Mold Occurrence and Aflatoxin B$_1$ and Fumonisin B$_1$ Determination in Corn Samples in Venezuela

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Fumonisins are mycotoxins produced mainly by *Fusarium moniliforme* and *Fusarium proliferatum*, which have been associated with several animal and human diseases. Aflatoxins are hepatotoxic, mutagenic, and teratogenic metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Both have been reported at high levels in corn. This study was pursued to determine mold, aflatoxin B$_1$ (AFTB$_1$), and fumonisin B$_1$ (FB$_1$) levels in white and yellow corn. Mold levels were determined using potato dextrose agar and identification of the main genus of molds present in corn, AFTB$_1$ levels by immunoaffinity chromatography, and FB$_1$ levels by a Bond-Elut SAX cartridge and HPLC. AFTB$_1$ and FB$_1$ occurrences were 16.6 and 83.78%, respectively. The yellow corn presented higher mold incidence than the white corn. *A. flavus* and *F. moniliforme* were isolated. The positive results show the importance of this study, corn being the main cereal consumed in the Venezuelan diet.

Keywords: *Aflatoxin; fumonisin; mold; corn*

INTRODUCTION

Fumonisins are a mycotoxin group produced mainly by *Fusarium moniliforme* and *Fusarium proliferatum*. These toxins, of which the types B$_1$ (FB$_1$), B$_2$ (FB$_2$), and B$_3$ (FB$_3$) are the most common, have recently been isolated and characterized by Gelderblom et al. (1988). The presence of high levels of *F. moniliforme* and fumonisins in corn in the Transkei region has been correlated with a high incidence of esophageal cancer and is thought to be a causal agent of equine leuko-encephalomalacia (ELEM) (Marasas et al., 1988), porcine pulmonary edema, and hepatocarcinogenicity in rats (Norred and Voss, 1994; Colvin and Harrison, 1992; Voss et al., 1993).

Aflatoxins, potent carcinogenic and toxic metabolites produced by the fungal species *Aspergillus flavus* and *A. parasiticus*, can contaminate animal feeds as a result of the currently unavoidable invasion by molds before or during harvest or because of improper storage of feeds. Studies of the levels of both mycotoxins in food have grown in response to the increasing reports of the incidence of diseases in farm animals of nonmicrobial origin and the potential human risk.

F. moniliforme and *F. proliferatum* are two of the most common species of the *Fusarium* genus isolated from the seed of corn, sorghum, rice, and other cereals (Bullerman and Draughan, 1994). Cagampang (1994) and Katta (1994) studied the incidence of *F. moniliforme* and fumonisin in corn, finding that the *Fusarium* content of dent corn, popcorn, and sweet corn ranged from 8.4 to 36.2% on average; in some samples, 100% kernel infection with *Fusarium* was observed.

Levels between 1.3 and 27 μg/g of FB$_1$ and 0.1 and 12.6 μg/g of FB$_2$ have been associated with ELEM (Kellerman et al., 1990), and fumonisin levels of 10.2 μg/g in good corn to 63.2 μg/g in moldy corn have been associated with high esophageal cancer rates in Transkei, South Africa (Rheeder et al., 1992). Levels of 235 and 350 ng/g of FB$_1$ have been reported by Trucksess et al. (1995) for canned and frozen corn in the United States. Maize shipments imported by South Africa from the United States and Argentina during 1992 were contaminated with FB$_1$, the level in the Argentinean corn (0.31 μg/g) being lower than that in the American corn (2.35 μg/g) (Stockenström et al., 1998).

Recently Stack (1998) evaluated levels of FB$_1$ and its hydrolysis products (HB) in tortillas and masa from the Texas−Mexico border; average amounts of FB$_1$ and HB$_1$ in tortillas were 157 and 82 ng/g, respectively. Average amounts of FB$_1$ and HB$_1$ in masa were 262 and 64 ng/g, respectively. The results of Stack's study suggest that FB$_1$ and HB are present in tortillas consumed by a population experiencing an increasing incidence of neural tube defects.

