Journal of
Microbial & Biochemical Technology

Fuentes et al., J Microb Biochem Technol 2018, 10:3
DOI: 10.4172/1948-5948.1000399

Research Article Open Access

Presence of Mutagens and Carcinogens, Called Aflatoxins, and their Hydroxylated Metabolites in Industrialized Food for Dogs

Stephania Fuentes D[1], Magda Carvajal M[1*], Silvia Ruiz V[2], Nallely Cecilia Martínez R[1], Ariadna Azucena Gómez C[1] and Francisco Rojo C[3]

[1]*Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México, Mexico*
[2]*Departamento de Probabilidad y Estadística, Instituto de Investigaciones en Matemáticas Aplicadas y en Sistemas, Universidad Nacional Autónoma de México, Mexico*
[3]*Facultad de Química, Universidad Nacional Autónoma de México, Mexico*

Abstract

Introduction: The Aflatoxin contamination in dog food poses a serious health threat for dogs and it affects pet food industry, veterinarians and owners. Pets that are long-lived and healthy consumers contribute to sales, so any reduction in product quality has an effect on profits or even a company's survival. Pet food safety is the responsibility of the pet food industry.

Aims: To determine the type and amount of aflatoxins in 29 samples of dry food and 24 brands of canned food for dogs**.**

Methodology: The chemical extraction method used immunoaffinity columns with antibodies for total aflatoxins, and the quantification was performed with liquid chromatography and fluorescence detection. The method was validated, so the results were considered to be reliable once the recovery percentage was applied.

Results and Discussion: With respect to dry food, the average Aflatoxins (µg kg^{-1}) contamination was AFB_1 (1.6), B_2 (0.1), AFG_1 (28.2), AFG_2 (1.3), AFM_1 (1.8), AFM_2 (0.2), P_1 (1.7), Aflatoxicol (28.6), and Total aflatoxins (59.1), and the average of dry food samples was 7.9 µg kg^{-1} total aflatoxins. Canned food contained AFB_1 (14.2), AFB_2 (2.3), AFG_1 (60.4), AFG_2 (4.5), AFM_1 (2.1), AFM_2 (4.6), AFP_1 (18.4), AFL (13.1), and AFt (119.5), and the average of all of the samples was 15.3 µg kg^{-1}. According to statistical analysis, significant differences (p-value) between dry food and canned food were observed for AFB_1 ($p<0.001$) and AFL ($p<0.001$). Canned food was more contaminated than dry food.

Conclusion: Aflatoxins are common carcinogens of food for dog. The dry food croquettes for dogs had 51.6% less aflatoxins, with an average of 7.9 µg kg^{-1} total aflatoxins, under the tolerable legal limit, and the canned food, more contaminated (15.3 µg kg^{-1}), and surpassed the tolerable limit for Codex Alimentarius. The addition of hydroxylated metabolites gaves the true ingestion measure of Aflatoxins.

Keywords: Aflatoxins; Carcinogens; Contamination; Dogs

Abbreviations: %=Percentage; % CV=Variation coefficient percentage; < LOD=Below limit of detection; >LOD=Above limit of detection; °C=Centigrades; ACN=Acetonitrile; AFB_1=Aflatoxin B_1; AFB_2=Aflatoxin B_2; AFG_1=Aflatoxin G_1; AFG_2=Aflatoxin G_2; AFL=Aflatoxicol; AFM_1=Aflatoxin M_1; AFM_2=Aflatoxin M_2; AFP_1=Aflatoxin P_1; AFt=Total aflatoxins; ATF=trifluoroacetic acid; b_1=Value of the slope; bo=Ordinate to origin; CF=AF spiked concentration; CA=AF spiked concentration in the spiked sample; CU=basal AF concentration in a non-spiked sample; g (s)=Gram (s);

HPLC=High Performance Liquid chromatography; HPLC-FL=High Performance Liquid chromatography and Fluorescence; H_2O_d=distilled water; IAC=Immuno Affinity Columns; IC(β)=Confidence interval for the slope to origin; LOD=Limits of Detection; LOQ=Limits of Quantification; MeOH=Methanol; min=minute; mL , mL^{-1}= milliliter; mg=Milligrams; mm=Millimeters; MO=Missouri; NaCl=Sodium chloride; ng=Nanograms; nm= Nanometers; OH=Hydroxyl; % CV=Coefficient percentage; % R=Recovery percentage; PBS=Phosphate buffered saline; pH=Hydrogen potential; R^2= Coefficient determination; rpm=Revolutions per minute; RT =Retention time; SD=Standard deviation; $S_{y/x}$=Standard deviation of the regression; µL=microliters; µg=micrograms; UV=Ultraviolet; v/v=Volume to volume; WI=Wisconsin; µg kg^{-1}=Micrograms per kilogram; µg L^{-1}=Micrograms per liter; µL=Microliters; µL=Microliters

Introduction

Dog (*Canis familiaris*) domestication began 15,000 yrs ago in Asia. The ancient evidence of dog domestication is a mandible dated 12,000 years ago that was discovered in a cave in Iraq [1]. The dog was thought to have evolved from a mixture of a wolf and jackal, but according to modern anatomic, genetic and behavioral evidence, modern scientists agree that the dog exclusively derived from the grey wolf (*Canis lupus)* [2]. Dogs evolved into 350 different breeds in a mutually beneficial relationship with humans [3]. Pets are often regarded as family members by their owners, and a person may develop strong relationships with animals throughout his or her lifetime. Pet interactions and ownership have been associated with both emotional and physical health benefits [4,5]. Dogs and cats continue to be the most popular pets and are found in at least one out of every three US households, which creates a large market for the pet food industry [6]. The lifespan of small breeds is shorter, although some survive longer due to artificial selection [7]. The

***Corresponding author:** Magda Carvajal M, Departamento de Botánica. Instituto de Biología, Ciudad Universitaria, Universidad Nacional Autónoma de México, Ciudad Universitaria, Coyoacán, México, E-mail: magdac@ib.unam.mx; Tel: +5255 2523 8197

Received May 08, 2018; **Accepted** June 06, 2018; **Published** June 13, 2018

Citation: Stephania Fuentes D, Magda Carvajal M, Silvia Ruiz V, Nallely Cecilia Martínez R, Ariadna Azucena Gómez C (2018) Presence of Mutagens and Carcinogens, Called Aflatoxins, and their Hydroxylated Metabolites in Industrialized Food for Dogs J Microb Biochem Technol 10: 76-86. doi: 10.4172/1948-5948.1000399

lifespan of dogs is approximately 12 yrs, although some have lived up to 20 yrs [8]. Adult dogs are more affected by degenerative diseases related to age and breed. Large dogs experience fast growth, which makes them susceptible to cancer, but small breeds can also suffer from cancer and cardiac diseases [9].

One out of every 3 dogs dies due to cancer, and the breeds with the highest risk of cancer and their life expectancies ages are Boxers (10.5 yrs), Golden Retrievers (12 yrs), Rottweilers (10 yrs) and Bernese Mountain Dogs (8 yrs).

Dogs with a high risk of developing cancer are Boston Terriers (13 yrs), English Bulldogs (8 yrs), Scottish Terriers (13 yrs) and English Cocker Spaniels (12 yrs).

