Fumonisin Content in Masa and Tortillas from Mexico

Mary A. Dombrink-Kurtzman* and Timothy J. Dvorak

Mycotoxin Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604

Fumonisins, a family of mycotoxins produced by *Fusarium moniliforme* and *Fusarium proliferatum*, are found in maize worldwide and have been associated with animal diseases. There is concern that high dietary intake of a maize-based diet may expose people in Mexico and Central America to fumonisins. Nixtamalized maize products from Mexico and the United States were examined to evaluate methods for quantitation of the different forms of fumonisins. The chelating reagent EDTA (exceeding the calcium concentration by a factor of 1.36) was added to enhance extraction of fumonisins because calcium remained in the samples as a result of processing. It was expected that the majority of the fumonisin detected would be in the hydrolyzed form, yet the highest level of hydrolyzed fumonisin B_1 detected was 0.1 ppm. The amount of fumonisin B_1 was significantly higher in Mexican samples (mean = 0.79 ppm) than in samples purchased in the United States (mean = 0.16 ppm).

Keywords: *Fumonisin B_1; maize; Mexico; nixtamalization*

INTRODUCTION

Fusarium moniliforme and *Fusarium proliferatum*, fungi found frequently in corn (maize), produce a family of related compounds called fumonisins (Nelson et al., 1993), which are capable of causing equine leukoencephalomalacia (ELEM) (Marasas et al., 1988; Thiel et al., 1991), porcine pulmonary edema (Harrison et al., 1990), and hepatotoxicity and nephrotoxicity in rats (Voss et al., 1993). Fumonisins are considered a serious health risk to humans on subsistence diets containing fungus-contaminated corn. Consumption of fumonisin-contaminated corn has been correlated with a higher incidence of human esophageal cancer in the Transkei area in South Africa (Rheeder et al., 1992) and in Linxian County of the Henan province in China (Chu and Li, 1994). Significantly higher levels of both fumonisins B_1 (FB_1) and B_2 (FB_2) have been found in samples from the high rate of esophageal cancer areas in Transkei, South Africa (Rheeder et al., 1992). The levels of FB_1 (up to 117.5 ppm) and FB_2 (up to 22.96 ppm) were among the highest levels of these toxins reported in naturally contaminated corn. High levels of FB_1 (18−155 ppm) have also been found in samples that showed heavy mold contamination in households in areas of the Henan province where high occurrence of esophageal cancer has been reported; FB_1 was even found in samples without visible mold contamination at levels of 20−60 ppm (Chu and Li, 1994). The incidence of neural tube defects (NTDs) in these rural areas in Transkei, South Africa, and the Henan province, China, is also very high, 61.3 and 51.7 per 10000 births, respectively (Venter et al., 1995; Xiao et al., 1990). Central nervous system malformations, especially NTDs, are one of the most frequent congenital anomalies and have been the object of intense epidemiological studies. Over the past 100 years in the United States, cases of ELEM and higher rates of NTDs have occurred at the same points in time (MacMahon and Yen, 1971; Marasas et al., 1984). Recently, Stevens and Tang (1997) described the inhibition of folate uptake by FB_1 and suggested that some birth defects may be related to dietary exposure to fumonisin.

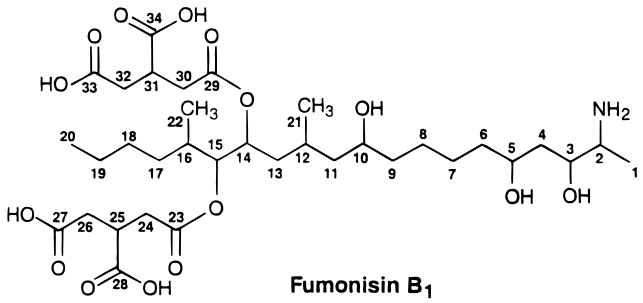

Figure 1. Structure of FB_1. Alkaline hydrolysis of FB_1 cleaves the tricarballylic side chains at C-14 and C-15 to produce HFB_1. FB_2 lacks the C-10 hydroxyl.