High incidences of *A. flavus* and aflatoxins in corn have been reported in Venezuela (Martínez and Alvarado, 1986), Colombia (Cespedes and Díaz, 1997), and Costa Rica (Mora, 1988; Viquez et al., 1994).

This study was conducted to evaluate the incidence of molds and levels of aflatoxins and FB$_1$ in corn intended for human consumption harvested in Venezuela.

MATERIALS AND METHODS

Caution. Fumonisins are known carcinogens. Consequently, fungal cultures and solvent extracts should be handled with extreme care.

Samples. Whole yellow corn was collected in October 1993 as single random purchases from retail outlets in Caracas, Venezuela; whole white corn was withdrawn from trucks at grain elevators in accordance with the COVENIN 612-82 norm (1982). A subsample of ~2 kg was prepared for analysis by

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grinding 1 kg in a disk mill (Quaker model 4E, Philadelphia, PA). The remaining sample was used for mycological analysis.

Mold Enumeration. Total count of molds in whole corn grains was performed by mixing 11 g of each sample with 99 mL of peptone water (0.1%) containing 0.01% Tween 80. The samples were shaken vigorously for 2 min and decimal dilutions prepared. Petri dishes containing potato dextrose agar (PDA) supplemented with NaCl (7.5%) and oxytetracycline (40 mg/L) were surfaced plated in duplicate with 0.1 mL portions from each dilution. The PDA plates were incubated at room temperature for 5 days. The results are expressed as colony forming units per gram (CFU/g) of seed.

Mold colonies were transferred to PDA and their identities verified by microscopical observations following the taxonomic keys of Raper and Fennell (1965), Fassatiova (1986), and Samson et al. (1995).

Aflatoxin Determination. The aflatoxin analysis was performed using an immunoaffinity column chromatography method (Aflatest-P, Vicam Corp.). For each sample, 50 g was added to 100 mL of methanol/water (85+15) and homogenized for 1 min in a blender. The samples were then filtered through Whatman No. 4 paper. A 10 mL portion of the filtrate was diluted with 40 mL of distilled water to reduce the methanol concentration to 30%. The dilution was filtered using a microfiber glass filter, and a 10 mL portion was transferred to the affinity column. The column was washed with distilled water using a syringe to push air through the column to remove any interfering compounds. Aflatoxins were eluted using 1.0 mL of HPLC grade methanol and collected in a vial. The eluate was resuspended with 100 μL of chloroform/acetone (9+1), and volumes of 2, 4, 8, and 10 μL were spotted on TLC plates without fluorescent indicator. Standard solutions (2, 4, 5, 8, and 10 μL) at spotting concentrations of 0.5 μg/mL for B_1 and G_1 and 0.15 μg/mL for B_2 or G_2 were spotted, and the plates were developed in an unequilibrated tank for 1 h with chloroform/acetone (9+1) and examined under long-wave UV light (363 nm) to determine the presence or absence of aflatoxins.

Quantification of aflatoxins was accomplished by visual comparison of the samples' fluorescence intensity with reference standards (Sigma Chemical Co., St. Louis, MO). The identity of AFTB$_1$ was confirmed by preparing the aflatoxin–water adduct, using a trifluoroacetic acid procedure following AOAC methodology (26.083). The use of TLC in the determination of AFT instead of HPLC was due to the experience of more than 15 years of working with the method; the average recovery of AFTB$_1$ is on the order of 96−98%, and the limit of quantification for B$_1$ is 2 ng/g.