Dogs with a medium risk of developing cancer are Irish Setters (12 yrs), Schnauzers (Standard 12 yr; Miniature 15 yrs), Labrador Retrievers (12½ yrs) and mixed breeds.

Dogs with a low risk of developing cancer are Beagles (13 yrs), Poodles (Standard 12 yrs; Miniature 15 yrs), Collies (12 yrs) and Dachshunds (15.5 yrs). These life spans can be related to aflatoxin (AF) contamination of dog food and the amount ingested by the different breeds [9].

Aflatoxins

Aflatoxins (AFs) are secondary metabolites that chemically correspond to a bisdihydrodifuran or tetrahydrobisfuran bound to a coumarin substituted by a cyclopentanone or lactone [10-12]. AFs are divided into two subgroups that produce blue or green fluorescence [11,13,14]. AFs are produced by molds and do not affect fungal growth or reproduction, but can intoxicate animals and humans [15]. The physicochemical properties of AFs are well known [16-19] (Table 1) (Figure 1).

Aflatoxins are the most important mycotoxins in food at the worldwide scale [20-22]. Not all molds produce aflatoxins. The main AF-producing fungi are *Aspergillus flavus* [23-26], *A. parasiticus* [27-34], and *A. nomius* [35,36], but not all strains of these species. Other fungi have this property as well [37-39]. These mycotoxicogenic molds are distributed worldwide in warm, tropical, subtropical and temperate climates with high humidity. Only Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2) are naturally synthesized by toxigenic fungi. The other AFs (M_1, M_2, P_1, Q_1, G_{2a}, B_{2a}) and Aflatoxicol (AFL) are hydroxylated metabolites and are products of microbial or animal metabolism [14, 40-43].

Aflatoxins can be produced before or after harvest in many foods that contain cereal grains, oil seeds, edible nuts and spices [44]. The most toxic and important AF is AFB_1 [45], which can synthesize AFB_2, AFG_1 and AFG_2 [46]. The other AFs are called hydroxylated metabolites, which are products of animal or microbial metabolism. These include AFM_1 [47], AFM_2 [48], which is biotransformed from AFB_2, AFP_1, AFQ_1, AFG_{2a}, AFB_{2a}, and AFL [49,50], which is a very toxic metabolite formed by the selective reduction of a cyclopentancarbonile from AFB_1. When AFB_1 is ingested, the liver reduces its toxicity by adding a hydroxyl group, forming hydroxylated metabolites to make AFs soluble in water to facilitate their excretion by milk, feces, urine, biliary salts, and so on. All AFs in cereals and other vegetable ingredients, as well as their hydroxylated metabolites present in dairy products, meats, eggs, and so on, can contaminate the ingredients used to make industrialized food for pets [42].

AFs cause liver diseases, hemorrhages, immunosuppression and vomiting [51]. These mycotoxins are mutagenic and carcinogenic. Dogs exposed to 0.5-1 mg AFs/kg of body weight die in days from vomiting, depression, polydipsia, polyuria and hepatitis [52]. Anorexia, lethargy, jaundice, disseminated intravascular coagulation and death have been described in dogs that have ingested between 0.05-0.3 mg AF/kg of food within 6-8 weeks [53]. Dogs ingest aflatoxins because balanced food generally contains contaminated cereals [54].

Nutrients in balanced food for dogs

Nutrition is fundamental for dog's health, and scientific knowledge of the requirements, digestion, composition, nutrient profiles, and metabolism guides adequate food formulations for dogs and pets [55]. Dogs require different types of nutrients to survive: amino acids from proteins, fatty acids, carbohydrates, vitamins, minerals and water [56].

Dry food for dogs contains between 6-12% humidity and >88% dry matter. Among their ingredients are cereals; meat derivatives of bovines, pigs, poultry or fish; dairy products; and supplemental vitamins and minerals [57]. All of these ingredients are susceptible to aflatoxin contamination, especially dairy products; viscera, such as the liver; cereals, especially maize; and so on.

A summary of the global balance of food production (954 million tons) in 2012 ranked 134 countries. China was the lead producer, with 198.3 million tons; the USA produced 168.460 million tons as the second-leading producer; and Brazil was the third leading producer. Asia (356), Europe (207), the USA and Canada (188), Latin America (137), the Middle East and Africa (56), other (10) and Mexico produced 28.536 million tons [58].

There is no regulatory legislation for the minimum specifications for puppy food, so industries use the norms of international organizations, such as the National Research Council (NRC) and American Association of Feed Control Officials (AAFCO), to determine the nutritional needs of animals. The nutritional guidelines have been previously reported [59]. The AF content is not considered in the industrialization of dog food, so the purpose of this study is to determine the amount and type of AFs in dog foods and the meaning of their presence for canine health.

Methods and Materials

Sampling

Dry and canned food samples from Mexico City markets and the metropolitan area were purchase from October 22, 2014, to January 10, 2015 (Table 1).

Chemical extraction of AF

Fifty grams of samples from both presentations, dry and canned food, were independently blended (Waring ETL laboratory blender 7010S model WF 2211214, Torrington, CT, USA) with 100 mL of a mixture of methanol: H_2O_d (80:20 v/v) and two grams of NaCl to clarify food from the two presentations. The blended mixtures were centrifuged (ALC 4235 cool working system) at 4300 rpm for 15 min, and the supernatant was retained. Two milliliters of the supernatant was

Sample N°	AF risk ingredients						AF protective ingredients															
	Cereals: maize, sorghum rice, wheat, hulls	Oilseeds paste, soybean or canola	Meat bone and fat flour	Pigments (1)	Artificial flavors	Milk power, dairy	Prebiotic yeast (1)	Linoleic acid	Alumino silicates	Gluco-mannans Biosaf	Vitamins and minerals	*Yucca schidigera, (3),* folic acid	Maize gluten	Antioxidants			Etoxiquin	Sodium bisulphyte	Propianate		Flax-seed Omega 3 & 6
														BHA	BHT	TBHQ			Ca	Na	
1	X		X		X	X	X	X	X		X			X	X						
2	X	X	X	X			X	X			X			X	X						
3	X	X	X	X							X	X		X	X			X	X		
4	X	X	X								X			X	X						
5	X	X	X	X			X		X		X		X	X	X						
6	X	X	X				X		X			X	X	X	X						
7	X	X	X	X							X		X	X	X						
8	X	X	X		X								X	X	X	X					
9	X	X	X	X							X		X	X	X	X					
10	X	X	X	X		X		X			X	X	X	X	X		X				
11	X	X	X	X	X		X	X	.	X	X	X	X	X	X	X	X				
12	X	X	X	X				X			X	X		X	X						
13	X	X	X	X							X	X		X	X						
14	X		X			X	X							X	X						X
15	X	X	X			X	X														X
16	X	X	X			X	X			X	X	X		X	X						X
17	X	X	Xe	X		X	X			X	X	X									X
18	X	X	X			X					X	X	X	X	X						X
19	X		X				X				X	X	X	X	X				X	X	X
20	X		X				X				X	X							.		X
21	X		X				X				X										X
22	X	X	X	X							X			X				X			
23	X	X	X			X					X			X	X						
24	X	X	X	X							X	X		X	X						
25	X		X								X			X	X						
26	X	X	X								X	X	X	X	X						
27	X	X	X								X	X	X	X	X						
28	X		X				X				X										X

(1) Red 40, green 5, yellow 6, blue 1 & 2. (2) Beer yeast and *Lactobacillus acidophilus*, *Sacc. cerevisiae* (3) Yucca helps against arthritis.

