Biochemistry & Analytical Biochemistry
ISSN: 2161-1009

Martínez-Ruiz et al., Biochem Anal Biochem 2018, 7:4
DOI: 10.4172/2161-1009.1000366

Research Article Open Access

Mutagens and Carcinogens Called Aflatoxins and Their Hydroxylated Metabolites in Food for Domestic Cats

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Abstract

Aflatoxins (AFs) in cat food are a serious threat to cat health. The exposure of cats to AFs can cause damage depending on the exposure time and dosage, as well as the diet, nutritional state, age and sex of the cat. The first acute effect of AFs is liver damage, including cellular necrosis, hemorrhage, fibrosis, cirrhosis, immunosuppression, respiratory infection, anorexia, and fever. Chronic exposure to AFs can lead to hepatitis, cirrhosis and cancer in the liver, kidneys, lungs, colon and nervous system.

Domestic cats can tolerate up to 0.55 mg of AFB1 per kg of body weight, which is the Lethal Dose 50% (LD50) that causes acute toxicity. Subacute aflatoxicosis (0.5-1 mg of AF/kg of food for cats) produces anorexia, drowsiness, jaundice, intravascular coagulation, hematomas, hemorrhagic gut and death in 2 to 3 weeks. For cats, hepatotoxic effects occur with chronic exposure to AFs at 0.05-0.3 mg of AF/kg of food over 6 to 8 weeks.

Cat food samples, including 21 croquette samples and 32 semi-liquid food samples in envelopes and cans, were purchased from Mexico City markets from October 22, 2014 to January 10, 2015. A method for the analysis of AFs that included immunoaffinity columns, derivatization and quantification by HPLC was validated and used.

For the croquettes, the best sample brands had no basic AFs; however, when hydroxylated AF metabolites were considered, the average AF contamination increased from 4.98 to 20.87 μg kg^{-1}, values that are still within the AF tolerance level of 20 μg kg^{-1}. For the semi-liquid cat food samples in cans and envelopes, 23 of the 53 samples, or 43%, had no basic AFs; however, they were all contaminated when AF hydroxylates were assessed, with concentrations ranging from 2.76 to 15,974 μg kg^{-1}. The protective ingredients used in cat food differ from those used in dog food, as no hydrated sodium calcium aluminosilicates, glucomannans, are used, and only one sample had flaxseed omega -3 and -6 fatty acids.

Keywords: Aflatoxins; Carcinogens; Food contamination; Cats

Introduction

Origin

Cats, Felis silvestris subspecies catus [1], are felid carnivores that originated 65 million years ago in the Eocene [2]. Recent genetic evidence revealed the direct origin of domestic cats-5 wild female cats that associated with man 10,000 years ago in the Middle East [3-5].

Cats began their domestication as mealtime companions who fed from the plagues of mice that infested warehouses of the first farmers [4,6]. One of the first evidence of the origin of cats is from 9500 years ago in Cyprus [7]. Domestic cats come from the mountain cats of Europe, but they are tamer with different colors [8].

In Egypt, cats represented the goddess Bastet or Bastes, who symbolized light, warmth, sun energy, mystery, night and the moon and who brought males and animals fertility, healed diseases and took care of the souls of the dead [8]. Cats were present in Great Britain during the Stone age. Later, they were taken in ships to control the rats and to serve as good-luck amulets. During the Middle Ages, cats were associated with witchery due to the luminescence of their eyes and were burned with witches [9]. In Japan, the neko cat was a symbol of good fortune. Cats were also sacred to Muslims [10], and they ate rats, helping to control the black plague, which was transmitted by fleas [11].

Balanced food for cats

The total pet food market for 2017 was predicted to achieve USD $ 957 000 [12]. The USA is the most important pet food market, followed by Europe, Japan and Brazil. The USA and Europe comprise 80% of the pet food market. Providing balanced food for cats is a big business; in the USA, Pet Smart earned approximately USD $ 35000 million in 2017 [13]. With 700,000 tons per year, the Mexican market is the 10th largest market in the world for pet food, and it is increasing each year; 75% of pet food consumption in Mexico is from the imported Pedigree and Whiskas trademarks, followed by Nestlé México, which produces Cat Chow. Twenty-three percent of Mexican homes own cats [14].

Aflatoxins (AF)

The word aflatoxin comes from Aspergillus (A), flavus (fla) and toxin, which means poison [15]. Aflatoxins (AFs) are the most important mycotoxins in human foods and animal feed worldwide [16].

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Received: November 01, 2018; **Accepted:** November 20, 2018; **Published** November 27, 2018

Citation: Martínez-Ruiz NC, Carvajal-Moreno M, Rojo-Callejas F, Fuentes-Daza S, Gómez-Carrión A, et al. (2018) Mutagens and Carcinogens Called Aflatoxins and Their Hydroxylated Metabolites in Food for Domestic Cats. Biochem Anal Biochem 7: 366. doi: 10.4172/2161-1009.1000366

AFs are secondary metabolites produced mainly by the fungus Aspergillus. The fungal species that have been identified as producers of aflatoxin B1 (AFB1) include *A. pseudotamarii* (Japan and South America), Emericella astellata (South America), *E. olivicola* (Southern Europe) and *E. venezuelensis* (South America). AFB1 and AFB2 producers include Aspergillus flavus (ubiquitous), *A. ochraceoroseus* and *A. rambellii* (Africa), and producers of AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) include *A. nomius* (USA, Thailand, and South America), *A. parvisclerotigenus* (Africa), *A. parasiticus* (ubiquitous), *A. bombycis* (Japan and Indonesia), *A. arachidicola* (South America) and *A. minisclerotigenes* (USA, Africa, Australia and South America) [17].

