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Research Article

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Simultaneous Occurrence of Aflatoxins and Fumonisins in Corn Intended for the Pet Feed Industry and for Human Consumption

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Abstract

In this study, 24 samples of corn intended for the pet feed industry (PFI) and 24 samples of corn for human consumption (HC) commercialized in São Paulo, Brazil were analyzed for aflatoxins B_1, B_2, G_1 and G_2, and fumonisins B_1 and B_2 by high performance liquid chromatography. The concentrations of total aflatoxins in positive samples of PFI (33.3%) and HC (70.8%) corn samples ranged from 0.5 to 3.9 µg/kg and 0.5 to 41.8 µg/kg, respectively. For fumonisins, the concentrations in positive samples of PFI (100%) and HC (83.3%) corn samples ranged from 685 to 11,379 µg/kg and 157 to 6,495 µg/kg, respectively. Overall the aflatoxin levels complied with regulations for food products in Brazil (20 µg/kg), except for one sample of HC corn. However, the high incidence of fumonisins indicates the need for better agricultural practices to avoid contamination of corn, especially in the PFI.

Keywords

Mycotoxins, Aflatoxin B_1, Fumonisin B_1, Corn kernels, Occurrence, Quantitative

Introduction

Mycotoxins are secondary metabolites produced by fungi that grow in food products worldwide, leading to a great variety of toxic effects in vertebrates, including humans [1]. Toxigenic fungi may contaminate food products at different phases of production and processing, mainly under favourable humidity and temperature conditions [2]. In Brazil, the most common toxigenic fungi found in cereals include species from the genera *Aspergillus* and *Fusarium*. *Aspergillus* species, mainly *A. flavus*, *A. parasiticus* and *A. nomius*, produce aflatoxins, which have high toxicity, teratogenicity, mutagenicity and carcinogenicity [3].

There are four major aflatoxins, namely B1, B2, G1 and G2, which can contaminate feed and foodstuffs. However, aflatoxin B1 (AFB1) is the most commonly occurring and has the higher toxigenic potential, with the liver as the main affected organ [2]. The occurrence of AFB1 in food products is a public health concern because the International Agency for Research on Cancer (2015) [4] classified AFB1 in Group 1 - human carcinogen. AFB1 can produce liver tumors in Fisher rats at levels as low as 50 µg/kg [5]. Fumonisins are a group of structurally related mycotoxins produced mainly by Fusarium verticillioides. Although 28 fumonisin analogs have been identified since 1988, fumonisin B1 (FB1) is the most prevalent and also the most toxic, FB1 is considered a possible carcinogen in humans (group 2B) by the International Agency for Research on Cancer (2015) [4]. Since their identification, fumonisins have been associated with animal diseases such as equine leukoencephalomalacia and porcine pulmonary

edema, although at concentrations nearly 1,000 higher than the toxic levels of AFB1 [3].

Outbreaks related to pet food contaminated with aflatoxins have been described in several countries, including USA [6] and Israel [7], with corn as the usual source of aflatoxins in the feed. Although outbreaks caused by mycotoxins in pet animals have not been described in Brazil, a few cases of natural aflatoxicosis in dogs were reported in Rio Grande do Sul [8]. Moreover, previous studies reported aflatoxin contamination in the 12% of 4 of samples collected in Santa Catarina State presenting at least one type of mycotoxin [9]. Fumonisin-related outbreaks in pet animals have not been described in the literature.

Brazilian corn production is around 75 million tons per year, of which approximately 65% is destined for the animal feeding industry. Previous reports have indicated that the incidence of aflatoxins in Brazilian corn is highly variable, with mean levels of up to 460 µg/kg [10, 11]. For fumonisins, higher incidences (up to 100%) were reported in corn, with mean levels ranging from 20-22,600 µg/kg [10-12]. However, there is very little information on the simultaneous occurrence of aflatoxins and fumonisins in Brazilian corn kernels. The objective of the present trial was to evaluate the simultaneous occurrence of fumonisins and aflatoxins in corn kernels available for the pet feed industry (PFI) and for human consumption (HC) in São Paulo, Brazil.