Fumonisins inhibit sphingolipid biosynthesis by interfering with the enzyme sphinganine (sphingosine) *N*-acyltransferase (ceramide synthase), with the result that sphinganine accumulates and the biosynthesis of ceramides and complex sphingolipids is blocked (Wang et al., 1991). The elevation of sphinganine has been observed after both in vivo and in vitro exposure to fumonisins [reviewed by Merrill et al. (1996)]. FB_1 has also been shown to induce cell cycle arrest in the G1 phase in normal-type African green monkey kidney cells (CV-1) but not in COS-7 cells, which are CV-1 cells transformed by the simian virus 40 (SV40) large T antigen (Wang et al., 1996).

Fumonisins are often present in corn used for animal feed and can occur in corn products destined for human consumption.

Alkaline hydrolysis of FB_1 (Figure 1) cleaves the tricarballylic side chains at C-14 and C-15 to produce hydrolyzed FB_1 (HFB_1). Typically, FB_1 is present at

* Author to whom correspondence should be addressed [telephone (309) 681-6254; fax (309) 681-6686; e-mail dombrink@mail.ncaur.usda.gov].

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~70% of the total fumonisins detected. Fumonisin content (generally <1 ppm) in alkaline-processed food has been reported (Hopmans and Murphy, 1993; Maragos et al., 1997; Murphy et al., 1996; Scott and Lawrence, 1996; Stack, 1998), but none of these methods incorporated ethylenediaminetetraacetic acid (EDTA) to aid in the extraction of fumonisins. Sydenham et al. (1995) included EDTA for extraction of FB_1 and HFB_1 from naturally contaminated corn, but their procedure for nixtamalization was under conditions (no heating) that differed from the alkaline processing that is used commercially to produce masa and only one concentration of EDTA was tested. Although the distribution of fumonisins has been determined in products obtained from naturally contaminated corn used for dry-milling (Katta et al., 1997) and ethanol fermentation and wet-milling operations (Bennett et al., 1996), the fate of fumonisins has not been examined in a commercial process of nixtamalization.

Tortillas have long been a staple food in Mexico and Central America, and maize has been the traditional cereal for preparation of tortillas in these countries. In Mexico, 72% of the total maize production is used for human food, whereas in the United States ~10% of the corn produced is used for human food (Rooney and Serna-Saldivar, 1987). Annual consumption of maize in Mexico per person has been estimated to be 410 pounds (Watson, 1988). Thus, populations in Mexico and Central America may potentially be at risk due to their high daily consumption of maize and maize-derived products.

Serna-Saldivar et al. (1990) have presented a comprehensive review of the technology and chemistry involved in preparing alkaline-cooked maize products. Conditions for alkaline cooking (nixtamalization) are dependent on the corn varieties and growing conditions (Trejo-Gonzalez et al., 1982). The traditional method for producing tortillas involves boiling whole corn in water containing 1% $Ca(OH)_2$ (lime) for ~1 h, followed by steeping for 16 h. Lime treatment improves niacin availability. The cooking liquid is discarded, and the cooked kernels (nixtamal) are washed to remove the pericarp and excess lime. The nixtamal is ground to yield masa, which is baked or fried to produce tortillas or chips (Serna-Saldivar et al., 1990). The high pH of the alkaline system appears to promote ionization of starch hydroxyl groups, producing Ca−starch cross-links (Bryant and Hamaker, 1997). This treatment can hydrolyze parent FB_1 to HFB_1.

Nixtamalized products produced in Mexico have not previously been examined for fumonisins. It is important that nixtamalized corn products be examined for the presence of fumonisins (native and hydrolyzed) because high levels of foods based on masa are consumed. This study describes a comparison of several analytical methods for quantifying fumonisins in masa and tortillas purchased in Mexico and the United States. The main objective of this study was to improve methods for analyzing the forms of fumonisin in nixtamalized corn. The second objective was to analyze commercially produced masa and tortillas to determine the amount of FB_1, HFB_1, and FB_2 present in samples produced in Mexico and those manufactured in the United States.

MATERIALS AND METHODS

Analytical Standards. Pure standards of FB_1 and FB_2 were purchased from Sigma Chemical Co. (St. Louis, MO). HFB_1 was prepared by mild alkaline hydrolysis of FB_1, provided by R. D. Plattner, USDA, ARS, NCAUR, Peoria, IL, and was >99.8% pure by mass spectrometric analysis.

