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Fumonisin in Tortillas Produced in Small-Scale Facilities and Effect of Traditional Masa Production Methods on This Mycotoxin

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Four small tortilla plants were visited in Cameron County, Texas, where observations were made on their production methods. Samples of liquids and solids were collected at each stage of the nixtamalization process, and the pH was recorded. Samples were analyzed for fumonisin B_1 (FB_1) using an immunoaffinity column/HPLC method chosen for its sensitivity for FB_1. It was found that production methods were highly variable among the producers visited, with major differences particularly in the amount of lime added and boiling times. As reported by others working in Mexico and Central America, FB_1 was found in some tortillas. This led to studies of the effects of the various recipes and across a greater range of initial FB_1 concentration/damaged corn than has typically been reported. Five initial concentrations of FB_1 were tested using irradiated corn kernels inoculated with *Fusarium verticillioides* MRC 826 as the source of FB_1. The amount of FB_1 detected in the masa and tortillas decreased as the concentration of $Ca(OH)_2$ increased, and boiling time had no apparent effect. Unexpectedly, as the initial concentrations were increased in the corn prior to nixtamalization, greater percentage reductions in FB_1 were observed.

KEYWORDS: **Fumonisin B_1; nixtamalization; alkaline hydrolysis; corn; tortillas**

INTRODUCTION

The fumonisin mycotoxins have been found in a wide range of corn-based foods and feedstuffs. Fumonisins B_1, B_2, and B_3 are the most abundant naturally occurring fumonisins in warm corn-producing areas worldwide. FB_1 has been shown to cause some animal diseases associated with *Fusarium verticillioides*-contaminated feeds such as leukoencephalomalacia in horses, pulmonary edema syndrome in pigs, and hepatocellular carcinoma in rats (*1*). In 2001, the Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA) established a Provisional Maximum Tolerable Daily Intake (PMTDI) for fumonisin of 2.0 μg/kg of body weight per day (*2*). In 2003 the International Agency for Research on Cancer (IARC) classified FB_1 as a Group 2B carcinogen (possible human carcinogen) (*3*). The presence and recovery of fumonisins in processed foods are affected by the milling and cooking of corn as well as other ingredients present in the recipes (*4*−*6*). Questions about the fate and distribution of contaminants in commercial food products have been typically addressed in response to consumer and regulatory concerns in Europe or North America. When food products are made at home by traditional methods or in small-scale production facilities, the fate of mycotoxins is less likely to be studied. JECFA estimated that the average FB_1 exposure in Africa exceeded the PMTDI, and that in Latin America it was exceeded for ∼30% of the population (*2*). Under African conditions, corn meal is boiled, and cooking does not materially reduce exposure to this toxin (*7*). The situation in Latin America is certainly different because a high percentage of corn consumption is from nixtimalized corn foods.

Nixtamalization is a process by which corn is boiled in a lime solution to produce masa for tortillas, which is known to reduce exposure to aflatoxin (*8*), deoxynivalenol, and zearalenone (*9*) and to fumonisins (*10*). A study of fumonisin in home-produced tortillas and masa collected in Mexico reported fumonisin B_1 (FB_1) in some samples (*11*, *12*). In nixtimal, FB_1 is hydrolyzed to HFB_1 and then to various other known and unknown products (*10*, *12*). Typically, HFB_1 either is not found or is found close to the detection limit (*10*, *11*). HFB_1 is much less potent than FB_1 in vitro (*13*, *14*) and in rodent assays (*15*). Additionally, Voss et al. (*16*) found that nixtamalized ground culture material was found to be less toxic in vivo. On this evidence, concern about residual fumonisin degradation products made from nixtimal is likely small in comparison to FB_1. There is one report of tortillas obtained from a community in rural Guatemala containing extraordinary amounts (185 μg/g) of

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Table 1. Comparison between Starting Ingredients and Processing Methods at Four Tortilla Producers from Texas

tortilla producer	corn used (kg)	lime used (kg)	water used (L)	concn of lime water (mM)	pH	cooking time (min)	steeping time (h)	baking temp (°C)
factory A	780	6.20	600	140	11.0	7	18	250
factory B	1100	16.30	2250	100	11.1	150	16	250
factory C	1100	10.10	1800	80	10.8	10	16	375
factory D	2650	20.55	1800	150	10.6	120	18	232

HFB_1. In this case, the authors reported that the crucial water-washing step was limited by a severe water shortage in the community (*17*). On the basis of the amount of HFB_1 reported, initial FB_1 could not have been <20 μg/g, indicating that poor-quality, very damaged corn was used. Similarly high values were not seen in other nearby communities (*17*, *18*).

In 1990, a cluster of infants born with neural tube birth defects was reported in at least one county on the Texas−Mexico border. Preliminary epidemiological evidence suggested that the neural tube birth defects were associated with tortilla consumption by the mothers during pregnancy (*19*, *20*) Recently, fumonisin has been demonstrated to cause neural tube defects in mouse embryos in vitro and in vivo (*20*, *21*). This prompted an investigation of the methods used to produce tortillas in the affected area as well as an examination of the impact of the recipes used on FB_1 concentration across a wider range of starting concentrations than has typically been reported.

MATERIALS AND METHODS

Reagents. All analytical grade reagents, HPLC grade solvents, and food grade lime, $Ca(OH)_2$ (95% purity), were purchased from Sigma Chemical Co., St. Louis, MO.

Analytical Standards. *F. verticillioides* strain MRC 826 was obtained from Professor W. F. O. Marasas. Commercial white corn was obtained from Bee Agricultural, Beeville, TX, and was used to prepare masa and tortillas in the laboratory.