Materials and Methods

Sampling procedures

Corn intended for the PFI was collected during the same 6-month period, from a large scale supplier of corn located in Porto Ferreira city, state of São Paulo (4 samples from different batches per month), totaling 24 samples. The supplier received corn from several producers in the state of São Paulo by the time of sampling, stored the product for a period of time and distributed it for at least 8 pet feed factories in the state. Sampling procedures followed the recommendations of Food and Agriculture Organization [13]. Incremental portions of corn were collected at different intervals during the transference of kernels from trucks to the storage silos, until reaching 16 kg of corn for each sample. The kernels were collected in sterile polypropylene bags, identified and kept at room temperature until analysis.

Samples of packaged corn kernels for HC were collected in the cities of Araras, Leme, Pirassununga and Porto Ferreira, all located in the Northeast of the state of São Paulo. In each city, samples from the brands that showed the greatest trade volume in supermarkets were collected from January to June 2008 (4 samples per month, one per city), totalling 24 samples of corn. The sampling unit was made up of original closed packages of at least 500 g of kernels, collected from different batches as indicated in the label, avoiding batch repetition. Samples were identified, including data on the manufacturer, batch and/or manufacturing date and expiring date. Products were stored in their respective packages and kept at room temperature (same supermarket conditions) until the moment of analysis.

Extraction of aflatoxins and fumonisins

The extraction and purification of aflatoxins (B_1, B_2, G_1 and G_2) and fumonisins (B_1 and B_2) in PFI and HC samples were performed using immunoaffinity columns, following manufacturer recommendations (Aflatest® or Fumonitest®, Vicam, Watertown, MA, USA). Previously, the total amount of corn samples collected in supermarkets and a 2.5 kg sub-sample of corn collected in the supplier were grinded in a hammer mill (Marconi, Piracicaba, Brazil) until a particle size of nearly 14 mesh, and mixed thoroughly. An aliquot of each sample (50 g) was weighted in an Erlenmeyer flask containing 5 g of sodium chloride and 100 ml of methanol/water (80:20, v/v) were added. The flask was placed in an orbital shaker (Tecnal, Piracicaba, Brazil) for 30 minutes, the flask content was filtered and 10 ml of the filtrate were placed in a Becker. 40 ml of ultra-pure water (Milli Q, Millipore, Bedford, MA, USA) were added and the mixture was filtered in a 1.5 µm microfiber filter. Two 10 ml aliquots were then passed through the immunoaffinity columns (Aflatest® or Fumonitest®) at flow rate of 1-2 drops/sec. After washing with 10 ml of ultra-pure water, aflatoxins or fumonisins were eluted from their respective columns with 1 ml of methanol, being each eluate collected in an amber vial. The aflatoxins and fumonisins eluates were evaporated to dryness under nitrogen flow.

Determination of aflatoxins

AFB_1 and AFG_1 in the final extracts were derivatized by adding 200 µL of n-hexane and 200 µL of trifluoroacetic acid (TFA) to the aflatoxin dried extract [14]. The mixture was kept at 40 °C for 10 min, evaporated to near-dryness and diluted in 1 ml of methanol: water (50:50, v/v). Final extracts were filtered through a 0.45 µm PTFE membrane and 20 µL were injected into a Shimadzu (Kyoto, Japan) 10VP high performance liquid chromatograph (HPLC) with a 10 AXL fluorescence detector (excitation at 360 nm and emission above 440 nm). A Shim-Pack CLC-ODS Sil column (4.6 X 250 mm, 5 µm) and a Shim-Pack pre-column (4 X 10 mm, 5 µm CLC G-ODS) were used. The isocratic mobile phase consisted of methanol-water (45:55, v/v) with a flow rate of 1.0 ml/min. Calibration curves were prepared using standard solutions of aflatoxins B_1, B_2, G_1 and G_2 (Sigma, St Louis, MO, USA) previously evaluated according to Scott (1990) [14]. The individual aflatoxins solutions were mixed in convenient volumes to reach working solutions at the concentrations of 2.5, 5.0, 10.0 and 20.0 ng/ml of each aflatoxin. Aflatoxins working solutions were prepared with TFA similarly to the sample extracts. The retention times were approximately 4.2 min for AFG_1 (converted to AFG_{2a}), 5.0 min for AFB_1 (converted to AFB_{2a}), 7.1 min for AFG_2 and 9.7 min for AFB_2 (Figure 1).

