Surveillance of Fumonisins in Maize-Based Feeds and Cereals from Spain

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A survey has been carried out to determine the levels of fumonisins in 171 samples of maize-based feeds and cereals available in Spain. Also, the samples were examined for mold count and fungal species. *Aspergillus*, *Penicillium*, and *Fusarium* were the most frequent genera, and *Fusarium* and *Aphanocladium* had the highest individual percentage counts. Regarding *Fusarium* species, *F. moniliforme* (47.4%) was the predominant species; *F. proliferatum* (5.3%) and *F. subglutinans* (7.0%) were isolated at low frequency. The high-performance liquid chromatography−*o*-phthaldialdehyde fluorescence method was used for the analysis of fumonisins. The highest levels of fumonisins were detected in maize. Overall, fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) were detected in 79.5 and 14.6%, of samples respectively, with average FB_1 levels of 3.3 μg/g and average FB_2 levels of 1.7 μg/g. Low levels of fumonisins in wheat, barley, and soybean were detected. This would appear to be the first report of concentrations of fumonisins in these commodities.

Keywords: *Barley; fumonisins; maize; soybean; wheat*

INTRODUCTION

Research over the past 20 years has demonstrated that *Fusarium* molds are capable of producing a range of mycotoxins, including trichothecenes, zearalenone, fusarins, and moniliformin (Marasas et al., 1984). Since 1988, fumonisin mycotoxins have been identified as being produced by certain *Fusarium* species, primarily of the Liseola section, most notably *Fusarium moniliforme* (Bezuidenhout et al., 1988; Nelson et al., 1992). Ten fumonisins have so far been isolated from *F. moniliforme*, although fumonisins B_1, B_2, and B_3 are the major fumonisins produced in nature (Cawood et al., 1991).

Grains naturally contaminated with *F. moniliforme* and its culture material have been associated with a range of diseases, including leukoencephalomalacia in horses (Marasas et al., 1988), pulmonary edema in swine (Harrison et al., 1990), hepatotoxicity in various animal species (Kriek et al., 1981), and liver cancer in rats (Gelderblom et al., 1988). Correlation studies indicate that there may be a relationship between esophaegal cancer rates in certain parts of the world and the occurrence of *F. moniliforme* or its toxins in maize (Sydenham et al., 1990).

Fumonisins have recently been evaluated by the International Agency of Cancer Research (IARC). Although the IARC concluded that there was insufficient evidence in humans to classify the fumonisins as carcinogens, there was sufficient evidence in experimental animals for the carcinogenicity of cultures of *F. moniliforme* containing significant amounts of fumonisins. The overall evaluation was that toxins derived from *F. moniliforme*, including fusarin C, are possibly carcinogenic to humans and classified as Class 2b carcinogens, a classification similar to that of ochratoxin A (IARC, 1993).

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Our objectives were to determine the fungal flora and the levels of fumonisins in maize-based feeds and raw commodities to assess the dietary exposure to this group of mycotoxins.

MATERIALS AND METHODS

Sources of Samples. Products were collected during 1994, 1995, and 1996. Samples were obtained from some agricultural cooperatives, some factories, and some equine stalls. A total of 171 samples were collected: maize (55), swine feed (47), barley (29), horse feed (20), wheat (17), poultry feed (2), and soybean (1).

Representative samples (1 kg) were transported in plastic bags to our laboratory. Once in the laboratory, they were divided into subsamples (200 g) for each analysis (mycoflora and fumonisin determinations) and stock. They were processed immediately, or they were stored in sealed plastic bags at 4 °C and were analyzed within 1−2 days.