Table 1: AF risk and protective ingredients in dog croquettes.

dissolved in 14 mL of Phosphate Buffered Saline (PBS) at 7.4 pH, and this mixture was slowly passed individually over an immunoaffinity column (Easi-Extract R-Biopharm Rhône LTD, UK) for total aflatoxins (AFt). The column was washed with 20 mL of H_2O_d and eluted by gravity with 1.5 mL of pure HPLC methanol, followed by 1.5 mL of H_2O_d with reflux. Three milliliters of the eluate was received in an amber vial and dried in an oven (Novatech BTC 9100, Houston Texas, USA) at 40ºC. Next, 200 µL of the eluate was derivatized to increase its fluorescence, and 60 µL of the eluate was finally injected in triplicate for quantification using liquid chromatography with fluorescence detection (HPLC-FL). The physicochemical properties of aflatoxins required for quantification have been reported [16].

Derivatization

Derivatization is a process used to increase the AF fluorescence of samples or standards to generate calibration curves to transform AFB_1 and AFG_1, which are not very fluorescent, into their highly fluorescent hemiacetals, AFB_{2a} and AFG_{2a}. AFB_2 and AFG_2, are fluorescent, do not undergo any transformation reactions during derivatization and are not affected by this reaction due to their saturated structure [40,60,61].

The dried eluate was resuspended in 200 µL of acetonitrile (ACN), and 800 µL of a previously prepared derivatizing solution with 5 mL of trifluoroacetic acid (ATF) (Sigma-Aldrich, St. Louis MO, USA), 2.5 mL of glacial acetic acid (Merck, Naucalpan, Edo. Mex., México) and 17.5 mL of deionized water were added, and the mixture was shaken (Vortex G-560, Bohemia, NY, USA) for 30 sec.

The amber vials were placed in a steam water bath at 65°C for 10 min [40,60]. Later, at room temperature, 200 µL of the mixture was applied in an insert, and 60 µL of the mixture was injected in a liquid chromatograph with a 20 µL loop in triplicate for quantification by HPLC with fluorescence (HPLC-FL).

Liquid chromatography conditions

The chromatographic system used was an Agilent Series 1200 HPLC (Agilent Technologies, Inc., USA) and consisted of an isocratic pump (Model G1310A), fluorescence detector (Model G1310A Series DE62957044, Agilent Technologies, Inc., USA) set to an excitation wavelength from 357-360 nm and an emission maximum of 450 nm, and autosampler (G1329A Series DE64761666). The chromatography column used was a VDS Optilab VDSpher 100 C18–E 5 µm 250 x 4.6 mm maintained at room temperature (22°C) with a mobile phase of water:ACN:methanol (65:15:20 v/v/v) that was degasified for 30 min by vacuum filtration and added at a flux of 1.0 mL min^{-1}.

Validation of the extraction method

Validation of the analytical methods and analyses of the dog food samples were performed using known parameters [62-64]. Validation of the method assured that the equipment was calibrated and working properly [65]. For validation, the following criteria were considered: the linearity of the calibration curves, Limits of Detection (LOD), Limits of Quantification (LOQ) and recovery percentage.

Linearity of the system (calibration curves)

The linearity of the system is the capacity of the analytical method to obtain results that are directly proportional to the concentration of the analyte (AF) in a defined range. The linearity of the system is obtained through a mathematical treatment of the obtained results in the analyte analysis. The range selection and number of experimental points are related to the method of application [64]. A parameter is the coefficient of determination (R^2), which should be near 1 [66].

Solutions with different concentrations of the eight AFs were prepared from a 1000 ng (=1 µg mL^{-1}) AF stock. The 0.25 mg AFM standards were diluted with benzene: acetonitrile (98:2 v/v) according to a previously reported methodology [67] to prevent the decomposition of pure AFs.

a. The spectrophotometer (Genesys 10 UV Thermo Electron Corporation; Madison, WI, USA) was calibrated before the experiments to measure the absorbance of the AF standard solutions from 357-360 nm.

b. The following formula was applied to calculate 1000 ng stock solutions of each AF concentration [67]:

AF (µg mL^{-1}) = absorbance × molecular weight × 1000 × correction factor of the equipment

extinction coefficient.

c. Twelve concentrations (0.01, 0.05, 0.1, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 ng) of the 8 different AFs were independently created from the 1000 ng stock solution. These standard dilutions were then used to plot the analytic signal (the area below the curve of each chromatographic peak) against the AF concentrations. The curve equation and statistical parameters were obtained. The slope value (b1), ordinate to origin (bo), determination coefficient (R^2), confidence interval for the slope to origin (IC(β)), variation coefficient percentage (%CV), Standard Deviation (SD), Limits of Detection (LOD) and Limits of Quantification (LOQ) were calculated using Excel 2003.

Limits of Detection (LOD) and Quantification (LOQ)

The LOD of the equipment was established in relation to the noise in the chromatogram. The LOD equals the AFM_1 concentration that demonstrates a signal that is three times greater than the noise. The LOQ equals the AFM_1 concentration that is 10 times greater than the noise **[68]**. To calculate the LOD, the following equation was used:

$$LOD = \frac{3.3 \times S(y/x)}{b_1}$$

The LOQ was calculated using the following equation:

$$LOQ = \frac{10 \times S(y/x)}{b_1},$$

where S(y/x) is the standard deviation of the regression and b_1 is the value of the slope [68].

Recovery percentages

The recovery percentage is a measure of the accuracy of the method and expresses the proximity between the theoretical and experimental values. The recovery percentage is the percentage of difference between the average concentration of AF (analyte) from a spiked sample and the concentration measured in the sample with no spiking divided between the spiked concentration [62].

$$\% R = [(CF - CU)/CA] \times 100$$

where % R is the recovery percentage, CF is the spiked and basal AF

Sample N°	AF risk ingredients				Protective ingredients against Aflatoxins.											
	Vegetables	Artificial pigments (1)	Meats,bone and fat flour	Fungicides	Prebiotic yeast (2)	Linoleic acid	Alumino silicates	Vitamins and minerals	Y. *schidigera* (3), folic acid	Maize gluten	Antioxidants			Etoxiquin	Sodium bisulphyte	Calcium propianate	Flax-seed Omega 3 & 6
											BHA	BHT	TBHQ				
29			X		X	X	X				X	X					
30	X		X			X					X	X					
31			X	X							X	X			X	X	
32	X		X								X	X					
33			X								X	X					
34			X		X		X			X	X						
35			X							X	X	X					
36			X							X	X	X	X				
37			X							X	X	X	X				
38			X			X				X	X	X	X				
39			X			X				X	X	X		X			
40			X			X			X		X	X					
41		X	X					X (4)			X	X	X				
42		X	X					X (4)									
43		X	X					X (4)									
44	X (5)	X	X			X		X			X	X					
45		X (1)	X					X									
46				X				X	X		X	X					X
47	X (6)		X			X		X									
48			X														
49	X		X		X			X									
50	X (7)	X	X														
51	X (7)	X	X					X		X							
52	X (7,8)	X (1)	X				X	X		X	X	X	X				
53	X (7)		X					X	X	X							
Average																	

1) Artificial pigments. Yellow (5) and (6), and Sodium nitrite (for color retention), *2) Lactobacillus acidophilus*, *Sacc.cerevisiae*. *3) Yucca schidigera vs arthritis* 4) Xanthan gum, Carrageenan, magnesium proteinate, dried yam, Guar Gum, Zinc sulfate. 5) Cereals, 6) Canola and alfalfa. 7) Maize, rice, wheat and soybean. 8) Dry green pea and carrot.

