoxynivalenol, zearalenone, fumonisins, ochratoxin A, and aflatoxins in commercial dry dog food. Mycotoxin Res. 26, 147–153.

Bruchim, Y., Segev, G., Sela, U., Bdolah-Abram, T., Salomon, A., Aroch, I., 2012. Accidental fatal aflatoxicosis due to contaminated commercial diet in 50 dogs. Res. Vet. Sci. 93, 279–287.

Denli, M., Perez, J.F., 2010. Ochratoxins in feed, a risk for animal and human health: control strategies. Toxins (Basel) 2, 1065–1077.

EC, 2006. Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Off. J. Eur. Union 229, 7–9.

EC, 2009. Regulation (EC) No 767/2009 of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC. Off. J. Eur. Union, 1–36.

EFSA, 2004. Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to ochratoxin A (OTA) as undesirable substance in animal feed. EFSA J. 101, 1–36.

EFSA, 2006. Opinion of the scientific panel on contaminants in the food chain on a request from the Commission related to ochratoxin A in food. EFSA J. 365, 1–56.

EFSA, 2011a. Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. EFSA J. 9, 1–185.

EFSA, 2011b. Scientific Opinion on the risks for public health related to the presence of zearalenone in food. EFSA J. 9, 1–123.

EFSA, 2013a. Aflatoxins (sum of B1, B2, G1, G2) in cereals and cereal-derived food products. EFSA Supporting Publications EN-406, pp. 1–11.

EFSA, 2013b. Deoxynivalenol in food and feed: occurrence and exposure. EFSA J. 11, 1–56.

EFSA, 2014. Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. EFSA J. 12, 1–107.

EFSA, 2017a. Risks for animal health related to the presence of zearalenone and its modified forms in feed. EFSA J. 15, 4851–4974.

EFSA, 2017b. Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. EFSA J. 15, 4718–5063.

EFSA, 2018a. Effect on public health of a possible increase of the maximum level for 'aflatoxin total' from 4 to 10 μg/kg in peanuts and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs. EFSA J. 16.

EFSA, 2018b. Risks for animal health related to the presence of fumonisins, their modified forms and hidden forms in feed. EFSA J. 16, 5242–5386.

FEDIAF, 2018a. Code of Good Practice for labeling of Pet Food. The Pet Food Industry, pp. 1–80.

FEDIAF, 2018b. European Facts & Figures 2018. The European Pet Food Industry, Brussels, Belgium.

FEDIAF, 2018c. Guide to Good Practice for the Manufacture of Safe Pet Foods. The European Pet Food Industry, pp. 3–72.

Frehse, M.S., Martins, M.I., Ono, E.Y., Bracarense, A.P., Bissoqui, L.Y., Teixeira, E.M., Santos, N.J., Freire, R.L., 2015. Aflatoxins ingestion and canine mammary tumors: There is an association? Food Chem. Toxicol. 84, 74–78.

Gajecka, M., Zielonka, L., Gajecki, M., 2015. The effect of low monotonic doses of zearalenone on selected reproductive tissues in pre-pubertal female dogs–a review. Molecules 20, 20669–20687.

Gazzotti, T., Biagi, G., Pagliuca, G., Pinna, C., Scardilli, M., Grandi, M., Zaghini, G., 2015. Occurrence of mycotoxins in extruded commercial dog food. Anim. Feed Sci. Technol. 202, 81–89.

IARC, 2002. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Aflatoxins. World Health Organization, Lyon. 1-130. Available at.

JECFA, 1998. Joint FAO/WHO Expert Committee on Food Additives. Safety Evaluation of Certain Food Additives and Contaminants. WHO Food additives series 40. World Health Organization.

Kaushik, G., 2015. Effect of processing on mycotoxin content in grains. Crit. Rev. Food Sci. Nutr. 55, 1672–1683.

Kempe, R., Saastamoinen, M., Hyyppä, S., 2004. Composition, digestibility and nutritive value ofcereals for dogs. Agric. Food Sci. 13, 5–17.