Approximately 18 AFs have been chemically characterized as dihydrofuran-coumarin, and they are subdivided into two groups based on their structure. Group 1 contains difuran-coumarin-cyclopentanones (AFB1), aflatoxin B2 (AFB2), AFB2 derivatized (AFB2a), aflatoxin M1 (AFM1), aflatoxin M2 (AFM2), AFM2a and aflatoxicol (AFL), and Group 2 comprises difuran-coumarin-lactones (AFG1, AFG2, AFG2a, AFGM1, AFGM2, AFGM2a and AFB3) [18]. AFB2 and AFG2 have saturated difurans, and AFB2a and AFG2a have a hydrated difuran moiety. B- and G-type AFs produce long-wave ultraviolet light with excitation at 225-365 nm and emission at 425-450 nm, and they can be observed with a fluorescent lamp that produces blue (B-type AFs) or green (G-type AFs) light (Figure 1) [19,20].

Only AFB1, AFB2, AFG1 and AFG2 are naturally formed in foods due to contamination with aflatoxigenic Aspergillus. Other AFs (AFM1, AFM2, aflatoxin P1 (AFP1), aflatoxicol (AFL), etc.) are produced as hydroxylated metabolites by microbial or animal metabolisms [21]. AFB1 is the most toxic and abundant AF in foods. The order of AF toxicity is AFB1>AFG1>AFB2>AFG2 [19]. All these AFs contaminate many foods, such as cereals, oilseeds, spices, cotton, dry fruits and derived products [18].

The biotransformation [22] and biosynthetic routes of AFB1 were described [23].

Physicochemical properties

AFs are odorless and flavorless, they are resistant to high temperatures of over 200°C (260-320°C), and they can cause damage at trace concentrations of micrograms per kilogram (parts per billion). They are mutagens, carcinogens and teratogens that can cause malformations, and the most toxic AFs are AFB1 and AFG1 [24,25]. The physicochemical properties (molecular weight, excitation (absorbance) and emission wavelengths and extinction coefficient) of AFs have been reported (Table 1) [24].

AFs are solid crystals that change from white to yellow; they are soluble in water with difficulty and are very soluble in organic solvents, such as alcohol, chloroform, acetonitrile, acetone and benzene.