Table 2: AF risk and protective ingredients in canned dog food.

concentration, CU is the basal AF concentration in a non-spiked sample, and CA is the AF spiked concentration in the spiked sample [62].

The arithmetic average, standard deviation, percentage of variation coefficient and confidence interval were calculated. To obtain accurate measurements, the AFs of the samples of dog food in 1 g of dried food diluted in PBS (1:4 v/v) were individually spiked with three different concentrations (5, 20 and 40 μg kg^{-1}) of the eight individual AF standards (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2, and AFP_1) and AFL. One aliquot without spiked AF was used as the control, which gave the basal contamination level. The samples were individually processed using the R-Biopharm extraction method [69]. The AFs were purified and concentrated using an IAC, derivatized, and quantified by HPLC-FL, and the percentage of recovery for each AF was obtained. After the derivatization mixture was cooled to room temperature, triplicates of each sample (60 μL) were injected onto the HPLC-FL.

Statistical analysis

We compared the levels of aflatoxins in dry food and canned food for dogs using a t-test with different variances to test the equality of means.

Aflatoxin	LOD (ng g^{-1})	RT Range in min	R^2
AFB_1	0.5	7.085-8.849	0.9986
AFB_2	0.05	17.452-20.228	0.9817
AFG_1	0.5	7.681 -9.541	0.9898
AFG_2	0.5	11.215-14.513	0.9946
AFM_1	0.1	8.514-8.769	0.9834
AFM_2	0.05	20.208-22.447	0.9946
AFP_1	0.05	15.563-19.318	0.9960
AFL	0.01	3.032-5.569	0.9978

LOD=Limit of detection, RT=Retention time in min, R^2=Coefficient of determination of aflatoxin standards.

Table 3: Validation of the extraction method.

Sample	Aflatoxins (μg kg^{-1}) in dry food for dogs									
	AFB_1	AFB_2	AFG_1	AFG2	AFM_1	AFM_2	AFP_1	AFL	AFt	Average per sample
1	2.2	0	110.9	2.1	0	0	1.1	24.8	141.1	17.6
2	0	0	54.4	2.0	4.2	0	1.0	25.7	87.3	10.9
3	2.2	0	105.0	2.6	6.1	4.0	1.4	41.6	162.9	20.4
4	5.0	<LOD	5.8	1.2	0	0	1.2	25.3	38.5	4.8
5	1.4	0	20.3	0.5	0	0	0.2	16.3	38.7	4.8
6	<LOD	0	8 .4	2.7	0	0	0.7	29.4	41.2	5.2
7	1.9	<LOD	28.7	1.1	5.5	<LOD	0.6	26.5	64.3	8.0
8	<LOD	0	<LOD	0	<LOD	0	0.6	20.4	21.0	2.6
9	5.5	0	12.6	1.7	0	0	0.9	20.6	41.3	5.2
10	<LOD	0	9.7	1.0	0	0	0.6	17.7	29.0	3.6
11	1.0	<LOD	28.8	5.3	7.2	0	1.8	30.3	74.4	9.3
12	<LOD	0	0	0	2.3	0	0.7	22.5	25.5	3.2
13	1.7	0	11.8	<LOD	3.9	0	0.9	22.2	40.5	5.1
14	0	0	0	1.7	0	0	0.5	29.9	32.1	4.0
15	2.3	0.2	53.5	3.8	1.4	0	15.7	33.0	109.9	13.7
16	0	0	12.3	2.5	7.0	0	1.2	25.6	48.6	6.1
17	<LOD	0	23.3	1.4	6.3	0	1.0	26.4	58.4	7.3
18	0	0	5.8	0.7	2.7	0	1.6	19.5	30.3	3.8
19	3.6	0	30.0	0.6	0	0	0.8	19.8	54.8	6.9
20	2.0	0	13.1	0.5	0	0	1.6	19.1	36.3	4.5
21	2.1	0	79.1	0.5	0	0	1.1	19.9	102.7	12.8
22	3.4	0.1	91.1	1.7	0	0	2.0	38.5	136.8	17.1
23	9.5	0	34.3	1.3	0	0	0.8	22.5	68.4	8.6
24	0	0	6.5	0.8	0	0	1.9	141.4	150.6	18.8
25	<LOD	0	11.9	<LOD	0	0	1.3	26.9	40.1	5.0
26	2.5	0	53.8	0.8	0	0.8	2.0	32.0	91.9	11.5
27	0.9	0	0	0.8	1.1	<LOD	1.7	20.5	25.0	3.1
28	0	<LOD	7.4	0.6	2.4	0.8	0.8	0.8	12.8	1.6
29	0	2.6	0	0.6	1.4	1.0	0.9	28.8	35.3	4.4
Average	**1.6**	**0.1**	**28.2**	**1.3**	**1.8**	**0.2**	**1.7**	**28.6**	**59.1**	**7.9**

AFB_1=Aflatoxin B1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2, AFM_1=Aflatoxin M_1, AFM_2=Aflatoxin M_2, AFP_1=Aflatoxin P1, AFL=aflatoxicol, AFt=Total aflatoxins.

< LOD=Below Limit of Detection, >LOD=Above Limit of Detection

Table 4: Aflatoxin (μg kg^{-1}) types in dry food (croquettes) for dogs.

Samples	Aflatoxin average (µg kg^{-1}) in canned feed for dog.									Averageper sample
	AFB_1	AFB_2	AFG_1	AFG_2	AFM_1	AFM_2	AFP_1	AFL	AFt	
30	0	0	0	0	2.4	0	1.1	19.1	22.6	**2.8**
31	0	0	<LOD	0	0	0	0.7	37.3	38.0	**4.8**
32	0	0	0.7	0	0	0	1.3	35.8	37.8	**4.7**
33	<LOD	0	0	0.7	0	0	1.9	34.8	37.4	**4.7**
34	<LOD	0	0	0	0	0	1	27.3	28.3	**3.5**
35	<LOD	0	0	0	0	0	0.2	20.5	20.7	**2.6**
36	<LOD	0	0	0	0	0	0.4	27.3	28.1	**3.5**
37	0	0	0	0	0	0	0.2	35.8	36.0	**4.5**
38	0	21.9	0	16.8	0	27.9	133.7	0	200.3	**25.0**
39	0	22.9	0	0	0	21.3	27.5	0	71.7	**9.0**
40	<LOD	4.5	0	2.7	0	49.6	192.5	0	249.3	**31.2**
41	0	0	0	<LOD	0	6	0.7	0	**6.7**	**0.8**
42	2.7	0.3	77.3	21.5	9.7	0.6	30.1	0	142.2	**17.8**
43	11.4	<LOD	196.5	21.4	4.9	0.3	<LOD	0	234.5	**29.3**
44	20.9	0	374.5	11.0	0	0	0.7	0	407.1	**50.9**
45	**32.5**	2	38.2	6.3	0	1.6	47.0	5.3	132.9	**16.6**
46	21.8	0	87.3	2.5	9.1	0	0	1.6	122.2	**15.3**
47	27.0	0	74.0	0.6	9.6	0	0	0	111.2	**13.9**
48	16.6	3.9	134.6	1.0	0	0	0	4.8	160.9	**20.1**
49	3.8	0	154	5.6	9.7	0	0.6	0	173.7	**21.7**
50	**144.2**	<LOD	169.4	16.6	0	0	0.2	0	330.4	**41.3**
51	28.9	0	89.2	0	0	1.2	2.1	11.6	133.0	**16.6**
52	18.9	0	26.2	0.8	0	0	<LOD	38.5	84.5	**10.6**
53	13.1	0	27	0	4.7	0.6	0.3	13.6	59.3	**7.4**
Average	**14.2**	**2.3**	**60.4**	**4.5**	**2.1**	**4.6**	**18.4**	**13.1**	**119.5**	**15.3**