Fumonisin Determination. The fumonisins do not possess any chromophores and therefore do not absorb UV or visible light, nor do they fluoresce, making too difficult their determination by TLC. In this study, each corn sample was analyzed only for FB$_1$, according to the HPLC method by Stack and Eppley (1992). Briefly, 50 g of ground samples was extracted with 100 mL of methanol/water (3+1) for 2 min in an Osterizer and filtered through Whatman No. 4 paper. Ten milliliters of the filtrate was applied to a Bond-Elut strong anion-exchange (SAX) cartridge previously equilibrated with 2.5 mL of methanol/water (3+1), the cartridge was washed with 25 mL of pure methanol, and the fumonisin was eluted with 14 mL of methanol/acetic acid (199+1). The eluate was evaporated to dryness, the residue was redissolved in 200 μL of *O*-phthaldialdehyde (OPA) (40 mg of OPA was disssoled in 1 mL of methanol and diluted with 5 mL of 0.1 M sodium tetraborate; 50 μL of 2-mercaptoethanol was added, and the mixture was stored in the dark for less than 1 week), and 20 μL was chromatographed on a Waters C$_{18}$ analytical column of 10 cm length and 8 mm diameter with a radial compression module model Z (Waters Associates). A flow rate of 1.5 mL/min was used. The fluorescing derivatives were detected by a Waters fluorescence spectrometer model 420 (excitation at 338 nm, emission at 425 nm) and registered in a Waters model 730 recorder with a speed chart of 0.5 cm/min. Identification

Table 1. Mold Incidence and Aflatoxin B$_1$ and Fumonisin B$_1$ Levels in Corn Samples

sample	kind of corn	mold count (CFU/g)	aflatoxin B$_1$ (ppb)	fumonisin B$_1$ (ppb)
1	yellow	2.6×10^4	5	806
2		3.2×10^5	ND[a]	301
3		1.1×10^6	33	2088
4		3.2×10^5	ND	102
5		3.3×10^3	ND	585
6		NA[b]	ND	891
7		5.8×10^4	ND	480
8		NA	ND	40
9		NA	50	572
10		NA	37.5	1677
11		1.0×10^5	ND	3859
12		ND	37.5	1309
13		ND	ND	371
14		ND	ND	685
15		ND	ND	10941
16		ND	ND	ND
17		ND	ND	15050
18	white	4.9×10^5	ND	502
19		9.0×10^3	ND	602
20		1.3×10^5	ND	3433
21		3.0×10^3	ND	1850
22		7.0×10^3	5	1373
23		4.0×10^3	ND	1311
24		9.0×10^3	ND	25
25		2.6×10^4	ND	572
26		3.7×10^4	ND	773
27		2.1×10^4	ND	363
28		1.0×10^3	ND	ND
29		4.0×10^3	ND	511
30		1.0×10^2	ND	379
31		2.0×10^2	ND	ND
32		1.1×10^4	ND	788
33		9.0×10^3	ND	1423
34		2.2×10^3	ND	326
35		2.6×10^3	ND	ND
36		1.8×10^3	ND	ND
37		5.5×10^3	ND	ND

[a] ND, not detected. [b] NA, not analyzed.

and quantification of the FB$_1$ were performed by comparison of the retention time and peak area observed in the samples with those observed for FB$_1$ standard (Sigma Chemical Co.).

According to the authors, the average recovery from corn was 67% for FB$_1$ at added levels of 0.5, 1.0, and 2.0 μg/g of FB$_1$ with relative standard deviations of 5% for FB$_1$, and the limit of quantification for the metabolite is 10 ng/g.

Evaluation of the Production of Aflatoxin and Fumonisin. *A. flavus* and *Fusarium* sp. isolates from corn were inoculated in flasks containing 50 g of polished rice previously sterilized (Scott et al., 1970) and incubated at room temperature for 12 days. One hundred milliliters of chloroform and 12.5 mL of water were added and filtered through a Whatman No. 4 paper. The filtrate was collected and evaporated until a final volume of 5 mL. Aliquots of 5, 10, 15, and 20 μL were spotted on TLC plates to evaluate the presence of aflatoxins, whereas for fumonisin the toxin was extracted using the procedure described in Stack and Eppley (1992).