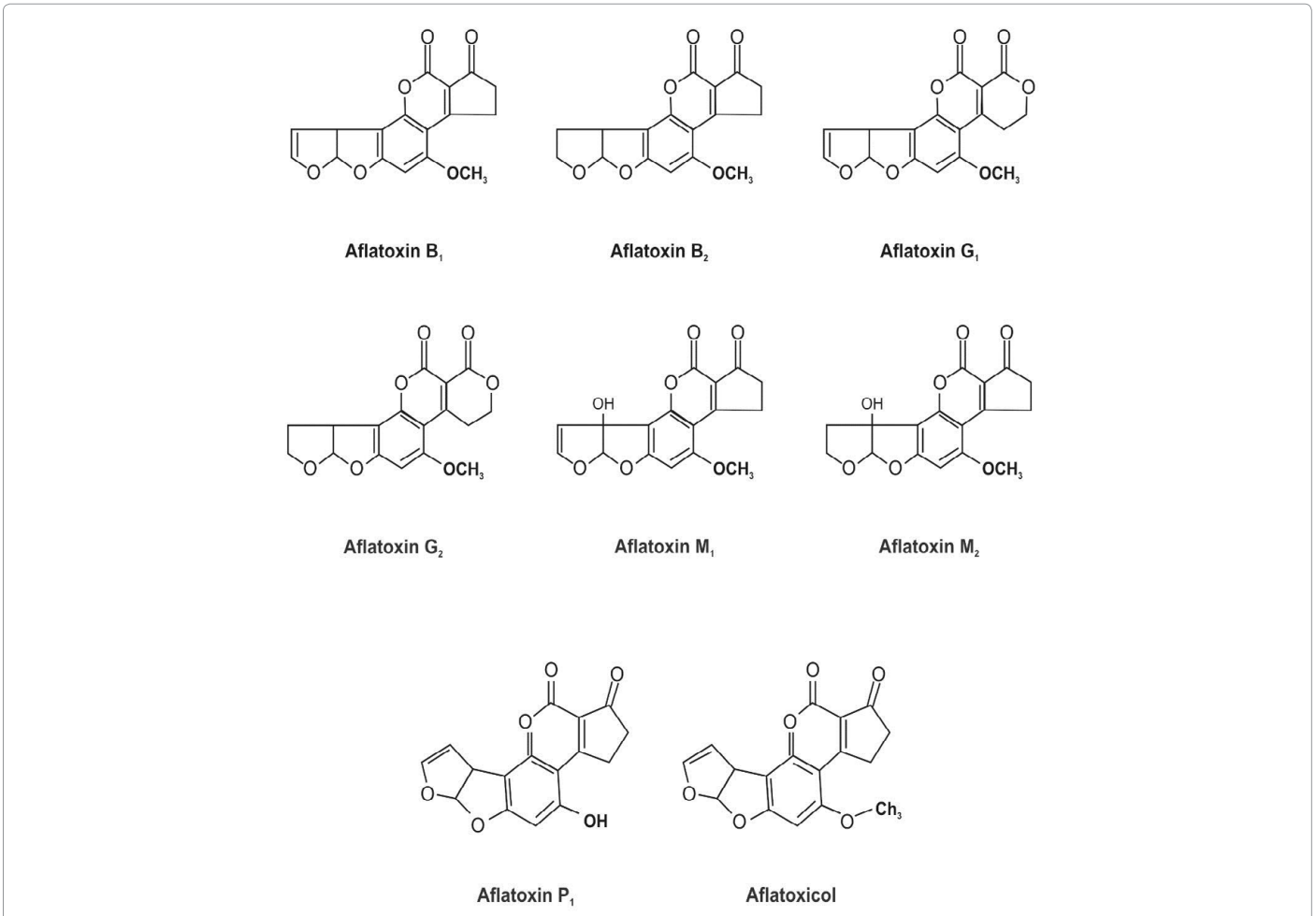

Figure 1: Chemical structure of basic aflatoxins (AFB1, AFB2, AFG1 and AFG2), and their hydroxylated metabolites AFM1, AFM2, AFP1 and Aflatoxicol [20].

Aflatoxin	Molecular weight	Excitation of wave length (Absorbance)	Emission of wave length	Extinction coefficient
AFB_1	312	362	425	21,800
AFB_2	314	362	425	24,000
AFG_1	328	362	450	17,700
AFG_2	330	362	450	17,100
AFM_1	328	357	425	21,250
AFM_2	328	357	425	22,900
AFP_1	298	362	425	15,400
AFL	314	325	425	14,100

Table 1: Physicochemical properties of the studied Aflatoxins [24].

AFs dissolved in chloroform or in a mixture of benzene:acetonitrile (98:2 v/v) are stable for years when stored in the dark and cold, but their stability decreases over time in methanol [26,27].

Sub index 1 or 2 of AF type B, G, M or P indicates their mobility in thin layer chromatography, which depends on their molecular weight, which ranges from 298 (AFP1) to 330 (AFG2 and AFM2), giving different retention coefficients by which to identify them [19].

Effects of aflatoxins on cats

The presence of mycotoxins in pet feed, including food for dogs, cats, fowl, fish, reptiles and rodents, is a serious threat to pet health. Cereals, dry fruits and dairy products are used as ingredients, and they are often contaminated [28].

The exposure of animals to AFs can cause severe damage depending on the exposure time and dosage, as well as the diet, nutritional state, age and sex of the animal [29]. The first acute effect of AFs is structural and functional liver damage, including cellular necrosis, hemorrhage, fibrosis and cirrhosis; other additional effects include hepatic encephalopathy, immunosuppression, respiratory infections, gastrointestinal hemorrhage, anorexia and fever. Chronic exposure to AFs can lead to hepatitis, cirrhosis and cancer mainly in the liver but also in other organs, such as the kidneys, lungs, colon and nervous system [30].

Domestic cats can tolerate AFB1 at concentrations up to 0.55 mg kg^{-1} of body weight, which is the Lethal Dose 50% (LD50) that causes acute toxicity [31].

After ingestion, AFs are absorbed and transported to the liver by the circulatory system. Later, they become toxic reactive epoxides that react with cellular macromolecules such as DNA and RNA enzymes as well as other proteins, causing damage [32]. Subacute aflatoxicosis (0.5-1 mg of AF/kg of pet food) is characterized by anorexia, drowsiness, jaundice, intravascular coagulation, hematomas and hemorrhagic gut, like the alterations caused by warfarin [32], and death occurs within 2 to 3 weeks. The hepatotoxic effects are like those produced by chronic exposure to AFs at 0.05-0.3 mg of AF/kg of pet food over 6 to 8 weeks [31].

Chronic AF poisoning appears after one or two months and manifests as a decrease in productivity along with weight gain, hair loss, anemia, abdominal swelling, mild jaundice, depression, anorexia, abortion and leg swelling. All studied species experience biliary duct proliferation in response to AFB1; hepatocyte changes (more vacuoles, fat degeneration and parenchymal loss) that cause necrosis in the liver also occur, depending on the species [29,33].

The aims of the present study are to accurately identify and quantify eight AFs (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2, AFP1 and AFL) in cat food samples from markets across the metropolitan area of Mexico City using previously validated AF extraction and quantitative methods with known linearity, limits of detection and quantification and recovery percentages.

Methodology

Sampling

Cat food samples, including twenty-one dry food or croquette samples and thirty-two semiliquid food samples in envelopes or cans, were purchased from markets in the metropolitan area of Mexico City from October 22, 2014 to January 10, 2015. The physicochemical properties of aflatoxins required for quantification have been reported previously (Table 2) [24].