AFB_1=Aflatoxin B1, AFB_2=Aflatoxin B_2, AFG_1=Aflatoxin G_1, AFG_2=Aflatoxin G_2, AFM_1=Aflatoxin M_1, AFM_2=Aflatoxin M_2, AFP_1=Aflatoxin P1, AFL=aflatoxicol, AFt=Total aflatoxins.

Table 5: Type and concentration of aflatoxins (µg kg^{-1}) in canned feed for dogs.

Aflatoxins (µg kg^{-1})									
AFB_1	AFB_2	AFG_1	AFG2	AFM_1	AFM_2	AFP_1	AFL	AFt	Average
	Canned food (higher contamination)								
14.2	2.3	60.4	4.5	2.1	4.6	18.4	13.1	119.5	15.3
Croquettes (less contamination)									
1.6	0.1	28.2	1.3	1.8	0.2	1.7	28.6[a]	59.1	7.9
	Different contamination in food presentation								
12.6	2.2	32.2	3.2	0.3	4.4	16.7	- 15.5[b]	640.4	7.4

a=Only case where dry food was more contaminated with AFL than canned food, there was more biotransformation of basic aflatoxins to the AFL hydroxylate metabolite.
b=Only difference between dry food with more AFL hydroxylate biotransformation than canned food with less AFL.

Table 6: Average of Aflatoxins (µg kg^{-1}) in croquettes and canned food for dogs.

For each product (dry and canned food), we performed a Kruskal-Wallis analysis to determine the differences of the concentrations of AFB_1, AFB_2, AFB_1, AFG_2, AFM_1, AFM_2, AFP_1 and AFL among the samples. If statistically significant differences were found, we performed pairwise Wilcoxon Rank sum tests to determine where the differences were.

The general purpose of this study was to determine the AF concentrations of industrialized food for dogs through validation of the method of extraction and quantification of the basic AFs (AFB_1, AFB_2, AFG_1, AFG_2) and their hydroxylated metabolites (AFM_1, AFM_2, AFP_1 and AFL).

Results and Discussion

Sampling

All of the ingredients of dry food, (Table 1) and canned dog food (Table 2) were obtained and analyzed, and the ingredients were divided between AF risk or AF protective ingredients. The validation of the method is presented in Table 3. After quantifying the AFs (Tables 4 and 5), the ingredients explained the contamination and amount of AF discovered. Among the AF risk ingredients of dog food are cereals, such as corn and rice, and leguminosae, such as soybean, which are frequently contaminated with AFs. Artificial pigments pose a cancer risk [70]. Red (40), Yellow (5), and Yellow (6) have been found to be contaminated with benzidine or other carcinogens. At least four dyes; Blue (1), Red (40), Yellow (5), and Yellow (6) cause hypersensitivity reactions. Numerous microbiological and rodent studies of Yellow (5) were positive for genotoxicity. Toxicity tests performed on two dyes; Citrus Red (2) and Orange B also suggest safety concerns, but Citrus Red (2) is used at low levels and only in some Florida oranges and Orange B has not been used for several years [70]. All dog food contains derivatives from all types of meat, and viscera, such as the liver, are

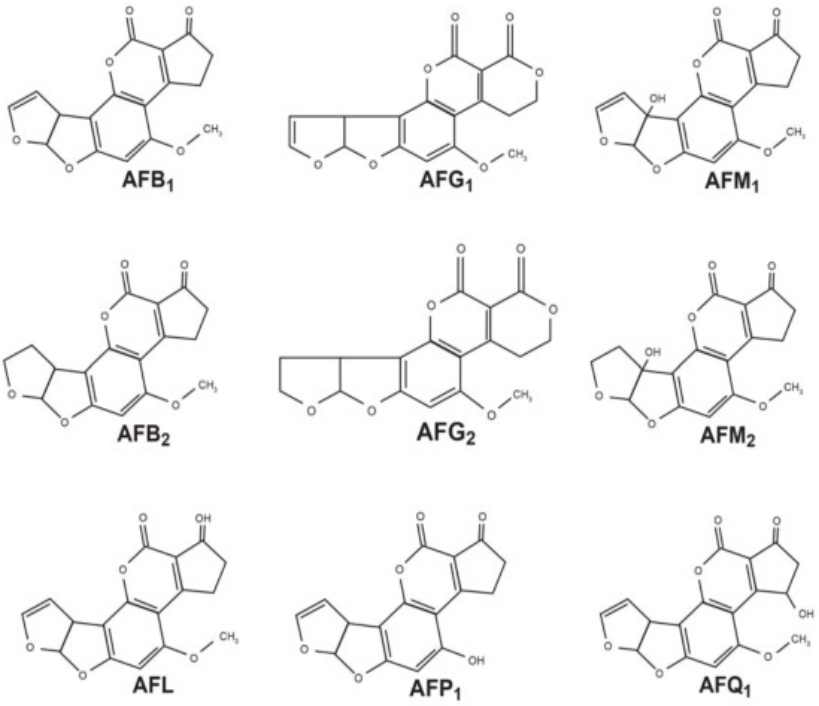

Figure 1: Chemical structures of the different types of Aflatoxin.

Aflatoxin	t-test	p-value
AFB_1	-4.7899	**< 0.001**
AFB_2	-1.9009	0.061
AFG_1	-2.3187	0.023
AFG_2	-3.2517	0.002
AFM_1	-0.3676	0.714
AFM_2	-2.5625	0.013
AFP_1	-2.6663	0.009
AFL	5.4426	**< 0.001**

Table 7: Aflatoxin t-test and p-value statistics for differences of croquettes and canned food for dogs.

usually contaminated with AFs [71]. Aflatoxins can be present in milk from dairy cows, meat from swine or chicken, and eggs if the animals consume sufficient amounts AF-contaminated feed [72]. To estimate the risk associated with mycotoxin exposure, we needed to determine the dose a pet could consume in food on a daily basis for their entire life with no adverse effect (i.e., NOAEL) [73]. The effects of mycotoxins on pets are severe and can lead to death. In 1952, a case of hepatitis in dogs was directly linked to the consumption of moldy food [74]. Following the discovery of AF, the agent responsible for the 1952 case was identified as AFB_1 [75] and the symptoms of aflatoxicoses in dogs were also elucidated [75,76].