Kowalska, K., Habrowska-Gorczynska, D.E., Urbanek, K.A., Dominska, K., Piastowska-Ciesielska, A.W., 2018. Estrogen receptor alpha is crucial in zearalenone-induced invasion and migration of prostate cancer cells. Toxins (Basel) 10.

Leung, M.C., Diaz-Llano, G., Smith, T.K., 2006. Mycotoxins in pet food: a review on worldwide prevalence and preventative strategies. J. Agric. Food Chem. 54, 9623–9635.

Luzardo, O.P., Bernal-Suarez, M.D., Camacho, M., Henriquez-Hernandez, L.A., Boada, L.D., Rial-Berriel, C., Almeida-Gonzalez, M., Zumbado, M., Diaz-Diaz, R., 2016. Estimated exposure to EU regulated mycotoxins and risk characterization of aflatoxin-induced hepatic toxicity through the consumption of the toasted cereal flour called "gofio", a traditional food of the Canary Islands (Spain). Food Chem. Toxicol. 93, 73–81.

Maia, P.P., Bastos, Pereira, de Siqueira, M.E., 2002. Occurrence of aflatoxins B1, B2, G1 and G2 in some Brazilian pet foods. Food Addit. Contam. 19, 1180–1183.

MAPAMA, 2018. Datos de producción de piensos 2017. Spanish Ministry of Agriculture, Fisheries, and Food, Madrid, pp. 1–43.

Marroquin-Cardona, A.G., Johnson, N.M., Phillips, T.D., Hayes, A.W., 2014. Mycotoxins in a changing global environment–a review. Food Chem. Toxicol. 69, 220–230.

Martins, M.L., Martins, M.H., Bernardo, F., 2003. Fungal flora and mycotoxins detection in commercial pet food Revista Portuguesa de Ciencias Veterinarias 98, pp. 179–183.

Meucci, V., Luci, G., Vanni, M., Guidi, G., Perondi, F., Intorre, L., 2017. Serum levels of ochratoxin A in dogs with chronic kidney disease (CKD): a retrospective study. J. Vet. Med. Sci. 79, 440–447.

Mulunda, M., Ndou, R.V., Dzoma, B., Nyirenda, M., Bakunzi, F., 2013. Canine aflatoxicosis outbreak in South Africa (2011): a possible multi-mycotoxins aetiology. J. S. Afr. Vet. Assoc. 84, E1–E5.

Newberne, P.M., Butler, W.H., 1969. Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: a review. Cancer Res. 29, 236–250.

Newman, S.J., Smith, J.R., Stenske, K.A., Newman, L.B., Dunlap, J.R., Imerman, P.M., Kirk, C.A., 2007. Aflatoxicosis in nine dogs after exposure to contaminated commercial dog food. J. Vet. Diagn. Invest. 19, 168–175.

Osweiler, G., 2019. Trichothecene Toxicosis. In: S. Line (Ed.), MSD Veterinary Manual. Merck Sharp & Dohme Corp., Kenilworth, NJ, USA.

Pinotti, L., Ottoboni, M., Giromini, C., Dell'Orto, V., Cheli, F., 2016. Mycotoxin contamination in the EU feed supply chain: a focus on cereal byproducts. Toxins (Basel) 8, 45.

Poundstone, W., 2011. Priceless: The Myth of Fair Value (and How to Take Advantage of It). Hill and Wang, New York. Available at.

Razzazi-Fazeli, E., Bohm, J., Grajewski, J., Szczepaniak, K., Kubber-Heiss, A.J., Iben, C. H., 2001. Residues of ochratoxin A in pet foods, canine and feline kidneys. J. Anim. Physiol. A. Anim. Nutr. 85, 212–216.

Ruiz-Suarez, N., Camacho, M., Boada, L.D., Henriquez-Hernandez, L.A., Rial, C., Valeron, P.F., Zumbado, M., Gonzalez, M.A., Luzardo, O.P., 2015. The assessment of daily dietary intake reveals the existence of a different pattern of bioaccumulation of chlorinated pollutants between domestic dogs and cats. Sci. Total Environ. 530–531, 45–52.