Chemical extraction of AFs from cat food

Fifty grams of samples of each food type, dry or semiliquid enveloped or canned food, was independently blended (Waring ETL laboratory blender 7010S model WF 2211214, Torrington, CT, USA) with 100mL of a methanol:H2Od (80:20 v/v) mixture and two grams of NaCl to clarify food from the two food types. The blended mixtures were centrifuged (ALC 4235 refrigerated centrifuge) at 4300 rpm for 15 min, and the supernatants were retained. Two milliliters of each supernatant were dissolved in 14 mL of phosphate buffered saline (PBS) at 7.4 pH, and each mixture was slowly passed over an immunoaffinity column (Easi-Extract R-Biopharm Rhone LTD, UK) for total aflatoxins (AFt) [34,35]. The column was washed with 20 mL of H2Od and gravity eluted with 1.5 mL of pure HPLC-grade methanol, followed by 1.5 mL of H2Od with reflux. Three milliliters of each eluate were collected in an amber vials and dried in an oven (Novatech BTC 9100, Houston Texas, USA) at 40°C. Next, 200 µL of each eluate was derivatized to increase fluorescence, and 60 µL was then quantified in triplicate using liquid chromatography with fluorescence detection (HPLC-FL).

Derivatization for HPLC quantification

Derivatization with trifluoroacetic acid (TFA) is used to increase the fluorescence of AF samples or standards; in this process, AFB1 and AFG1, which are not very fluorescent, are transformed into their highly fluorescent hemiacetals, AFB2a and AFG2a. AFB2 and AFG2, which are fluorescent, do not undergo any transformation reactions during derivatization and are not affected by this reaction due to their saturated structures [36-38].

Each dried eluate was resuspended in 200 µL of acetonitrile (ACN) (JT Baker N° 75-05-8, Xalostoc, State of Mexico), and 800 µL of a previously prepared derivatizing solution containing 5 mL of trifluoroacetic acid (ATF) (Sigma-Aldrich, St. Louis MO, USA), 2.5 mL of glacial acetic acid (Merck, Naucalpan, Edo. Mex., Mexico) and 17.5 mL of deionized water was added, followed by shaking (Vortex G-560, Bohemia, NY, USA) for 30 sec. The amber vials were then placed in a steam water bath at 65°C for 10 min [36,37]. Later, HPLC

Croquettes	Semiliquid food, in envelopes or canned
Whiskas Mars, Mexico	**Whiskas Mars, Mexico**
1. Whiskas (meat)	22. Vapoured boiled salmon.
2. Whiskas (Supreme) fish	23. Supreme (mini beef filets vapor boiled).
3. Whiskas beef meat (Kitten)	24. Supreme (tuna baked mini filets).
4. Whiskas (chicken-milk)	25. Boiled turkey.
5. Whiskas (Supreme) Salmon	26. Steamed white fish.
Chow Purina	27. Supreme steamed chicken mini filets.
6. Cat chow delimix	28. Mix parrilla steam boiled
7. Cat chow con calorías reducidas.	29. Steam boiled beef brochette.
8. Cat chow (kitten) Milk, beef and fish.	30. Steam boiled turkey and viscera.
9. Cat chow (homecare)	31. Kitten, beef meat. Whiskas
10. Gatina home flavors. Purina	32. Supreme (baked salmon mini filets).
Other brands	33. Boiled chicken.
11. Magic cat	34. Vapor boiled chicken.
12. Mr cat	35. Temptation (salmon).
13. Minino plus. Neovia. Malta de México SA de CV	36. Cat milk.
20=14 Minino. Neovia 14=15 Nucat. Nupec	**Fancy feast, Purina-Nestlé**
	37. Mousse with ocean fish.
19=16. Optimo feline. Nupec 15=17. Catsky. Canis	38. Farm delights in gourmet sauce.
	39. Pathé gourmet. 40. Poultry mousse.
16=18. Pal gato. Pumascota Mercado Libre	41. Tuna fish mini filets.
	42. Salmon mini filets.
17=19. Minino dúo Fish & beef. Malta Texo de México	43. Minifilets with chicken.
18=20. OL´ROY. Comerc. Mexico-Am.S de RL de SV	
21. Royal canin (weight control)	**Felix Nestlé-Purina.**
	44. Turkey pathé and viscera.
	45. Marine sensations.
	46. Chicken in sauce.
	47. Chicken and salmon filets in sauce.
	48. Salmon pathé.
	49. Salmon and turkey filets in sauce.
	50. White fish sensations in sauce.
	51. Turkey sensations in sauce.
	52. Tuna fish sensations in sauce.
	K/D
	53. Feline renal health with chicken K/D

Table 2: Samples of dry food (croquettes) and semiliquid food, in envelopes or canned, food for cats analyzed for the presence of Aflatoxins.

quantification with fluorescence detection (HPLC-FL) was performed as described previously.

The AF standards (Sigma-Aldrich, St. Louis MO, USA) were derivatized, and different concentrations were prepared to construct calibration curves; the AFs eluted from the samples were also derivatized.

Liquid chromatography conditions

The chromatographic system used was an Agilent Series 1200 HPLC (Agilent Technologies, Inc., USA) that consisted of an isocratic pump (Model G1310A), a fluorescence detector (Model G1310A Series DE62957044, Agilent Technologies, Inc., USA) set to an excitation wavelength of 357-360 nm and an emission maximum of 450 nm and an autosampler (G1329A Series DE64761666). The VDS Optilab VDSpher 100 C18–E chromatography column (5 µm;250 x 4.6 mm) was maintained at room temperature (22°C) with a mobile phase of water:ACN:methanol (65:15:20 v/v/v) and degasified for 30 min by vacuum filtration. The flow rate was 1.0 mL min^{-1}. The Chem Station 32 software program was used for chromatographic quantification.