Among the protective ingredients used against AFs are maize gluten, which controls AFs [77]; hydrated sodium calcium aluminosilicate [78]; prebiotic yeast [79]; probiotic bacteria [80]; ascorbic acid **[81]**; linolenic acid [82]; glucomannans [83]; vitamins and minerals [84]; *Yucca schidigera* [85]; antioxidants, including phenols and butylated hydroxytoluene (BHT) [86]; ethoxyquin [87]; sodium bisulphyte [88]; sodium propionate [89]; and flax seed omega 3 and 6 [90].

Dry food is the main product of the pet food industry and is used because of its storage and feeding convenience. Dry foods are protected against spoilage due to their low water content. The resulting extruded material has a moisture content of approximately 25% before drying and a final moisture content of 8-10% after drying. At this level of moisture, mold formation is inhibited [91-93]. Thermal inactivation processes are not sufficient to control the pre-formed aflatoxins in the ingredients. Mycotoxins are chemically and thermally stable, so commonly used food manufacturing techniques do not destroy them [53,94]. Aflatoxins are stable up to their melting point of approximately 250°C and are not destroyed completely by boiling water, autoclaving, or a variety of food and feed processing procedures [95,96].

Validation of the chemical method

The linearity (calibration curves) of the AFs and hydroxylated metabolites (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2, AFP_1 and AFL), limit of detection, coefficient of determination and percent of recovery were obtained, so the method was considered to be validated (Table 3).

Aflatoxin contamination

The results in dry food are presented (Table 4), with an AFt average of 7.9 ng g^{-1}, and in the canned food (Table 5), an AFt average of 15.3 ng g^{-1} was determined**.** A comparison of the average AFs is shown in Table 6**,** with canned food being more contaminated than dry foods, except in regard to AFL, for which dry food was more contaminated.

Conclusion

In the comparison analysis of the levels of AFB_1 and AFL in dry food and canned food for dogs, there was a significant difference in the t-test and p-values. The results are shown in the Table 7. For AFB_1, AFG_1, AFG_2, AFM_2 and AFP_1, the levels found in canned food were significantly greater than those found in dry food, whereas for AFL, the result was the opposite. Although companies add control ingredients to reduce AFs, some AFs still remain.

For the aflatoxin t-test and p-value, significant differences between dry food and canned food only appeared in respect to AFB_1 and AFL (Table 7).

For each type of food, dry and canned, Kruskal-Wallis analysis was performed. The values of the statistics and their significant values were significantly different for AFB_1, AFG_2 and AFL (Table 8).

For dry food, we found differences in AFB_2, AFG_1, AFG_2, AFM_2 and AFL.

We conclude that the chemical method of analysis was validated, so the results were reliable once the recovery percentage was applied. Canned food was more contaminated than the dry food, so the amount of water must play and important role.

Aflatoxin contamination in dog food poses a serious health problem for dogs and affects the entire pet food industry. Long-lived, healthy consumers (pets) cannot avoid aflatoxin contamination of their food, and breakdowns in product quality can have catastrophic effects on a company's profits or even its viability. Pet food safety is the responsibility of the pet food industry.

Acknowledgment

The authors would like to thank the following personnel from the Instituto de Biología, Universidad Nacional Autónoma de México (IBUNAM): Noemí Chávez from the Secretaría Técnica, Joel Villavicencio, Jorge López, Alfredo Wong, Celina Bernal, Diana Martínez and Julio César Montero. Additionally, we would like to thank Georgina Ortega Leite and Gerardo Arévalo for library information.

References

1. Vilà C, Savolainen P, Maldonado JE, Amorim IR, Rice JE et al. (1997) Multiple and ancient origins of the domestic dog. Science 276: 1687-1689.
2. Lindblad-Toh K, Wade CM, Lander ES\ (2005) Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature 38: 803-819.
3. Klütsch CFC, Savolainen P (2011) Geographical origin of the domestic dog. John Wiley & Sons, Ltd. ELS.
4. Milani MM (1996) The importance of the human–animal bond. J Amer Vet Med Assoc 209:1064-1065.
5. Adams CL, Conlon PD, Long KC (2004) Professional and veterinary competencies: Addressing human relations and the human–animal bond in veterinary medicine. J Vet Med Education 31: 66-71.
6. APPMA (2006) American Pet Products Manufacturers Association. National Pet Owners Survey 2005/2006. In: APPMA News, Accessed abril 2018.
7. Galis F, Van Der Sluijs I, Van Dooren TJM, Metz JA, Nussbaumer M (2007) Do large dogs die young?. J Exp Zool B Mol Dev Evol 308: 119-126.
8. Nowak RM (1999) Walker´s mammals of the world. (6thedn), The John Hopkins University Press. Baltimore, Maryland USA.
9. Wolf NS, Austad S (2010) Introduction: Lifespans and pathologies present at death in laboratory animals. In: Wolf N. (eds). Comp Biol Aging: 1-26.
10. Jaimez J, Fuente CA, Vázquez BI, Franco CM, Cepeda A, et al. (2000) Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. J Chromatogr A 882: 1-10.
11. Leeson S, Diaz G, Summers J (1995) Publication announcement: Poultry metabolic disorders and mycotoxins. University Books, Ontario, Canada.
12. Palmgren MS, Hayes AW (1987) Aflatoxins in Food. In: Mycotoxins in foods. Food Science and Technology, A Series of monographs Ed Academic Press. San Diego, USA.
13. Nakai VK, Rocha LOD, Gonçalez E, Fonseca H, Ortega EMM, et al. (2008) Distribution of fungi and aflatoxins in a stored peanuts variety. Food Chem 106: 285-290.
14. Rastogi SC, Heydorn S, Johansen JD, Basketter DA (2001) Fragrance chemicals in domestic and occupational products. Contact Dermatitis 45: 221–225.
15. CAST Council for Agricultural Science and Technolgy (2003) Risks assessment and regulations for mycotoxins. Chapter 8. In: Mycotoxins: Risks in Plant, Animal and Human systems. Task Force Report N° 139: 104-128.
16. OPS, Organización Panamericana de la Salud. (1983) Criterios de Salud Ambiental 11. Publicación Científica N° 453. Organización Mundial de la Salud. USA.
17. Reddy SV, Farid W (2006 a) Properties of aflatoxin and its producing fungi.
18. Rustom IYS, López-Leiva MH, Nair BM (1993) Effect of pH and heat treatment on the mutagenic activity of peanut beverage contaminated with aflatoxin B_1. Food Chem 46: 37-42.
19. Rustom IYS (1997) Aflatoxin in food and feed: Occurrence, legislation and inactivation by physical methods. Food Chem 59: 57-67.
20. Diener UL, Cole RJ, Sanders TH, Payne GA, Lee SL et al. (1987) Epidemiology of aflatoxin formation by Aspergillus flavus. Annual Rev Phytopath 25: 249-270.
21. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, et al. (2004) Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80: 1106-1122.
22. Strosnider H, Azziz-Baumgartner E, Banziger M, Bhat RV, Breiman R, et al. (2006) Workgroup report: Public health strategies for reducing aflatoxin exposure in developing countries. Environ Health Perspect 114: 1898-1903.
23. Link HF (1809) Observationes in Ordines plantarum naturales. Dissertatio prima, complectens Anandrarum ordines Epiphytas, Mucedines Gastomycos et Fungos Der Gesellschaft Naturforschender Freunde zu Berlin. Magazin für die neuesten Entdeckungen in der gesamten Naturkunde 3: 1-42.
24. Scheidegger KA, Payne GA (2003) Unlocking the secrets behind secondary metabolism: A review of Aspergillus flavus from pathogenicity to functional genomics. J Toxicol-Toxin Rev 22: 423-459.
25. Richard JL, Payne GA (2003) Mycotoxins in plant, animal, and human systems. Task Force Report No. 139. Council for Agric Sci Technol.
26. Payne GA (1998) Process of contamination by aflatoxin producing fungi and their impacts on crops. In: Mycotoxins in Agriculture and Food Safety. Sinha KK and Bhatnagar D. Marcel (eds). Dekker, Inc. New York. USA.
27. Speare AT (1912) Fungi parasitic upon insects injurious to sugar cane. Pathology and Physiological Series, Bulletin No. 12. Honolulu: Hawaiian Sugar Planters' Assoc Exp Stat. Path Phys. Series Bull 12:1-62.
28. Cullen JM, Newberne PM (1994) Acute hepatotoxicity of aflatoxins. In: Eaton DL, Groopman JD, eds. The toxicology of aflatoxins: human health, veterinary, and agricultural significance. 3–26.San Diego: Academic Press, USA.