Determination of fumonisins

Fumonisin dried extracts were re-diluted with 200 µL of acetonitrile-water (50:50, v/v) and filtered using a 0.45 µm PTFE membrane. A 100-µL aliquot was placed in a test tube. After that, 200 µL of *o*-phthaldialdehyde (OPA) reagent (prepared using 40 µg of OPA diluted in 5 mL of sodium tetraborate solution 0.1 M and 50 µL of 2-mercaptoethanol) were added. After 2 minutes, 20 µL were used for quantification of the toxins in the same HPLC system described before, with a

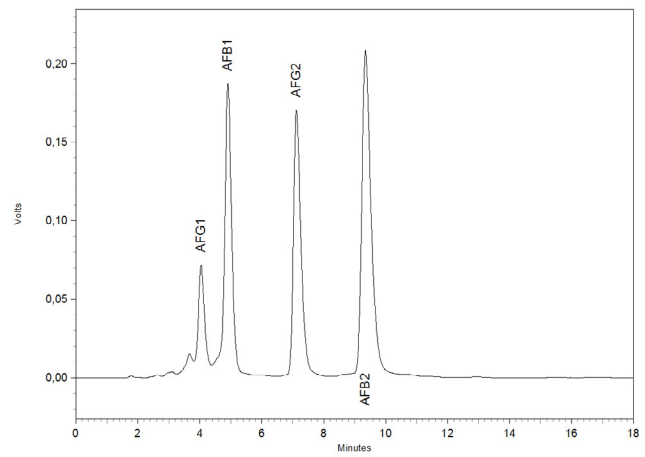

Figure 1: Chromatogram showing the retention times of aflatoxin standards (20.0 ng/ml of each compound): AFG1 (converted to AFG2a) (nearly 4.2 min), AFB1 (converted to AFB2a) (nearly 5.0 min.), AFG2 (nearly 7.1 min.) and AFB2 (nearly 9.7 min.).

reverse phase C_{18} column (150 x 4.6 mm, particle size 5 µm – Phenomenex, Torrance, USA) kept at constant temperature of 30 °C. Mobile phase was made up of acetonitrile-water-acetic acid (50:50:1, v/v), and constant flow of 1.0 ml/min was used. Detection of derivatized fumonisins was performed using the same HPLC system as described for aflatoxins, under fluorescence from 335 to 440 nm for excitation and emission, respectively. The calibration curves were performed from successive chromatographic measurements of FB_1 and FB_2 standards in triplicate at concentrations of 0.313, 0.625, 1.25, 2.5 and 5.0 µg/ml of each fumonisin. Retention time was approximately 9.5 minutes for FB_1 and 27.4 minutes for FB_2 (Figure 2).