Validation of the extraction method

Validation of the analytical methods and cat food sample analyses were performed using known parameters [39-41]. Validation of the method ensured that the equipment was calibrated and working properly [42]. For validation, the following criteria were considered: the linearity of the calibration curves, the limits of detection (LOD) and quantification (LOQ) and the recovery percentage.

Linearity of the system (calibration curves)

The linearity of the system indicates 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 mathematical treatment of the results obtained during analyte analysis. The selected range and number of experimental points depend on the application method [41]. The coefficient of determination (R2) parameter should be near 1 [43].

Solutions with different concentrations of eight AFs were prepared from a 1000 ng (1 µg mL^{-1}) AF stock. AFM standards (0.25 mg) were

diluted with benzene: acetonitrile (98:2 v/v) (Merck, Naucalpan, Edo. Mex. México) according to a previously reported method [44]. Each mixture was homogenized in an orbital shaker (Vortex G-560, Bohemia NY, USA).

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

b. The following formula was used to calculate the concentration of each AF in the 1000 ng stock solution [44]:

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 mL^{-1}) of the 8 different AFs were created independently 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. Each curve equation and its statistical parameters were obtained. The slope value (b1), ordinate to origin (bo), determination coefficient (R2), confidence interval for the slope to origin (IC(β)), variation coefficient percentage (% CV), standard deviation (SD), limit of detection (LOD) and limit 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 chromatogram noise. The LOD is the AF concentration that gives a signal three times greater than the noise. The LOD is the smallest analyte concentration that can be detected by the chromatography system. The LOQ is the AF concentration that gives a signal 10 times greater than the noise [44]. 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 b1 is the value of the slope [45].

Recovery percentages

The recovery percentage is a measure of the accuracy of the method that expresses the proximity between the theoretical and experimental values. The recovery percentage is the difference between the average AF concentration (analyte) of a spiked sample and the concentration measured in a sample with no spiking divided by the spiked concentration [46].

% R=[(CF-CU)/CA]x100

where % R is the recovery percentage, CF is the spiked AF concentration, CU is the basal AF concentration of the no spiked sample, and CA is the AF spiked concentration of the spiked sample [46].

The arithmetic average, standard deviation, percentage of variation coefficient and confidence interval were calculated. To obtain accurate measurements, the cat food samples (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 (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2, and AFP1) and AFL. One aliquot that was not spiked was used as a control to provide the basal contamination level. The samples were individually processed using the R-Biopharm extraction method [47]. The AFs were purified and concentrated using an IAC and were subsequently derivatized and quantified by HPLC-FL, after which the percentage of recovery was obtained for each AF. After each derivatization mixture cooled to room temperature, each sample (60 μL) was injected into the HPLC-FL in triplicate.

Statistical analysis

The R statistical program was used to perform non-parametric Kruskal-Wallis tests to determine the differences among the AF contents in different samples (cans and croquettes were examined separately). The Wilcoxon range test was applied to determine the significance of the differences.

We compared the levels of aflatoxins in croquettes and in canned or enveloped cat food. When the test of equal variance was rejected for different aflatoxins, we used a t-test with different variances to test the equality of means.

Results and Discussion

Validation of the chemical method

The method was validated with linear calibration curves for the AFs (AFB1, AFB2, AFG1, AFG2), and hydroxylated metabolites (AFM1, AFM2, AFP1 and AFL), the limit of detection, the coefficient of determination and 85-100% recovery was obtained (Table 3).

Sampling

Cat food samples, including dry food and semiliquid food in envelopes and cans (Table 2), were obtained and analyzed, and the ingredients were categorized as AF-risk or AF-protective ingredients (Tables 4a and 4b). Validation of the analytical method is presented in Table 3. After quantifying the AFs (Tables 5 and 6), the ingredients explained the contamination and amounts of AFs discovered. AF-risk ingredients in cat food include cereals, such as corn and rice, and legumes, such as soybeans, which are frequently contaminated with

Aflatoxin	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Retention time (min)	R^2	Recovery percentage
AFB_1	0.5	5.0	7.085- 8.849	0.9986	90
AFB_2	0.05	0.5	17.452- 20.228	0.9817	100
AFG_1	0.5	5.0	7.681-9.541	0.9898	100
AFG_2	0.5	5.0	11.215-14.513	0.9946	100
AFM_1	0.1	1.0	8.514-8.769	0.9834	98
AFM_2	0.05	0.5	20.208-22.447	0.9946	95
AFP_1	0.05	0.5	15.563-19.318	0.9960	96
AFL	0.01	0.1	3.032-5.569	0.9978	99
LOD: Limit of Detection, LOQ: LOD x 10; RT: Retention Time in minutes, R2: Coefficient of determination of aflatoxin standards.					

Table 3: Validation of the extraction method.