29. Horn BW, Greene RL, Sobolev VS, Dorner JW, Powell JH, et al. (1996) Association of morphology and mycotoxin production with vegetative compatibility groups in Aspergillus flavus, A. parasiticus, and A. tamarii. Mycologia 88: 574-587.

30. Horn BW, Greene RL (1995) Vegetative compatibility within populations of Aspergillus flavus, A. parasiticus, and A. tamarii from a peanut field. Mycologia 87: 324-332.

31. McAlpin CE, Wicklow DT, Platis CE (1998) Genotypic diversity of Aspergillus parasiticus in an Illinois corn field. Plant Dis 82: 1132-1136.

32. Geiser DM, Timberlake WE, Arnold ML (1996) Loss of meiosis in Aspergillus. Mol Biol Evol 13: 809-817.

33. Peterson SW (2008) Phylogenetic analysis of Aspergillus species using DNA sequences from four loci. Mycologia 100: 205-226.

34. Klich MA, Pitt JI (1988) Differentiation of Aspergillus flavus from A. parasiticus and other closely related species. Trans Brit Mycol Soc 91: 99-108.

35. Kurtzman CP, Horn BW and Hesseltine CW (1987) Aspergillus nomius, a new aflatoxin-producing species related to Aspergillus flavus and Aspergillus tamarii. Antonie van Leeuwenhoek 53: 147-158.

36. Horn BW, Moore GG, Carbone I (2011) Sexual reproduction in aflatoxin-producing Aspergillus nomius. Mycologia 103: 174-183.

37. Goto T, Wicklow DT, Ito Y (1996) Aflatoxin and cyclopiazonic acid production by a sclerotium-producing Aspergillus tamarii strain. Appl Environ Microbiol 62: 4036-4038.

38. Domsch KH, Gams W, Anderson TH (1980) Compendium of soil fungi. London, England: Academic Press, UK.

39. CRCC (2015) Centre de Recherche sur la conservation de collections.

40. Akiyama H, Goda Y, Tanaka T, Toyoda M (2001) Determination of aflatoxins B_1, B_2, G_1 and G_2 in spices using a multifunctional column clean-up. J Chromatogr A 932: 153-157.

41. Lindner E (1995) Foods Toxicology. 2nd Ed. Acribia SA Zaragoza, Spain.

42. Otta KH, Papp E, Bagócsi B (2000) Determination of aflatoxins in food by overpressured-layer chromatography. J Chromatogr A, 882: 11-16.

43. Williams J, Wilson D (1999) Informe sobre el problema de aflatoxinas de la castaña (Bertholletia excelsa) en Bolivia: Documento Técnico 71: 4-7.

44. FAO, Food and Agricultural Organization (2003) Manual over the application of the system of analysis of Hazard Critical Countrol Points (HCCP) in the prevention and control of mycotoxins. Department of Agriculture. FAO Study on Feeding and Nutrition.

45. Soriano del Castillo JM (Ed) (2007) Micotoxinas en alimentos. Vol. 1. Editorial Díaz de Santos, Burjassot. Valencia. España: 396.

46. Puschner B (2002) Mycotoxins. Veterinary clinics of North America: Small animal practice 32: 409-419.

47. Carvajal M, Bolaños A, Rojo F, Méndez I (2003a) Aflatoxin M_1 in pasteurized and ultrapasteurized milk with different fat content in Mexico. J Food Prot 66: 1885-1892.

48. Aquino S, Correa B (2011) Aflatoxins in pet foods: A risk to special consumers. In: A risk to special consumers. Intech Open 1: 53-74.

49. Carvajal M, Rojo F, Méndez I, Bolaños A (2003b) Aflatoxin B_1 and its interconverting metabolite Aflatoxicol in milk. Situation in Mexico. Food Addit Contam 20: 1077-1086.

50. Karabulut S, Paytakov G, Leszczynski J (2014) Reduction of aflatoxins B_1 to aflatoxicol: A comprehensive DFT study provides clues to its toxicity. J Sci Food Agric 94: 3134-3140.

51. Richard J (2007) Some major mycotoxins and their mycotoxicoses-an overview. Int J Food Microbiol 119: 3-10.

52. Bôhm J, Razzai-Fazeli E (2005) Effects of mycotoxins on domestic pet species. In: Diaz D, Ed. The Mycotoxin Blue Book. Nottingham University Press. Nottingham UK.

53. Leung M, Díaz-Llano G, Smith TK (2006) Mycotoxins in pet food: A review on wordwide prevalence and preventative strategies. J Agric Food Chem 54: 9623-9635.

54. Bastianello S, Nesbit JW, Williams MC, Lange A (1987) Pathological findings in a natural outbreak of aflatoxicosis in dogs. Onderstepoort J Vet Res 54: 635-640.

55. Dobenecker B, Hendriks W, Hesta M (2011) Nutritional guidelines for complete and complementary pet food for cats and dogs. FEDIAF. Bruxelles, Belgium.

56. Feuer D (2006) Your dog's nutritional needs. A science-based guide for pet owners. National Research Council of National Academies. Washington DC. Int J Food Microbiol 119: 3-10.

57. Profeco, Procuraduría Federal del Consumidor (2002) Alimentos para mascotas. Revista del Consumidor Nº 307.

58. Alltech (2013) Global feed summary.

59. Dobenecker B,), Hendriks W, Hesta M (2014) Nutritional guidelines for complete and complementary pet food for cats and dogs. FEDIAF. Bruxelles, Belgium.

60. Kok WTh (1994) Derivatization reactions for the determination of aflatoxins by liquid chromatography with fluorescence detection. J Chromatogr B 659: 127-137.