Mycoflora Determination. All samples were examined by standard techniques. Quantitative enumeration of fungal propagules was done on solid media using the surface-spread method. Serial dilutions in saline water (0.9%) were made, and 0.1 mL aliquots were inoculated on three plates in three different culture media: malt extract agar [20 g of malt extract (Difco), 1.0 g of peptone (Difco), 20 g of dextrose (Merck), and 25 g of agar (Difco), in 1 L of distilled water]; malachite green agar (Castellá et al., 1997) [15.0 g of peptone (Difco), 1.0 g of KH_2PO_4 (Panreac), 0.5 g of $MgSO_4 \cdot 7H_2O$ (Panreac), 20.0 g of agar (Difco), and malachite green 2.5 ppm in 1 L of distilled water]; Nash and Snyder (1962) medium [15.0 g of peptone (Difco), 1.0 g of KH_2PO_4 (Panreac), 0.5 g of $MgSO_4 \cdot 7H_2O$ (Panreac), 20.0 g of agar (Difco), and 1.0 g of pentachloronitrobenzene in 1 L of distilled water]. All three media were amended with 100 ppm of chloramphenicol and 50 ppm of streptomycin and incubated at 281 °C for 5−7 days. Only plates containing malt extract agar with 10−100 colony forming units (CFU) were used for counting, and the results were expressed as CFU per gram of sample. Total CFU per gram counts of samples were determined after 3, 5, and 7 days of incubation. On the last day of incubation CFU per gram counts for each strain considered to belong to a different genus were recorded (individual counts). Colonies were transferred

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to slants for ensuring precise counting and then to plates for identification. In corn samples, a direct plating technique using the above-mentioned culture media was also used. Occurrence of fungal species was calculated by taking into account all of the colonies developed in the different culture media. Taxonomic identification of different strains was made using macroscopic and microscopic morphological criteria in accordance with appropiate keys. The isolates belonging to the genus *Fusarium* were identified to species level (Nelson et al., 1983), and the remaining molds were identified to genus level. Yeasts were not identified and were considered a unique group.

Fumonisin Determination. Determination of FB_1 and FB_2 was carried out using the high-performance liquid chromatography (HPLC) technique described by Sydenham et al. (1992). Briefly, fumonisins were extracted from a sample of the culture material with methanol/water (3:1, v/v). The extract was purified on a strong anion-exchange (SAX) cartridge, and an aliquot was derivatized with *o*-phthaldialdehyde. The derivatized fumonisins were separated on a reverse-phase column, monitored by fluorescence detection, and quantified by comparison of peak areas with those obtained with reference standards. FB_1 and FB_2 standards were purchased from Sigma (St. Louis, MO).

Confirmation of FB_1 in barley, wheat, and soybean was done following a liquid chromatography/mass spectrometric (LC/MS) method carried out by R. D. Plattner. Briefly, the samples were extracted with 1:1 acetonitrile/water (5 mL/g sample) for 3 h. They were then filtered, and 2 g equivalents (10 mL) of extract was taken through SAX cleanup (Sydenham et al., 1992). The fumonisin-containing fraction was evaporated under a stream of nitrogen and redissolved in 200 μL of solvent (acetonitrile/water). Ten microliters of the extracts was injected into the HPLC without derivatization and eluted into the liquid chromatographic electrospray interface with a flow rate of 0.3 mL/min. A gradient program from methanol/water 35:65 to 95:5 was used. The elution solvent also contained 0.1% acetic acid, which helps both with the chromatography (less tailing) and with the electrospray ionization process.

RESULTS

Total fungal counts, mean individual percentage counts, and occurrence of genera are sumarized in Table 1. Fungal counts ranged from $<10^2$ (theoretical lower limit of the colony count technique) to 4.3×10^6 CFU/g. The lowest counts corresponded to the samples of wheat. Maize grain had the highest count (mean value $= 3.9 \times 10^5$ CFU/g). The rest of the samples had a mean value of $\sim 10^4 - 10^5$ CFU/g. The occurrence of fungi was defined as the percentage of samples in which each fungus was present. *Fusarium* counts and its occurrence are sumarized in Table 2.

The results of the fumonisin survey are given in Table 3. Fumonisins were detected in 79.5% (136 of 171) of all feed samples at total concentrations ranging from 0.2 to 24.9 μg/g. Among these positive samples, 111 contained FB_1 only, and 25 contained FB_1 and FB_2. FB_1 was always the major fumonisin in positive samples. More than 80.0% of the samples contained <5 μg/g combined fumonisins, whereas 43.9% of the samples contained <1 μg/g.