Maize Samples. Two masa samples (harina de maiz nixtamalizado), four samples of commercially prepared tortillas, and three samples of tortillas locally produced at tortillarias were collected in Mexico. Four masa samples, of which one had been manufactured in Mexico, were obtained in the United States. Two additional samples purchased in the United States included a corn meal sample produced in Venezuela and Incaparina (harina de maiz plus cottonseed flour) produced in Guatemala.

Determination of FB_1, FB_2, and HFB_1 in Masa, Tortillas, and Maize Products. Three different methods were used to determine the levels of FB_1, FB_2, and HFB_1 present. Protocol 1 was a modification of the extraction/purification procedure described by Rottinghaus et al. (1992). Briefly, 25 g of corn meal, masa, or ground tortillas was extracted with 100 mL of acetonitrile/water (1:1) by shaking for 1 h. The aqueous phase was separated by filtration, and 2 mL of extract was diluted with 5 mL of water (rather than 1% KCl in the original method) and applied to a tC_{18} Sep-Pak Vac solid-phase extraction (SPE) cartridge (containing 500 mg of sorbent; Waters Corp., Milford, MA) that had been preconditioned with 5 mL of methanol and 5 mL of water. The cartridges were washed with 5 mL of water, followed by 2 mL of acetonitrile/water (1:9). The fumonisins were eluted with 4 mL of acetonitrile/water (7:3) under vacuum and at a flow rate of <1 mL/min. In protocol 2, a strong-anion-exchange (SAX) sorbent was used for isolation of fumonisins, as described by Sydenham et al. (1996) for an AOAC−IUPAC collaborative study. Briefly, 20 g of corn meal, masa, or ground tortillas was extracted with 100 mL of acetonitrile/100 mM NaH_2PO_4 (pH 3.0) (1:1) by shaking for 1 h. The aqueous phase was separated by filtration, and 5 mL of extract (adjusted to pH 6−7 with 1 N NaOH) was applied to a BondElut SAX SPE cartridge (containing 500 mg of sorbent; Varian, Harbor City, CA) that had been preconditioned with 5 mL of methanol and 5 mL of methanol/water (3:1). The cartridges were then washed with 8 mL of methanol/water (3:1), followed by 3 mL of methanol. The fumonisins (FB_1 and FB_2) were eluted with 12 mL of methanol/acetic acid (99:1). Protocol 3 was based on a modification of a method that included EDTA in the extraction (Sydenham et al., 1995). Briefly, 25 g of corn meal, masa, or ground tortillas was extracted with 25 mL of methanol plus 25 mL of EDTA (initially 10 mM was used). The Ca content of the individual samples was measured using a Perkin-Elmer Plasma 400 ICP emission spectrometer (Norwalk, CT), and the samples were then extracted for fumonisin analysis by adjusting the concentration of EDTA (19.0−79.4 mM) so that it exceeded that of calcium by a factor of 1.36. The aqueous phase was separated by filtration, and 10 mL of the extract (adjusted to pH 2.5 with 0.1 N HCl) was applied to a BondElut C_{18} SPE cartridge (containing 500 mg of sorbent; Varian) that had been preconditioned with 5 mL of methanol and 5 mL of water. The cartridges were washed with 3 mL of water, followed by 5 mL of methanol/water (1:3). The fumonisins were eluted with 15 mL of methanol.

Analysis of Fumonisins by High-Performance Liquid Chromatography (HPLC). The eluates from each of the protocols were evaporated to dryness (under nitrogen), dissolved in 1 mL of methanol, and derivatized with naphthalene dicarboxaldehyde (NDA) as previously described (Bennett and Richard, 1994), with the following modification: 7 mL of acetonitrile/water/acetic acid (60:40:1) was used in place of acetonitrile/phosphate buffer (60:40). A reversed-phase (RP) C_{18} column, SGE (250GL4) column (250 mm × 4 mm) packed with 5 μm of ODS2-I-10/5 material, was used in the HPLC method for analysis of fumonisins. The mobile phase of 65% acetonitrile/acetic acid (99:1) + 35% water/acetic acid (99:1) was pumped at a flow rate of 0.7 mL/min. An excitation wavelength of 280 nm and an emission wavelength of 470 nm were used for fluorescence detection.