Sample	Aflatoxin risk ingredients					Protective ingredients against aflatoxins							
	Cereals: maize, rice, sorghumwheat, hulls	Oilseeds paste, soybean or canola	Meat bone chicken,egg pork, beef and fat flour	Pigments (1)	Milk power, dairy	Probiotic yeast (2), beer yeast	Vitamins and minerals	*Yucca schidigera, (3),* folic acid	Maize gluten	Antioxidants		Sodium bisulphate	Omega 3,6
										BHA	BHT		
1,2,6	X	X	X	X			X	X		X	X	X	
3,4,7	X		X	X			X	X		X	X	X	
5,9	X	X	X	X			X	X	X	X	X	X	
8	X	X	X	X	X		X	X	X	X	X	X	
10	X	X	X	X			X		X	X	X	X	
11,12	X		X	X			X	X		X	X	X	
13	X	X	X	X	X	X	X	X	X				
14	X	X	X	X	X	X	X	X	X				
15	X	X	X				X	X		X	X		
16	X	X	X				X	X		X	X		
17	X	X	X	X		X		X	X	X	X		X
18	X	X	X				X		X			X	
19	X	X	X	X	X	X	X	X					
20	X	X	X	X			X	X		X	X	X	
21	X	X	X	X		X	X	X	X			X	
Yellow, 5, 6; blue 2, red 40, natural caramel brown; titanium dioxide. (2) Bacilllus amyloliquefaciens (1 x 104 UFC/g minimum), Lactobacillus acidophilus (3.5 x 104 UFC/g minimum) and *Saccharomyces cerevisia*													

Table 4a: Analyzed dry food croquettes for cats.

Sample	Aflatoxin risk ingredients						Protective ingredients against aflatoxins					
	Cereals: maize, rice, sorghum wheat, hulls	Oilseeds paste, soybean or canola	Meat bone chicken, beef, pork, fish and fat flour	Pigments (1) or titanic dioxide	Artificial flavors	Milk power, dairy	Vitamins and minerals	*Yucca schidigera, (3),* folic acid	Maize gluten	Antioxidants		Sodium bisulphyte, of menadione
										BHA	BHT	
22-26	X	X	X	X	X		X			X	X	
27-35	X	X	X	X	X		X		X	X	X	
36						X	X			X	X	
37	X		X	X			X			X	X	X
38,39,43-46, 48-52	X	X	X	X			X	X				X
40			X	X	X		X					
41,42	X	X	X	X			X					X
47	X	X	X	X			X	X	X			X
53	X		X				X	X				
Pigments: Yellow, 5, 6; blue 2; red 40; red 3, titanium dioxide, iron oxide.												

Table 4b: Analyzed semi-liquid food in envelopes and canned food for cats.

Croquettesamples	Basic aflatoxins				Sum of 4 basic AFt	Hydroxylated aflatoxins				Sum of 8 AFt
	AFB_1	AFB_2	AFG_1	AFG_2		AFM_1	AFM_2	AFP_1	AFL	
1.	11.30	<LOD	24.40	1.26	36.96	<LOD	0	1.28	11.56	49.80
2.	3.80	0	0	0	3.80	0	0.81	0	20.06	24.68
3.	0.74	0	29.95	2.76	33.45	7.34	2.68	0	5.26	48.74
4.	0	0	<LOD	<LOD	0	0	0	<LOD	4.98	4.98
5.	1.01	0	0.94	<LOD	1.95	0	0	0.33	30.99	33.27
6.	2.52	<LOD	18.31	6.03	26.86	3.50	0	11.41	0.51	42.28
7.	<LOD	0.26	0.87	0.18	1.31	0	0.82	0.23	3.46	5.83
8.	<LOD	0	<LOD	0	0	1.49	12.30	0.22	4.40	18.40
9.	5.15	0	19.11	<LOD	24.26	0	0	0.29	61.59	86.14
10.	4.97	8.21	0	6.11	20.29	0	20.93	126.57	5.11	171.90
11.	2.61	<LOD	2.75	0.87	6.23	0.87	0	25.72	8.78	41.59
12.	0.92	2.10	0	2.16	5.18	0	11.66	0	21.93	38.76
13.	<LOD	0	<LOD	0	0	0	0	0.49	12.21	12.69
14.	2.07	0	2.98	0	5.05	0	0	0.32	23.74	29.10
15.	0	0	0	1.61	1.61	0	0.76	0	10.47	12.84
16.	1.39	0	0.61	2.02	4.02	0	<LOD	1.04	13.83	18.89
17.	<LOD	0	0	<LOD	0	0	0.81	0	20.06	20.87
18.	<LOD	1.72	0	1.03	2.75	0	3.68	0	15.74	22.17
19.	0	0	0	0	0	0	0	<LOD	19.26	19.26
20.	1.55	0	<LOD	0.84	2.39	0	0	0.64	36.41	39.45
21.	<LOD	0	<LOD	<LOD	0	7.42	0	<LOD	9.26	16.69
Average	2.54	0.68	6.25	1.56	11.03	1.03	2.72	9.36	16.17	36.11

Table 5: Aflatoxins (µg kg^{-1}) in dry food, croquettes for cats.