RESULTS AND DISCUSSION

The results of the occurrence of molds in corn are presented in Table 1. It is observed that mold incidence in corn ranged from 1.0×10^2 to 1.1×10^6 CFU/g. Mold incidence in yellow corn (from 2.6×10^4 to 1.1×10^6 CFU/g) was higher than in white corn (from 1.0×10^2 to 4.9×10^5 CFU/g). The main genera and mold species identified were *A. flavus*, *A. niger*, *Penicillium* sp., *Fusarium* sp., *F. moniliforme*, *A. ochraceus*, *Aspergillus* sp., and *Rhizopus* sp. According to these results it is very clear that some of them can be toxigenic, mainly *A. flavus* and *Fusarium* sp.

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Ninety percent (eight isolates) of the *A. flavus* isolated (nine isolates) to evaluate their toxigenic abilities produced $AFTB_1$ at levels of 20–1000 ng/g. Viquez et al. (1994) reported that 58% of the corn samples from Costa Rica were contaminated with *A. flavus* and suggested that this factor seemed to be an indicator of toxin levels. However, in our studies we have not found any relation between *A. flavus* incidence and aflatoxin levels. The *Fusarium* sp. and *F. moniliforme* isolated did not produce fumonisin on rice after 12 days of incubation at room temperature.

$AFTB_1$ was the only AFT detected in the samples, and its levels ranged from 5 to 50 ng/g in the positive samples. Of 37 samples analyzed only 6 were contaminated with $AFTB_1$ (16.6%). $AFTB_1$ incidence in yellow corn (23.5%) was higher than in white corn (5.0%). The results suggest that corn variety, agronomic practices, and environmental conditions are the main factors related to the susceptibility of the yellow corn colonized by *A. flavus*. Viquez et al. (1994) found a significant effect on aflatoxin levels due to differences in harvest and storage conditions as well as agricultural practices in each region of Costa Rica. Also in Venezuela, the yellow corn is harvested on a small scale by people with poor facilities to harvest and store. Martínez and Monsalve (1989) reported an aflatoxin presence of 21.3 % in white corn and only two samples had >20 ng/g of $AFTB_1$. Levels of $AFTB_1$ of 1650 ng/g in corn were reported in Guatemala by Canahui (1988).

In this study, the occurrence of FB_1 in corn was 83.78% (31/37), with levels ranging from 25 to 15050 ng/g. A representative chromatogram of the OPA peaks for FB_1 is presented in Figure 1. It was observed that 94.1 and 75% of the yellow corn and white corn samples were contaminated with FB_1. This is the first report of the presence of fumonisin in Venezuelan corn.

The correlation ($p < 0.05$) between fumonisin levels and damaged kernels, impurities, moisture, specific weight, kernels damaged by insects, grains damaged by heat, broken grains, and crystallized grains was determined (Table 2). A linear correlation between FB_1 concentrations in naturally contaminated corn and total damaged grains was observed. No correlation was found between fumonisin levels and the other parameters evaluated. The high levels of fumonisin in corn samples indicate that fumonisin was preformed in the fields.

Similar levels of FB_1 have been reported by Sydenham et al. (1990) in moldy corn in an area where there exists a high rate of esophageal cancer in South Africa. The occurrence of FB_1, FB_2, and FB_3 in Argentinean corn was reported by Sydenham et al. (1993). Combined fumonisin concentrations recorded in the samples ranged between 1585 and 9990 ng/g. The bulk of the samples (94%) had combined fumonisin levels in excess of 2000 ng/g.