Validation procedures

The limits of detection (LOD) and quantification (LOQ) were calculated for each method of analysis based on signal:noise ratio of 3:1 and 10:1, respectively. Linearity was evaluated by verifying the coefficient of determination (r^2) and

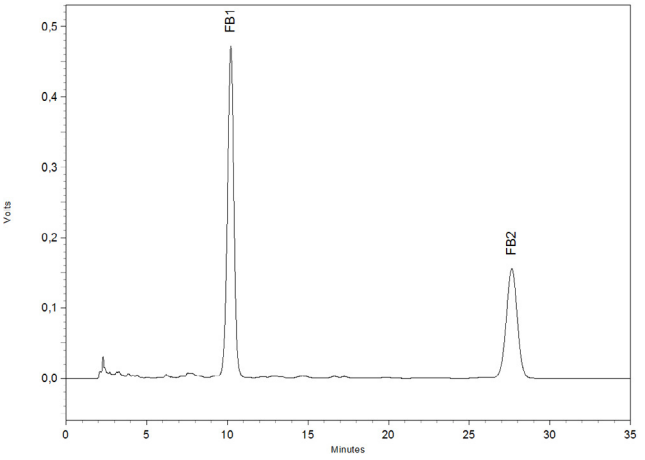

Figure 2: Chromatogram showing the retention times of fumonisin standards (5.0 µg/ml of each compound): FB1 (nearly 9.5 min), FB2 (nearly 27.4 min.).

visual inspection of residual plots of analytical curves built separately for each mycotoxin. The analytical methods used in the experiment were evaluated using commercially available corn samples previously analyzed for endogenous AFB_1 and FB_1. Triplicate samples were spiked with AFB_1 and FB_1 at levels of 2 and 20 µg/kg and 50 and 500 µg/kg, respectively. All these fortified samples were analyzed as described for samples collected in supermarkets and in the corn supplier.

Results and Discussions

Method performance

Recovery values [Table 1] for aflatoxins and fumonisins ranged from 77.5 to 103.7% and 78.2 to 93.4%, respectively. Relative standard deviations were lower than 13.2% for aflatoxins, and 15.7% for fumonisins, which comply with the requirements of the EC directive 401/2006 [15]. LOD and LOQ values for AFB_1, AFB_2, AFG_1 and AFG_2 were 0.15 and 0.5 µg/kg, respectively. For FB_1 and FB_2, LOD and LOQ were 10 and 30 µg/kg, respectively.

Aflatoxins and fumonisins in corn samples

Aflatoxins and fumonisins levels in PFI and HC corn samples are shown in [Table 2]. Corn intended for HC presented high incidence (70.8%) of total aflatoxins (B_1 + B_2 + G_1 + G_2) at levels ranging from 0.5 to 41.8 µg/kg (mean: 4.4 ± 9.8 µg/kg). Although a limited number of samples was analyzed (n = 24), one sample (4.1%) had total aflatoxins above the permitted maximum limit (20 µg/kg) adopted by Brazilian regulations [16], hence indicating health risks associated to dietary exposure to aflatoxins in Brazil. Another sample had 6.7 µg/kg AFB_1, which is higher than the limit adopted by the European Union (5 µg/kg for AFB_1 and 10 µg/kg for total aflatoxins) [17]. For fumonisins, the percentage of positive samples was even higher (83.3%), with levels ranging from 157 to 6,495 µg/kg (mean: 2,597 ± 1,958 µg/kg). 10 samples (41.7%) had concentrations above the limit for FB_1 and B_2 (2,000 µg/kg) [16].

Table 1: Performance of the analytical methods for aflatoxins and fumonisins in corn intended for human consumption and for the pet feed industry in São Paulo, Brazil.

Mycotoxin	Spiking level (µg/kg)	Recovery (%)1	Relative standard deviation (%)
AFB_1	2.0	88.0 ± 7.7	12.3
AFB_1	20.0	103.7 ± 2.9	3.1
AFB_2	2.0	90.3 ± 8.1	9.7
AFB_2	20.0	99.5 ± 4.5	5.3
AFG_1	2.0	84.2 ± 8.9	10.1
AFG_1	20.0	100.3 ± 4.3	4.7
AFG_2	2.0	92.4 ± 11.2	13.2
AFG_2	20.0	77.5 ± 4.7	6.5
FB_1	50	78.2 ± 8.0	10.4
FB_1	500	93.4 ± 6.5	6.9
FB_2	50	89.8 ± 12.2	15.7
FB_2	500	83.4 ± 7.1	8.3

[1]Values expressed as mean ± standard deviation of samples analyzed in triplicate.