Statistical Analysis. The data were analyzed by analysis of variance. Means were compared by the *F* test (SAS Institute Inc., 1989).

Table 1. Calcium Composition and Content of FB_1, HFB_1, and FB_2 in Masa and Tortillas from Mexico

sample	calcium (mg/kg)		fumonisin (μg g^{-1} of sample)
harina de maiz nixtamalizado	590	FB_1	1.80 ± 0.16
brand 1		HFB_1	ND[b]
Guadalupe, Nuevo Leon[a]		FB_2	1.38 ± 0.21
harina de maiz nixtamalizado	1550	FB_1	0.59 ± 0.20
brand 2		HFB_1	0.10 ± 0.03
Guadalajara, Jalisco		FB_2	0.11 ± 0.02
tortillas	560	FB_1	0.69 ± 0.01
brand 3		HFB_1	0.02 ± 0.02
Monterrey, Nuevo Leon		FB_2	ND
tortillas	830	FB_1	1.07 ± 0.18
brand 4		HFB_1	0.05 ± 0.02
Torreon, Coahuila		FB_2	0.18 ± 0.02
tortillas	790	FB_1	0.97 ± 0.33
made in Torreon,		HFB_1	0.02 ± 0.01
Coahuila		FB_2	0.05 ± 0.05
tortillas	810	FB_1	0.48 ± 0.02
brand 4		HFB_1	ND
Puebla		FB_2	0.07 ± 0.03
tortillas	820	FB_1	0.54 ± 0.03
made in Puebla		HFB_1	0.01 ± 0.01
		FB_2	0.11 ± 0.01
tortillas	1070	FB_1	0.25 ± 0
brand 4		HFB_1	0.01 ± 0.01
Chihuahua		FB_2	0.05 ± 0.01
tortillas	1020	FB_1	0.21 ± 0.10
made in Chihuahua		HFB_1	ND
		FB_2	0.07 ± 0.03

[a] Location within Mexico where sample was purchased. [b] ND, not detected (<0.01 μg g^{-1}).

Table 2. Calcium Composition and Content of FB_1, HFB_1, and FB_2 in Maize-Based Samples Purchased in the United States

sample	calcium (mg/kg)		fumonisin (μg g^{-1} of sample)
masa harina de maiz	1980	FB_1	0.04 ± 0.01
brand 5		HFB_1	0.02 ± 0.01
United States[a]		FB_2	ND[b]
corn tortilla mix	590	FB_1	0.38 ± 0.06
brand 6		HFB_1	0.10 ± 0.01
United States		FB_2	0.06 ± 0.01
instant corn masa mix	580	FB_1	0.07 ± 0.06
brand 1		HFB_1	ND
United States		FB_2	ND
harina de maiz nixtamalizado for making tortillas	680	FB_1	1.29 ± 0.18
brand 1		HFB_1	ND
Mexico		FB_2	0.17 ± 0
white corn meal precooked	2340	FB_1	0.04 ± 0
brand 7		HFB_1	ND
Venezuela		FB_2	0.01 ± 0
Incaparina (harina de maiz + cottonseed flour)	50	FB_1	0.97 ± 0.01
brand 8		HFB_1	ND
Guatemala		FB_2	0.14 ± 0.01

[a] Location where sample was manufactured. [b] ND, not detected (<0.01 μg g^{-1}).