Cans/envelopes	Basic aflatoxins				Sum of 4 basic AFt*	Hydroxylated aflatoxins				Sum of 8 AFt*
	AFB_1	AFB_2	AFG_1	AFG_2		AFM_1	AFM_2	AFP_1	AFL	
22	0	0	<LOD	<LOD	0	0	0	1.34	122.07	123.41
23	0	0	0	<LOD	0	<LOD	0	1.07	2.54	3.61
24	0	0	0	0	0	0	0	0.44	7.03	7.47
25	0	0	0	0.96	0.96	0	0	19.26	2.65	22.87
26	0	0	0	0	0	0	0	0.43	4.49	4.92
27	0	0	0	0	0	0	0	0.80	2.30	3.10
28	0	0	0	<LOD	0	0	0	0.43	6.69	7.13
29	0	0	0	<LOD	0	0	0	0.50	2.47	2.96
30	0	0	0	1.25	1.25	0	0	1.63	7.09	9.97
31	0	0	0	0.61	0.61	0	0	0.73	3.19	4.52
32	0	0	0	<LOD	0	0	0	0.25	5.45	5.70
33	0	0	0	<LOD	0	0	0	0.36	2.75	3.11
34	0.16	0	0	0	0.16	0	0	0.22	5.3	5.71
35	4.75	0	<LOD	0.79	5.54	0	0	<LOD	15,968	15,974
36 (1)	0	0	0.99	2.70	3.69	0	0	0.21	2.03	5.92
37	<LOD	0	0	0	0	0	0	0	15.64	15.64
38	<LOD	0	0	0	0	0	0	0	19.44	19.44
39	0.61	0	0.82	0	1.43	0	24.69	827.78	23.91	878
40	0	0	0	0	0	0	0	0	19.35	19.35
41	0	0	0	0	0	<LOD	0	0	15.49	15.49
42	8.95	0	16.07	0	25.02	3.78	0	0	556.06	585
43	0	0	0	<LOD	0	0	0	0.40	2.37	2.77
44	0	0	0	0	0	0	0	0	7.65	7.65
45	0	0	0	0	0	<LOD	0	0	10.56	10.56
46	0	0	0	0	0	0	0	0	15.07	15.07
47	0	0	0	0	0	0	0	0	9.92	9.92
48	0	0	0	0	0	0	0	0	20.23	20.23
49	0	0	0	0	0	0	0	0.74	4.60	5.34
50	0	0	0	0	0	0	0	30.56	11.92	42.48
51	<LOD	<LOD	0	<LOD	0	0	0	2.14	2.51	4.65
52	0	0	0	0	0	0	0	0.46	2.31	2.76
53	0.47	<LOD	<LOD	1.11	1.58	<LOD	0	1.27	5.43	8.27
Average	0.52	0	0.62	0.31	1.45	0.14	0.77	28.74	527.70	557.71
Whole milk, non fat dry milk, malt extract, lactase taurine and stabilizers.										

Table 6: Aflatoxin (µg kg^{-1}) contamination in semiliquid, cans and envelopes, food for cats.

Food	AFB_1	AFB_2	AFG_1	AFG_2	Sum of 4 AF	AFM_1	AFM_2	AFP_1	AFL	Sum of 8 AF
Croquettes	2.54	0.68	6.25	1.55	11.03	1.03	2.72	9.36	16.17	36.11
Cans/envelopes	0.52	0	0.62	0.31	1.45	0.14	0.77	28.74	527.70	557.71
Difference in dry food compared to semiliquid	+2.02	+0.68	+5.63	+1.24	9.58	+0.89	+1.95	-20.62	-511.53	-521.60

Table 7: Comparison of total AFB2 and AFG2 Aflatoxins in dry, semiliquid and canned food for cats.

AFs. Artificial pigments pose a cancer risk [48]. 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 suggested safety concerns, but Citrus Red 2 is used at low levels and only in some Florida oranges; Orange B has not been used for several years [48]. All cat food contains derivatives from all types of meat and viscera, such as the liver, which are usually contaminated with AFs [49]. Aflatoxins can be present in milk, meat from swine or chicken, and eggs if the animals consume sufficient amounts of AF-contaminated feed [50]. The risks associated with mycotoxin exposure can be related to the amount of food a pet can consume daily for their entire life with no adverse effect (NOAEL) [51]. The effects of aflatoxins on pets are severe and lead to death from hepatitis, with the causal agent typically being AFB1 [52,53].

Among the ingredients that protect against AFs, those commonly used in pet food include maize gluten [54]; hydrated sodium calcium aluminosilicate [55]; probiotic yeast [56]; probiotic bacteria [57]; ascorbic acid [58]; linolenic acid [59]; glucomannans [60]; vitamins and minerals [61]; folic acid sources [62]; antioxidants, including phenols and butylated hydroxytoluene (BHT) [63]; ethoxyquin [64]; sodium bisulfite [65]; sodium propionate [66]; and flax seed omega-3 and -6 fatty acids [67]. The most commonly used protective ingredients in cat food are maize gluten, antioxidants such as butylated hydroxytoluene (BHT) and ethoxyquin, sodium bisulfite, and vitamins and minerals. Protective ingredients that are never present in cat food include hydrated sodium calcium aluminosilicate, linoleic acid, glucomannans,

and flax seed omega -3 and -6 fatty acids. Probiotic yeast is found only in dry food, never in canned or other semi-liquid foods. Dry food is easier to store and is the main food type in the pet food industry. Dry foods have a low water content that is protective against spoilage. The resulting extruded material has a moisture content of approximately 25% before drying and a final moisture content of 8 to 10% after drying, which inhibits mold formation [68-70]. Thermal inactivation processes are not sufficient to control preformed aflatoxins in ingredients. Mycotoxins are thermally stable due to their chemical structure, so commonly used food manufacturing techniques do not destroy them. Aflatoxins are stable up to their melting point of approximately 250°C, and they are not destroyed by boiling water, autoclaving, or a variety of other food and feed processing procedures.