In this study no correlation was found between FB_1 and $AFTB_1$ contamination in corn. This result is in agreement with earlier findings (Chamberlain et al., 1993; Shetty and Bhat, 1997) but differs from a recent study (Yoshizawa et al., 1996) in which a negative correlation was found. The co-occurrence of FB_1 and $AFTB_1$ in Venezuelan corn was observed (19%). Shetty and Bahat (1997) reported for the first time the natural co-occurrence of FB_1 and $AFTB_1$ in Indian maize. The co-occurrence of mycotoxins is very important because the combined effects of the toxins can have synergistic or antagonistic effects in animals. The low co-occurrence
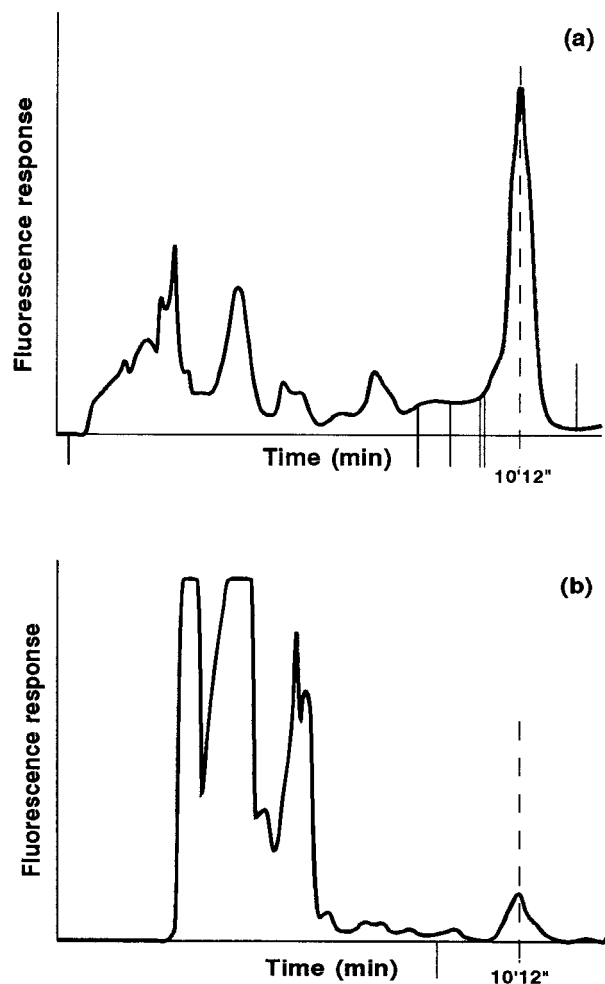

Figure 1. Representative chromatogram of the OPA peaks for FB_1: (a) standard; (b) sample.

Table 2. Correlation between Fumonisin B_1 Concentrations and Physical–Chemical and Biological Parameters in White Corn Samples for Industrial Purposes

parameter	correlation
moisture	0.3177
specific weight	0.3050
impurities	0.7307
damaged kernels	0.087
kernels damaged by insects and rodents	0.9936
microorganisms	0.1708
grains damaged by heat	0.5875
total damaged grains	+0.0341[a]
total healthy grains	−0.0371[b]
broken grains	0.5474
crystallized grains	0.1838

[a] Positive correlation ($p < 0.05$). [b] Negative correlation ($p < 0.05$).

of FB_1 and $AFTB_1$ can be explained because *A. flavus* and *F. moniliforme* infect maize ears by different routes. *A. flavus* is a nonpathogenic fungus colonizing through the silk ears and cracks in the pericarp of maize grain and need not be seedborne. *F. moniliforme* is endophytic to maize, entering the kernel through the pedicle to occupy the internal space distal to the tip cap and is primarily seedborne. Corn infection by *F. moniliforme* and *F. proliferatum* is mainly after physiological maturity of the grain, and fumonisin contamination is higher in samples with fungal infection mainly by these two molds (Chulze et al., 1996). *F. moniliforme* was

shown to inhibit kernel infection with *A. flavus* and aflatoxin production in laboratory conditions (Zummo and Scott, 1992).

It is very important to continue this research because of the high levels of incidence of fumonisin with respect to aflatoxins and also to determine the significance of these levels of fumonisin in corn.

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