DISCUSSION

The predominant fungi were *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. These species were found most frequently in animal feeds (Abarca et al., 1994; Bauduret, 1990; Bragulat et al., 1995; Chang-Yen et al., 1992; Halfon-Meiri et al., 1990) and had the highest individual counts (Bragulat et al., 1995). *Aspergillus* spp. had an elevated incidence in all of the samples (72.5%), but its individual percentage count

Table 1. Total Mold Counts and Mean Individual Percentage Counts on Malt Extract Agar and Occurrence of Genera Isolated

	total mold counts (CFU/g)		mean percentage counts (occurrence of genera or group isolated)														
sample[a]	range	mean value	*Aspergillus*	yeast	*Penicillium*	*Fusarium*	*Acremonium*	*Cladosporium*	*Aphanocladium*	*Alternaria*	*Mucor*	*Aureobasidium*	*Monascus*	*Rhizopus*	*Absidia*	MSD[c]	MSM[d]	*Paecilomyces*
maize	$5.7 \times 10^2 - 4.3 \times 10^6$	3.9×10^5	1.3	0.5	5.5	91.4	1.2	0.0008	0.02	0.001	0.005			0.0003	0.0003			
			(45.4)	(23.6)	(49.1)	(100)	(49.1)	(5.4)	(5.4)	(7.3)	(14.5)			(1.8)	(1.8)			
barley	$8.7 \times 10^2 - 2.4 \times 10^5$	3.0×10^4	10.4	67.0	3.0	0.1	11.8	4.5	0.5	0.5	0.03	2.0		0.004	0.01	0.004		0.004
			(82.8)	(93.1)	(55.2)	(13.7)	(27.6)	(55.2)	(13.8)	(34.5)	(10.3)	(48.3)		(3.4)	(6.9)	(3.4)		(3.4)
wheat	$2.3 \times 10^2 - 9.6 \times 10^3$	3.5×10^3	21.5	31.7	4.6	2.8	1.9	25.0	1.5	1.1	0.06	5.3	0.1	0.3		3.7	0.3	
			(70.6)	(94.1)	(52.9)	(29.4)	(58.8)	(70.6)	(11.8)	(47.1)	(5.9)	(23.5)	(5.9)	(11.8)		(23.5)	(5.9)	
soybean	1.3×10^4	1.3×10^4	6.3	87.1	1.0				5.1					0.5				
			(100)	(100)	(100)	(100)			(100)					(100)				
swine feed	$^b < 10^2 - 3.0 \times 10^6$	1.2×10^5	8.3	0.9	13.0	4.3	0.1	0.09	72.9	0.2	0.1		0.01	0.01	0.003	0.001		0.002
			(91.5)	(48.9)	(53.2)	(40.4)	(17.0)	(21.3)	(63.8)	(12.8)	(29.8)		(19.1)	(4.2)	(4.2)	(2.1)		(4.2)
horse feed	$2.0 \times 10^2 - 1.4 \times 10^6$	1.6×10^5	1.3	94.0	2.0	0.3	0.03	1.0	0.02	0.1	0.6	0.004	0.002	0.001	0.07		0.01	
			(85.0)	(85.0)	(60.0)	(45.0)	(20.0)	(45.0)	(15.0)	(20.0)	(10.0)	(5.0)	(10.0)	(5.0)	(10.0)		(10.0)	
poultry feed	$2.2 \times 10^4 - 4.2 \times 10^4$	3.2×10^4	13.5	18.8	48.7	15.7	0.5		2.2						0.5			
			(100)	(50.0)	(100)	(100)	(50.0)		(50.0)						(50.0)			
total	$^b < 10^2 - 4.3 \times 10^6$	1.8×10^5	2.9	12.4	6.5	63.1	1.2	0.3	13.3	0.1	0.09	0.07	0.003	0.003	0.001	0.01	0.001	0.0004
			(72.5)	(57.3)	(53.8)	(55.5)	(33.9)	(29.2)	(25.7)	(18.7)	(16.4)	(11.1)	(7.0)	(4.7)	(4.7)	(3.5)	(1.7)	(1.7)

[a] Number of samples: total, $n = 171$; maize, $n = 55$; barley, $n = 29$; wheat, $n = 17$; soybean, $n = 1$; swine feed, $n = 47$; horse feed, $n = 20$; poultry feed, $n = 2$. [b] Limit count of technique. [c] MSD, mycelium sterile dematiaceum. [d] MSM, mycelium sterile moniliaceum.