Despite the limitations in the sample collection, results for aflatoxins alone in HC corn in this trial were higher

than previous data reported in Brazil. Bento et al., [18] found aflatoxins in 19% and 23.8% of corn samples from Mato Grosso state harvested in 2009 and 2010 respectively, although at higher levels (1.0 to 108.7 µg/kg). 8% of 300 samples of freshly harvested corn collected in the reception and pre-drying steps in Paraná State had aflatoxins at mean concentration of 30.6 µg/kg [11]. However, the authors found fumonisins in 100% of the analyzed samples, with similar mean levels (2,082 µg/kg) as obtained in the present study. Moreover, the mean fumonisin level in corn intended for HC as presented in our work were in agreement with those found by Westhuizen et al. [12], in Santa Catarina state (mean: 2,870 µg/kg). In summary, the low levels of aflatoxin and high levels and incidence of fumonisin in corn reported in the present study is in accordance with previous studies indicating that producers of corn for HC have improved control practices to avoid contamination of corn grain with aflatoxin, but not fumonisin.

Table 2: Aflatoxins and fumonisins in corn intended for human consumption and for the pet feed industry in São Paulo, Brazil.

Mycotoxin	*n*[1]	%[2]	Range[3] (µg/kg)	Mean[4] (µg/kg)
Corn for human consumption:				
Aflatoxin B_1	17	70.8	0.5 - 38.0	3.5 ± 9.0
Aflatoxin B_2	2	8.3	0.5 - 0.6	0.6 ± 0.1
Aflatoxin G_1	5	20.8	0.5 - 4.5	2.3 ± 1.5
Aflatoxin G_2	2	8.3	0.5 - 0.7	0.6 ± 0.1
Total aflatoxins	17	70.8	0.5 - 41.8	4.4 ± 9.8
Fumonisin B_1	20	83.3	157 - 6,204	2,537 ± 1,949
Fumonisin B_2	12	50.0	30 - 291	100 ± 91
Total fumonisins	20	83.3	157 - 6,495	2,597 ± 1,958
Corn for pet feed industry:				
Aflatoxin B_1	8	33.3	0.5 - 1.6	0.8 ± 0.4
Aflatoxin B_2	0	0	-	-
Aflatoxin G_1	4	16.7	0.5 - 0.8	0.7 ± 0.1
Aflatoxin G_2	2	8.3	1.0 - 2.3	1.7 ± 0.9
Total aflatoxins	8	33.3	0.5 - 3.9	1.5 ± 1.1
Fumonisin B_1	24	100.0	539 - 9,707	3,275 ± 2,098
Fumonisin B_2	20	83.3	97 - 1,979	683 ± 586
Total fumonisins	24	100.0	685 - 11,379	3,845 ± 2,570

[1]Number of samples above the limit of quantification (LOQ) of the analytical methods (0.5 µg/kg for aflatoxins B_1, B_2, G_1 and G_2, and 30 mg/kg for fumonisins B_1 and B_2).

[2]Percentage of positive samples of the total analysed samples of corn for pet feed industry (n = 24) and for human consumption (n = 24)".

[3]Minimum and maximum levels quantified in analysed samples in duplicate.

[4]Values are reported as means ± SD, for samples containing quantifiable levels of each mycotoxin.

Samples of corn intended for PFI also presented low levels of aflatoxins, ranging from 0.5 to 3.9 µg/kg with a mean of total aflatoxins of 1.5 µg/kg [Table 2]. No sample had levels higher than the recommended level of aflatoxins for feed ingredients marketed in Brazil (50 µg/kg) [19]. However, fumonisins were detected in all samples analyzed, at levels ranging from 685 to 11,379 µg/kg (mean: 3,845 ± 2,570 µg/kg), which is higher than fumonisin levels found in samples of corn intended for HC. Importantly, there is no regulation for fumonisins in feedstuffs in Brazil, and the recommended level for aflatoxins (50 µg/kg) is not an action level.