Schleicher, M., Cash, S.B., Freeman, L.M., 2019. Determinants of pet food purchasing decisions. Can. Vet. J. 60, 644–650.

Scientific Working Group for Forensic, T., ientific Working Group for Forensic 2013. Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. J. Anal. Toxicol. 37, 452–474.

Scudamore, K.A., Hetmanski, M.T., Nawaz, S., Naylor, J., Rainbird, S., 1997. Determination of mycotoxins in pet foods sold for domestic pets and wild

birds using linked-column immunoassay clean-up and HPLC. Food Addit. Contam. 14, 175–186.
Singh, S.D., Baijnath, S., Chuturgoon, A.A., 2017. A comparison of mycotoxin contamination of premium and grocery brands of pelleted cat food in South Africa. J. S. Afr. Vet. Assoc. 88, e1–e4.
Singh, S.D., Chuturgoon, A.A., 2017. A comparative analysis of mycotoxin contamination of supermarket and premium brand pelleted dog food in Durban, South Africa. J. S. Afr. Vet. Assoc. 88, e1–e6.
Singh, S.D., Sheik Abdul, N., Phulukdaree, A., Tiloke, C., Nagiah, S., Baijnath, S., Chuturgoon, A.A., 2018. Toxicity assessment of mycotoxins extracted from contaminated commercial dog pelleted feed on canine blood mononuclear cells. Food Chem. Toxicol. 114, 112–118.
Stenske, K.A., Smith, J.R., Newman, S.J., Newman, L.B., Kirk, C.A., 2006. Aflatoxicosis in dogs and dealing with suspected contaminated commercial foods. J. Am. Vet. Med. Assoc. 228, 1686–1691.
Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Marin, D., Taranu, I., Tabuc, C., Nicolau, A., Aprodu, I., Puel, O., Oswald, I.P., 2012. Current situation of mycotoxin contamination and co-occurrence in animal feed–focus on Europe. Toxins (Basel) 4, 788–809.
Szczech, G.M., Carlton, W.W., Tuite, J., 1973. Ochratoxicosis in beagle dogs. Vet. Path. 10, 219–231.
USEPA, 2005. Guidelines for carcinogen risk assessment. U.S. Environmental Protection Agency, pp. 1–166. Available at.
WHO, 1998. Safety Evaluation of Certain Food Additives and Contaminants. Series No. 40. Food Additives. Geneva: WHO; 1998. pp. 359–469. Food Additives Series 40, pp. 359–469.
Wille, S.M.R., Coucke, W., De Baere, T., Peters, F.T., 2017. Update of standard practices for new method validation in forensic toxicology. Curr. Pharm. Des. 23, 5442–5454.
Witaszak, N., Stepien, L., Bocianowski, J., Waskiewicz, A., 2019. Fusarium Species and Mycotoxins Contaminating Veterinary Diets for Dogs and Cats. Microorganisms 7.
Wouters, A.T., Casagrande, R.A., Wouters, F., Watanabe, T.T., Boabaid, F.M., Cruz, C.E., Driemeier, D., 2013. An outbreak of aflatoxin poisoning in dogs associated with aflatoxin B1-contaminated maize products. J. Vet. Diagn. Invest. 25, 282–287.
Yip, K.Y., Wan, M.L.Y., Wong, A.S.T., Korach, K.S., El-Nezami, H., 2017. Combined low-dose zearalenone and aflatoxin B1 on cell growth and cell-cycle progression in breast cancer MCF-7 cells. Toxicol. Lett. 281, 139–151.
Yogendrarajah, P., Jacxsens, L., Lachat, C., Walpita, C.N., Kolsteren, P., De Saeger, S., De Meulenaer, B., 2014. Public health risk associated with the co-occurrence of mycotoxins in spices consumed in Sri Lanka. Food Chem. Toxicol. 74, 240–248.