Table 2. Mean Value of *Fusarium* Species Counts and Occurrence of Species Isolated

	F. moniliforme		*F. subglutinans*		*F. proliferatum*	
sample[a]	mean value	occurrence (%)	mean value	occurrence (%)	mean value	occurrence (%)
maize	3.6×10^5	50/55 (90.9)	1.2×10^2	6/55 (10.9)	9.0×10	4/55 (7.3)
barley	3.1×10	3/29 (10.3)		2/29 (6.9)		
wheat	6.5×10	2/17 (11.8)	3.9×10	2/17 (11.8)	3.1×10	2/17 (11.8)
soybean		1/1 (100)				
swine feed	5.0×10^3	16/47 (34.0)	2.3×10^2	1/47 (2.1)		2/47 (4.2)
horse feed	4.6×10^2	7/20 (35.0)		1/20 (5.0)		1/20 (5.0)
poultry feed	4.8×10^3	2/2 (100.0)				
total	1.2×10^5	81/171 (47.4)	1.1×10^2	12/171 (7.0)	3.2×10	9/171 (5.3)

[a] Number of samples: total, $n = 171$; maize, $n = 55$; barley, $n = 29$; wheat, $n = 17$; soybean, $n = 1$; swine feed, $n = 47$; horse feed, $n = 20$; poultry feed, $n = 2$.

Table 3. Range and Mean Fumonisin Levels Determined in Samples Analyzed

	FB_1			FB_2			$FB_1 + FB_2$		
sample[a]	positives/total (%)	range[b] (μg/g)	mean[c] (μg/g)	positives/total (%)	range[b] (μg/g)	mean[c] (μg/g)	positives/total (%)	range[b] ($\mu\gamma$/g)	mean[c] (μg/g)
maize	48/55 (87.3)	0.2−19.2	4.8	22/55 (40.0)	0.2−5.9	1.9	48/55 (87.3)	0.2−24.9	5.6
barley	21/29 (72.4)	0.2−11.6	1.9	1/29 (3.5)	0.5		21/29 (72.4)	0.2−11.6	1.9
wheat	8/17 (47.1)	0.2−8.8	2.9	1/17 (5.9)	0.2		8/17 (47.1)	0.2−8.8	2.9
soybeans	1/1 (100)	8.7		0/1			1/1 (100)	8.7	
swine feed	42/47 (89.4)	0.4−11.6	2.4	0/47			42/47 (89.4)	0.4−11.6	2.4
horse feed	14/20 (70.0)	0.4−23.6	3.5	1/20 (5.0)	0.3		14/20 (70.0)	0.4−23.6	3.6
poultry feed	2/2 (100)	1.1−1.4	1.3	0/2			2/2 (100)	1.2−1.4	1.3
total	136/171 (79.5)	0.2−23.6	3.3	25/171 (14.6)	0.2−5.9	1.7	136/171 (79.5)	0.2−24.9	3.6

[a] Number of samples: total, $n = 171$; maize, $n = 55$; barley, $n = 29$; wheat, $n = 17$; soybean, $n = 1$; swine feed, $n = 47$; horse feed, $n = 20$; poultry feed, $n = 2$. [b] Limit of detection: 0.06 μg of FB_1/g and 0.1 μg of FB_2/g. [c] Mean of positive samples.

was lower (2.9%) than those of *Penicillium* spp. (6.5%) and *Fusarium* spp. (63.1%).

Aphanocladium spp. were present in more than half of the swine feed (63.8%) and poultry feed (50.0%) samples and were the highest proportion of the total fungi recovered from swine feed.