61. Takahashi DM (1977) Reversed-phase high-performance liquid chromatographic analytical system for aflatoxins in wines with fluorescence detection. J Chromatogr 131:147-156.

62. García A, Soberón E, Cortés M, Rodríguez R, Herrera JL, et al. (2002) Métodos Analíticos. Guía de Validación. Colegio Nacional de Químicos Farmacéuticos Biólogos México AC. 123.

63. Pérez M, Ramírez G, Pérez M, Restrepo P (2007) Validación del método analítico para la determinación valsartán en un plasma humano por HPLC/UV con adición estándar empleando losartán como estándar interno. Revista Colombia Médica 38: 13-20.

64. Castillo B, González R (1996) Protocolo de validación de métodos analíticos para la cuantificación de fármacos. Revista Cubana de Farmacia 30: 17-22.

65. Sandoval S (2010) Validación de métodos y determinación de la incertidumbre de la medición: Aspectos generales sobre la validación de métodos. Instituto de Salud Pública. Santiago, Chile. 1-67.

66. USPC, United States Pharmacopeial Convention (2004) Validation of compendial methods. The United States Pharmacopeia. Rockville: National Publishing, Philadelphia 27: 2622-2625.

67. AOAC, Association of Official Analytical Chemists (2006) Natural toxins. In official methods of Analysis of AOAC International 18th ed., Horwitz W, Latimer GW Jr, Trucksess MW (eds). Gaithersburg (MD): AOAC International: 1-51.

68. Cruz-Rueda V (2016) Curso de Validación de Métodos Analíticos. Centro de Capacitación RH. World SA de CV.

69. R-Biopharm Rhone Ltd (2012) Easi-extract aflatoxin. Guía de uso para el usuario. Block 10 Todd Campus, West of Scotland Science Park. Acre Road, Glasgow G20 0XA. UK.

70. Kobylewski S, Jacobson MF (2012) Toxicology of food dyes. Int J Occup Environ Health 18: 220-246.

71. Aquino S, Corrêa B (2011) Aflatoxins in pet foods: A risk to special consumers, Aflatoxins - Detection, measurement and control. Dr Irineo Torres-Pacheco.

72. Robens JF, Richard JL (1992) Aflatoxins in animal and human health. Rev Environ Contam Toxicol 127: 69-94.

73. Boermans HJ, Leung MCK (2007) Mycotoxins and the pet food industry: Toxicological evidence and risk assessment. Int J Food Microbiol 19: 95-102.

74. Devegowda G, Castaldo D (2000) Mycotoxins: Hidden killers in pet foods. Is there a solution? In: Technical symposium on mycotoxins. Alltech Inc, Nicholasville, Kentucky, USA.

75. Newberne PM, Harrington DH, Wogan GN (1966) Effects of cirrhosis and other liver insults on induction of liver tumors by Aflatoxin in rats. Lab Invest 15: 962-969.

76. Ketterer PJ, Williams ES, Blaney BJ, Connolle MD (1975) Canine aflatoxicosis. Aust Vet J 51: 355-357.

77. Vega-Rojas LJ, Carvajal-Moreno M, Rojas-Molina I, Rojo-Callejas F, Ruiz-Velasco S, et al. (2016) The effect of maize germ on the presence of aflatoxins in corn flours treated with a thermo-alkaline process. J Microb Biochem Technol 8: 408-414.

78. Bingham AK, Huebner HJ, Phillips TD, Bauer JE (2004) Identification and reduction of urinary aflatoxin metabolites in dogs. Food Chem Toxicol 42: 1851-1858.

79. Pelkonen P, Lang M, Wild CP, Negishi M, Juvonen RO (1994) Activation of aflatoxin B1 by mouse CYP2A enzymes and cytotoxicity in recombinant yeast cells. Eur J Pharmacol 292: 67-73.

80. El-Nezami HS, Polychronaki NN, Ma J, Zhu H, Ling W, et al. (2006) Probiotic supplementation reduces a biomarker for increased risk of liver cancer in young men from Southern China. Am J Clin Nutr 83:1199-1203.

81. Netke SP, Roomi MW, Tsao C, Niedzwiecki A (1997) Ascorbic acid protects guinea pigs from acute aflatoxin toxicity. Toxicol Appl Pharmacol 143: 429-435.

82. Yan S, Liang Y, Zhang J, Chen Z, Chun-Ming, et al. (2015) Autoxidated linolenic acid inhibits aflatoxin biosynthesis in Aspergillus flavus via oxylipin species. Fungal Genet Biol 81: 229-237.

83. Dönmez N, Dönmez HH, Keskin E, Kısadere İ (2012) Effects of aflatoxin on some haematological parameters and protective effectiveness of esterified glucomannan in Merino rams. Sci World J 2012:342468.

84. Alpsoy L, Yildirim A, Agar G (2009) The antioxidant effects of vitamin A, C, and E on aflatoxin B1-induced oxidative stress in human lymphocytes. Toxicol Ind Health 25: 121-127.

85. Cravens RL, Goss GR, Chi F, DeBoer ED, Davis SW et al. (2015) Products to alleviate the effects of necrotic enteritis and aflatoxin on growth performance, lesion scores, and mortality in young broilers. J Appl Poult Res 24: 145-156.

86. Allameh A (1997) Comparison of the effect of low- and high-dose dietary butylated hydroxytoluene on microsome-mediated aflatoxin B1-DNA binding. Cancer Lett 114: 217-220.

87. Bammler TK, Slone DH, Eaton DL (2000) Effects of dietary oltipraz and ethoxyquin on aflatoxin B1 biotransformation in non-human primates. Toxicol Sci 54: 30-41.

88. Hagler WM, Hutchins JE, Hamilton PB (1982) Destruction of Aflatoxin in corn with sodium bisulfite. J Food Protect 45: 1287-1291.

89. Tsai W-YJ, Shao K-PP, Bullerman LB (1984) Effects of sorbate and propionate on growth and Aflatoxin production of sublethally injured Aspergillus parasiticus. J Food Sci 49: 86-90.

90. Mankevičienė A, Lugauskas A, Repečkienė J, Gruzdeviene E (2006) The effect of environmental conditions on the variation of fungi and mycotoxin contents in oil flax seed Ekologija 3: 64-70.

91. Zicker SC (2008) Evaluating Pet Foods: How confident are you when you recommend a commercial pet food? Top Companion Anim Med 23: 121-126.

92. Crane SW, Griffin RW, Messent PR (2000) Introduction to commercial pet foods, In: Small Animal clinical nutrition. Walsworth Publishing Company, Marceline, USA.

93. Miller EP, Cullor JS (2000) Food safety. In: Small animal clinical nutrition. Hand MS, Thatcher CD, Remillard RL, Topeka, KS, Mark Morris Institute, USA.

94. Kabak B, Dobson AD, Var I (2006) Strategies to prevent mycotoxin contamination of food and animal feed: A review. Food Sci Nutr 46: 593-619.

95. Feuell AJ (1966) Aflatoxin in groundnuts. Problems of detoxification. Tropical Sci 8: 61-70.

96. Van Der Zijden ASM, Blanch Kolensmid WAA, Boldingh J (1962) Isolation in crystalline form of a toxin responsible for turkey X disease. Nature London, 195: 1062.