Considering the 4 basic AFs found in croquettes, different sample brands had no AFs and appear to be the best food choices: 4) Whiskas (Chicken-milk); 8) Cat chow (kitten milk, beef and fish); 13) Neovia Malta de México SA de CV (Minino plus); 15) Canis (Catsky); 17) Malta Texo de México (Minino duo Fish and beef); and 21) Royal Canine (weight control).

Conversely, when hydroxylated aflatoxin metabolites were considered, different results were obtained: 4) Whiskas (Chicken-milk) had 4.98 μg kg^{-1}; 8) Cat chow (kitten milk, beef and fish) had 18.40 μg kg^{-1}; 13) Neovia Malta de México SA de CV (Minino plus) had 12.69 μg kg^{-1}; 15) Canis (Catsky) had 20.87 μg kg^{-1}; 17) Malta Texo de México (Minino duo Fish and beef) had 19.26 μg kg^{-1}; and 21) Royal Canine (weight control) had 16.69 μg kg^{-1} (Table 5). However, these foods are still within the AF tolerance level of 20 μg kg^{-1}.

Twenty-three of the 53 semi-liquid cat food samples from cans and envelopes, or 43%, had no basic AFs upon analysis; however, when the AF hydroxylates were considered, all the samples were contaminated with AFs, at concentrations ranging from 2.76 to 15,974 μg kg^{-1} (Table 6).

Most laboratories quantify only the 4 basic AFs. However, even though the AF hydroxylates are easier to eliminate, they stay in the body for some time, and AFM1 and AFP1 are still mutagenic and can cause damage. AFL is the most serious health risk because it can interconvert to AFB1, which is the most toxic AF. In general, most of the basic AFs are biotransformed into AFL or AFP1. The total amount of AFL (527.70 μg kg^{-1}) in the semiliquid canned cat food was higher than that in the croquettes; conversely, the latter had larger amounts of all AFs except for AFL (16.17μg kg^{-1}). The low number of other AFs detected in semiliquid food is likely due to the biotransformation of the other AFs to AFP1 and AFL (Tables 6 and 7).

Results of the statistical analysis

Statistically significant differences were observed for AFB1, AFB2, AFG1 and AFG2, with the average amounts of these aflatoxins being greater for croquettes in all cases. The differences in the AFM2 concentrations were not statistically significant at 5% but were at 10% (Table 8).

The Student t-test and p-values results are presented in Table 9. Kruskal Willis tests were used to find differences among the samples, with croquettes and can analyzed separately. Sample 35 (Temptation salmon), which had the antioxidants BHA and BHT, was the only sample with no basic AFs; however, it had high amounts of hydroxylates (AFt 15,973.88 μg kg^{-1}) of which 15,968 μg kg^{-1} were AFL, far exceeding the concentrations of other contaminating AFs. The risk of samples containing AFL is that it can interconvert to AFB1 inside the body and accumulate to concentrations of 0.5 to 1 mg of AF/kg of body weight,

Aflatoxin	Cans	Croquettes
AFB_1	<0.0001	0.0623
AFB_2	0.5009	0.4451
AFG_1	<0.0001	0.0317
AFG_2	<0.0001	0.0155
AFM_1	0.1583	0.1699
AFM_2	<0.0001	0.0437
AFP_1	<0.0001	0.0002
AFL	<0.0001	0.002

Table 8: p values for the Kruskal Willis tests.

Aflatoxin	t-test	p-value
AFB_1	2.6225	0.0102
AFB_2	2.5779	0.0109
AFG_1	2.1506	0.0351
AFG_2	2.5011	0.0150
AFM_1	1.5286	0.1309
AFM_2	1.9305	0.0576
AFP_1	-1.3449	0.1815
AFL	-1.3008	0.1963

Table 9: T-test unequal variances.

amounts that have been shown to cause alterations that can kill cats in 2 or 3 weeks [32]. We performed pairwise comparisons using Wilcoxon rank sum tests for the different aflatoxins. Table 8 shows these results for the croquettes; we did not find any significant differences for AFB2 and AFM1 among the brands.

Table 9 shows the pairwise comparison results for the envelopes and cans; only Sample 24 had an AFM2 concentration that was significantly different from zero. Moreover, we did not find any significant differences for AFB2 and AFM1 among the brands.

Conclusions

Considering the 4 basic AFs in croquettes, different sample brands had no AFs and appear to be the best food choices. Conversely, when hydroxylated aflatoxin metabolites were considered, the AF contamination was ranged from 4.98 μg kg^{-1} to 20.87 μg kg^{-1}; however, these foods are still within the AF tolerance level of 20 μg kg^{-1}. Twenty-three of the 53 semiliquid cat food samples from cans and envelopes, or 43%, had no basic AFs upon analysis; however, when the hydroxylates were considered, all the samples were contaminated with AFs, at concentrations ranging from 2.76 to 15,974 μg kg^{-1}.

The chemical method used to analyze the AFs was validated, and the AF concentration of dry and semiliquid cat food samples were measured. Croquettes had greater AF contamination than that found in semiliquid food, except for AFL. The protective ingredients used in cat food are different from those used in dog food, as hydrated sodium calcium aluminosilicates, glucomannans and flaxseed omega-3 and -6 fatty acids are not used. Aflatoxicol is a risky toxin and it was found in high amounts, as a biotransformed product from AFB1.

Acknowledgements

We would like to thank the following personnel at the Instituto de Biología, Universidad Nacional Autónoma de México (IBUNAM): Noemí Chávez (Technical Secretary), 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.

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