RESULTS AND DISCUSSION

Methods Development. Three different protocols were compared for quantifying FB_1, HFB_1, and FB_2. Only the eluate was analyzed for protocol 1 (Sep-Pak tC_{18} cartridge). Recoveries of FB_1, HFB_1, and FB_2, using protocol 1, were 105.3, 50.0, and 47.5%, respectively, at spiking levels of 1 μg g^{-1} FB_1, 1 μg g^{-1} HFB_1, and 500 ng g^{-1} FB_2. For protocols 2 (BondElut SAX cartridge) and 3 (BondElut C_{18} cartridge), all four fractions (application, first wash, second wash, and elution) were analyzed. Recoveries of FB_1, HFB_1, and FB_2 were 54.5, 0, and 40.5%, respectively, at spiking levels of 4 μg g^{-1} FB_1, 4 μg g^{-1} HFB_1, and 2 μg g^{-1} FB_2 in protocol 2. The failure to detect HFB_1 appeared to be due to the compound binding to the extracted matrix of the sample because it was not detected in the application fraction that had been spiked after it had been passed over the column and analyzed as an additional control. For protocol 3, duplicate analyses were performed; the amount of calcium present in the samples was determined, and EDTA was added to aid in the extraction of the fumonisins. Calcium concentrations (milligrams per kilogram) and identity of products are shown in Table 1 for the samples from Mexico and in Table 2 for samples purchased in the United States. The calcium concentrations ranged from 560 to 2340 mg/kg (0.056−0.234%) for the samples that had been nixtamalized. Recoveries of FB_1, HFB_1, and FB_2 were 86.0, 90.2, and 103.5%, respectively, at spiking levels of 2 μg g^{-1} FB_1, 2 μg g^{-1} HFB_1, and 1 μg g^{-1} FB_2, in protocol 3. The amount of FB_1 detected using protocols 1 and 2 (data not shown) was similar to the amount detected with protocol 3, but protocol 3 had the best recoveries and proved to be the optimal means for extracting HFB_1 from nixtamalized material. The lower recoveries of HFB_1 and FB_2 for protocol 1 indicated the importance of incorporating EDTA into the extraction solution because a C_{18} cartridge was used for both protocols 1 and 3. However, lower recoveries for protocols 1 and 2 were obtained only with nixtamalized samples that had been spiked, not with corn meal (data not shown). Thus, protocols that have been used to analyze corn and animal feed for fumonisins cannot be used for nixtamalized material without some modification.

A more efficient way to extract fumonisins from samples that had previously undergone alkaline hydrolysis involved determination of the calcium content and addition of an amount of EDTA that exceeded the concentration of calcium by a factor of 1.36 on a molar basis. This factor was based on the increased amount (~23%) of FB_1 recovered when the EDTA concentration was increased from 10 μM (Sydenham et al., 1995) to 20 μM. The amounts of FB_1 detected in duplicate samples using 10 and 20 μM EDTA were 1.59 and 1.96 μg g^{-1}, respectively. The amounts of HFB_1 and FB_2 detected using 20 μM EDTA also increased. The calcium content of masa is affected by levels of lime used in the processing, cooking−steeping temperatures, and characteristics of the maize (Pflugfelder et al., 1988). The highest levels of fumonisins, and the presence of HFB_1, were generally detected when EDTA was incorporated into the extract and a tC_{18} solid-phase extraction cartridge was used for cleanup prior to HPLC.

Fumonisin Levels in Masa and Tortillas. The amount of fumonisin detected (Table 1 for the Mexican products and Table 2 for the U.S.-purchased products for protocol 3) varied on the basis of the extraction procedure, the type of SPE column (tC_{18} or SAX), and the manufacturer of the columns. Another type of SPE column (Whatman ODS-3) was used, but it was less suitable (data not shown). All four fractions were collected when using the SAX column (protocol 2) because HFB_1 is not retained by the column. Although all four fractions were collected (protocol 3), the fumonisins were present only in the eluate.

The amount of FB_1 present in the samples of masa and tortillas produced in Mexico differed significantly

($P < 0.01$) compared to those products produced in the United States. The amount of FB_1 detected in the masa and tortilla samples from Mexico ranged from 0.21 to 1.80 ppm, with a mean of 0.79 ppm. The amount of FB_1 detected in the samples from the United States ranged from 0.04 to 0.38 ppm, with a mean of 0.16 ppm. The sample of harina de maiz nixtamalizado that had been purchased in the United States was included with the samples from Mexico because it had been manufactured in Mexico. Unfortunately, due to lack of sample remaining, it was only possible to analyze several of the samples in triplicate; the results agreed with the means obtained with the duplicate samples (protocol 3).

It was expected that the majority of the fumonisin detected would be in the hydrolyzed form, yet the highest level of HFB_1 detected was 0.1 ppm. There are several possible explanations for this: the process of nixtamalization may have been incomplete; more pericarp containing a greater amount of unhydrolyzed fumonisin may have been present; or the products may have been prepared by using an extrusion process rather than the traditional nixtamalization process. Tortillas prepared according to the traditional process have been reported to contain a lower amount of fiber due to the partial loss of pericarp and aleurone compared to tortillas prepared by using the extrusion process (Gomez Aldapa et al., 1996).