The incidence of this genera has not been reported in similar studies, with the exception of other surveys carried out in our laboratory (Abarca et al., 1994; Bragulat et al., 1995). *Acremonium* spp., *Cladosporium* spp., and *Mucor* spp. had moderate occurrences, in terms of the samples contaminated (33.9, 29.2, and 16.4%, respectively), but they were never >2% of the total fungal population. The remaining genera and groups were present individually in ≤11% of the samples and accounted for <1% of the fungi recovered.

Fusarium spp. were isolated in 55.5% of samples. The samples least contaminated with these fungi were barley. In maize, 100% of the samples had *Fusarium* spp., and these fungi comprised 91.4% of the total fungi recovered from this substrate. All of the *Fusarium* isolates from samples belonged to the Liseola section. *F. moniliforme* was isolated in 47.4% of the total samples, whereas both *F. proliferatum* and *F. subglutinans* were isolated in low frequency (5.3 and 7.0%, respectively). These results are in agreement with those of other authors (Abarca et al., 1994; Bragulat et al., 1995; Castellá et al., 1996; Chelkowski and Lew, 1992; Dutton and Kinsey, 1995; González et al., 1995).

Samples of maize had the highest levels of fumonisin B_1 and fumonisin B_2, ranging from 0.2 to 24.9 μg/g. Although *F. moniliforme* occurs ubiquitously in corn-producing areas, it predominates in warm, dry areas, where the incidence of *Fusarium* kernel rot is higher (Visconti, 1996). In line with this distribution of *F. moniliforme*, fumonisin contamination of maize is less common in the cooler climates of northern Europe and Canada, although individual samples from these areas may be positive for fumonisins (Kuiper-Goodman et al., 1996; Visconti, 1996). Results from our study are in agreement with those of studies on the occurrence of fumonisins in corn in other Mediterranean countries where the contamination levels and frequency of fumonisins-positive samples were considerably higher than those reported for the surveys in central European countries and the United States (Pittet et al., 1992; Shephard et al., 1996).

In our survey we detected FB_1 in 72.4% of the barley samples and in 47.1% of wheat samples, ranging from 0.2 to 11.6 μg/g (average = 1.9 μg/g) and from 0.2 to 8.8 μg/g (average = 2.9 μg/g), respectively. We also detected ~8.7 μg/g of FB_1 in a soybean sample. It has been confirmed by R. D. Plattner that traces of fumonisins are found in these samples (~0.4−1.1 μg/g). This is the first report of concentrations of fumonisins in these raw commodities, although Dutton and Kinsey (1995) reported FB_1 in triticale, and Patel et al. (1996) reported FB_1 in wheat noodles. Considering the absent or low pathogenicity of *F. moniliforme* isolates to wheat heads (Chelkowski et al., 1995), the detection of high concentrations of fumonisins in wheat grains is unlikely. However, this species is a frequent colonizer of the wheat kernel pericarp in seeds without any symptoms of fungal infection, and the isolates were able to produce fumonisins under laboratory conditions (Castellá et al., 1996; Nelson et al., 1992; Visconti and Doko, 1994). Thus, we think that *F. moniliforme* should be considered as a seed-transmitted pathogen of wheat.

Fumonisins were detected, too, in 89.4% of swine feed and in 70.0% of horse feed. This high incidence of FB_1 in swine feed could be due to its composition, which is very rich in maize. Only one swine feed and two horse feeds had levels of FB_1 higher than the levels recomended by the FDA (Miller et al., 1996). There are some reports of fumonisin contamination of animal feed not related with ELEM or PPE, and levels usually reported were <2μg/g (Dutton and Kinsey, 1995; Pittet et al., 1992; Shephard et al., 1996). Fumonisin levels in

animal feed implicated in outbreaks of ELEM or PPE are exceptionally high, with a maximun of 370 ppm of FB_1 (Wilkins et al., 1994).

We think that more information on the incidence of fumonisins in other raw commodities is needed to determine the health risk posed by these toxins for both humans and animals.

ABBREVIATIONS USED

FB_1, fumonisin B_1; FB_2, fumonisin B_2; IARC, International Agency of Cancer Research; CFU, colony forming units; HPLC, high-performance liquid chromatography; SAX, strong anion-exchange.

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