The comparison with our results with previous data is difficult because there is no report on the occurrence of aflatoxins or fumonisins in corn intended for the pet industry in Brazil. However, aflatoxin-contaminated pet feeds have been found in surveys conducted in the states of Minas Gerais [20] and Santa Catarina [9]. Pleadin et al. [21] confirmed the corn as the main source of aflatoxin contamination in feeds in Croatia, where high incidences of aflatoxins at levels ranging from 1.1 to 2,072 µg/kg were found in corn samples from different farms and feed factories. FB_1 levels in PFI or HC samples obtained in the presented study were higher when compared with those reported by Mngadi et al. [22], who

Table 3: Simultaneous occurrence of aflatoxins and fumonisins in corn intended for human consumption and for the pet feed industry in São Paulo, Brazil.

Sample number	Total aflatoxins[1] (µg/kg)	Total fumonisins[2] (µg/kg)
Corn for human consumption:		
1	4.0	431
2	0.9	1,396
3	0.7	1,376
4	0.8	4,685
5	0.7	5,276
6	0.9	3,092
7	1.7	6,495
8	1.0	4,279
9	4.6	4,022
10	2.6	4,183
11	41.8	467
12	7.3	488
13	0.6	404
Mean ± SD	5.2 ± 11.2	2,815 ± 2,144
Corn for pet feed industry:		
14	1.7	11,379
15	1.2	8,284
16	3.9	6,541
17	0.5	6,162
18	0.8	2,193
19	1.0	2,657
20	1.8	4,053
21	1.3	2,420
Mean ± SD	1.5 ± 1.1	5,461 ± 3,261

[1]Sum of aflatoxins B_1, B_2, G_1 and G_2.

[2]Sum of fumonisins B_1 and B_2.

found concentrations of 15 to 5,900 µg/kg in 23 animal feed from South Africa. The results also showed a contamination by fumonisins in 100% of corn samples evaluated. On the other hand, in Santa Catarina State, Scussel et al. [9], detected fumonisin in 11% of the 123 pet feed samples.

Despite the high incidence and levels of fumonisins in Brazil, there is no legally maximum limit for these mycotoxins in animal feed. The tolerance limit for FBs adopted by the European Union is 60,000 µg/kg for corn and corn products and 5,000 µg/kg for complementary and complete feeding-stuff for pet animals [23]. In our study, no corn sample had fumonisins levels above this limit. However, one sample had 11,379 µg/kg, which is above the U.S. Food and Drug Administration recommendation for total fumonisins (10,000 µg/kg) in corn [24].

The simultaneous occurrence of aflatoxins and fumonisins was found in 54.2% samples of corn intended for HC, and in 33.3% samples of corn for the PFI, as presented in [Table 3]. The levels of fumonisins were higher in corn destined for feed pet industry, hence indicating that better control practices are applied to corn kernels destined for HC, reflecting specially with agronomic practices and climatic conditions. The higher fumonisin contamination in corn for animal consumption can also be due to a greater period that corn is stored before being used in industry, in addition to worst conditions of storage.

Conclusion

The simultaneous occurrence of aflatoxins and fumonisins in corn reinforces the need for regulations for mycotoxins in feedstuffs in Brazil, to avoid potential health risks in feed supply industry.

Samples of corn intended for the PFI, and corn kernels commercially available for HC in the Northeast region of São Paulo State presented high incidences of aflatoxins and fumonisins. Although the mean concentrations of aflatoxins in corn samples were below the maximum limit established adopted in Brazil, high fumonisin levels were found in all samples, especially in corn intended for the PFI. There is a need for regulations for mycotoxins in feedstuffs in Brazil.

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