Detection of 1.0−1.8 ppm of FB_1 in some of the Mexican samples of masa and tortillas suggested that either fumonisin was present at high levels in the maize before processing or the pericarp had not been fully removed. Sydenham et al. (1995) have reported that maize kernels retained 31.1% of the original FB_1, predominantly as the intact toxin, when only partial removal of pericarp occurred. When the pericarp had been efficiently removed by alkaline hydrolysis, the FB_1 concentration was reduced by 94.9% in the solid fraction, and the majority of the original toxin remained in the aqueous fraction as HFB_1 (Sydenham et al., 1995). This would suggest that processing with complete removal of the pericarp and rinsing of the processed maize would reduce the chance of exposure to fumonisin. This is assuming that fumonisin-related products containing a blocked primary amine group (Howard et al., 1998), which would not be detected by routine analysis, are not formed as a result of the processing. Studies on the effects that time, temperature, and pH have on the stability of FB_1 in an aqueous model system have indicated that no significant losses in FB_1 occurred during processing at 100 °C, the highest temperature reached in the process of nixtamalization (Jackson et al., 1996). In the studies by Sydenham et al. (1995), alkali treatment of the maize was performed at room temperature, whereas in the commercial process of nixtamalization, the maize is boiled in $Ca(OH)_2$ and allowed to steep overnight.

Toxicity studies of nixtamalized *F. moniliforme* or *F. proliferatum* culture material have indicated that after processing, the material was still capable of causing hepatotoxicity and nephrotoxicity (Voss et al., 1996) or was even more toxic (Hendrich et al., 1993) when fed to rats. Culture material, not purified fumonisins, was used in both of these studies. Studies are currently underway to examine the various fractions produced during nixtamalization mimicking the commercial process, using normal-appearing corn that contains a known concentration of fumonisins.

Epidemiological data have identified NTDs as being more common among Mexican than Caucasian populations in North America (Harris and Shaw, 1995). The rates of NTDs are highest among those living in Mexico. A study of the prevalence of NTDs in Mexico and California noted that there was a gradient of risk, with the highest rate in Mexico (32.6 NTDs per 10000 live births), the lowest among U.S. Caucasians and second-generation Mexican Americans (5.8 and 6.8 NTDs per 10000 live births, respectively), and an intermediate rate among populations of Mexico-born Mexicans in the United States (16.0 NTDs per 10000 live births). It is widely accepted that these disorders (NTDs) are multifactorial in origin, having both genetic and environmental components in their development (Sever, 1995). Could dietary patterns be involved, considering that populations in Mexico and Central America consume a lot of maize? This makes it all the more imperative that maize and products derived from maize be examined in areas where maize forms a major part of the diet.

Conclusions. It was expected that the alkali treatment would hydrolyze the native fumonisin in the nixtamalized products so that only the hydrolyzed form would remain. However, this did not occur. The highest level of HFB_1 detected was only 0.1 ppm, and some of the Mexican samples of masa and tortillas contained 1.0−1.8 ppm of FB_1. A special concern, because of the high intake of maize-based products by people in Mexico, is the finding that the nixtamalized maize products produced in Mexico contained a significantly higher amount of FB_1 than did the samples manufactured in the United States. In addition to growing conditions and the type of cultivar, there are several possible explanations related to processing for these results. The processing of maize may have occurred under conditions of incomplete nixtamalization. Fumonisin may have been present at moderately high levels in the Mexican maize before processing. The pericarp, where most of the fumonisins are located, may have been only partially removed. If an extrusion process, rather than the traditional nixtamalization process, had been used, it is likely that more dietary fiber (pericarp) would remain in the finished product. This suggests that by being able to monitor the degree of pericarp removal, it should be possible to predict the amount of intact fumonisin remaining in the nixtamalized products. The best way to determine how successful processing is in reducing the amounts and types of fumonisins remaining is to analyze samples from each stage of the production. These studies are currently underway in our laboratory.

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