Field Observations. In November 2001, four small tortilla plants were visited in Cameron County, Texas. The recipes used in these plants to produce masa and tortillas were recorded. The corn used was grown locally. Information was obtained on the ratio of corn to lime to water used and the boiling, soaking, and rinsing practices. During each step of the nixtamalization process, the pH was recorded and samples were collected, which were subsequently analyzed for FB_1 content.

Production of Fumonisins in Corn Kernel Cultures. This followed the method of Avantaggiato et al. (*22*). Briefly, 10 g of corn kernels (Pioneer Hibred, Johnston, IA) were sterilized by γ-irradiation at 25 kGy minimum dose and 30 kGy maximum dose (MDS Nordion, Montreal, PQ) and placed in sterile glass Petri dishes lined with filter paper, and 6 mL of sterile water was added. After an overnight equilibration period, the filter paper was inoculated with a 1 mm^2 piece of a malt extract agar culture of *F. verticillioides* MRC 826. This strain produced mainly FB_1, trace amounts of FB_2, and no fusarins or moniliformin. The corn kernel cultures were incubated in the dark at 28 °C for 6 days. The kernels were then harvested and freeze-dried. The final concentration of FB_1 in the kernels was 12 μg/g.

Nixtamalization. Tortillas were prepared by boiling 1 part whole corn kernels in 2 parts lime solution (1%) for 20−45 min. This was followed by steeping overnight and washing, after which masa was pressed into tortillas and grilled on a flat iron (*23*). For each treatment, $Ca(OH)_2$ was dissolved in 2 L of water in a 6 L Erlenmeyer flask, followed by the addition of 1 kg of corn. Four different concentrations of lime were used: 3.4, 8.5, 177.0, and 216 mM. The mixture was heated to boiling and then boiled for either 15 min or 1 h (time to boiling, ∼30 min). The flask was allowed to cool overnight (18 h) after which the pH of the steep water was measured and samples were frozen for FB_1 analysis. The corn was rinsed in 4.3 L of water under gentle agitation. The treated corn was ground into masa using a Porkert 150 manual mill. During grinding, 250 mL of water was added to obtain moist, workable masa.

Five masa lots of different FB_1 concentrations were obtained by adding fumonisin-contaminated corn kernel meal prepared as above. For each treatment, six 30 g masa balls were pressed into tortillas using a manual cast iron tortilla press (Casa Herrera Inc., Los Angeles, CA). The tortillas were cooked over an iron grill for ∼4 min. Temperatures reached ∼190 °C (on the outer edge) and 200 °C (at the center of the grill). The tortillas were cooked to a water content of ∼40%, left to dry overnight (resulting in a final weight of ∼20 g), and then ground into a fine meal prior to FB_1 analysis. The remaining masa was divided into six 20 g balls and allowed to dry overnight for FB_1 analysis (final dry weight of ∼10 g).

Analysis of FB_1. FB_1 was quantified in the corn kernels, tortillas, and masa according to the method of Visconti et al., with a slight modification (*24*). Briefly, a 50 mL portion of acetonitrile/methanol/water (25:25:50, v/v/v) was used to extract ∼20 g samples of finely ground corn, tortillas, or masa. The samples were extracted by shaking for 20 min using an orbital shaker, centrifuged for 10 min at 4100*g*, and filtered (Whatman no. 4 filter paper, 15 mm), and the process was repeated.

The two extracts for each sample were combined, and 10 mL was diluted with 40 mL of PBS buffer (8 g of NaCl, 1.2 g of Na_2HPO_4, 0.2 g of KH_2PO_4, 0.2 g of KCl, pH adjusted to 7.00 with concentrated HCl in 1 L of water). This extract was filtered using microfiber filter paper (Whatman GA, 15 mm). Ten milliliters was passed through a Fumonitest immunoaffinity column (Vicam, Watertown, MA), and the column was washed with 10 mL of PBS buffer followed by FB_1 elution with 1.5 mL of methanol. The eluate was collected in 4 mL amber glass vials and dried under a gentle stream of nitrogen gas. Dried samples were kept at 4 °C until HPLC analysis.

For HPLC analysis, the dried samples were dissolved in 200 μL of acetonitrile/water (1:1, v/v); 50 μL of the extract was derivatized with 50 μL of OPA solution [20 mg of *o*-phthaldialdehyde, 2.5 mL (0.1 M) of sodium borate buffer, 0.5 mL of methanol, 20 μL of mercaptoethanol) and mixed for 30 s (*24*). Derivatization was carried out at room temperature. Exactly 3 min after the addition of OPA, 20 μL of the derivatized solution was injected into the HPLC system. Quantification was done using a Varian 5500 HPLC pump system fitted with a Rheodyne 7126 injector and a ThermoSeparation Products FL 3000 fluorescence detector set to detect at 335 nm excitation and 440 nm emission wavelengths. Separation of the compounds was achieved using a Synergi Max-RP (80 Å, 5 μm, 250 × 4.60 mm) HPLC column (Phenomenex, Torrence, CA) and isocratic elution with methanol/0.1 M phosphate buffer (77:23, v/v) adjusted to pH 3.35 with concentrated orthophosphoric acid. The mobile phase was pumped through the system at a flow rate of 1 mL/min. The limit of detection for FB_1 using this method was 25 ng/g.

RESULTS AND DISCUSSION

We found considerable variation in the proportions of corn, lime, water, and boiling times used in the small-scale commercial tortilla facilities in Cameron County (**Table 1**). Three recipes called for ∼1% lime/corn (w/w) and one for less at 0.6%; water was added at 1.3% in two recipes and at 0.5% (w/w) in the other. The concentrations of $Ca(OH)_2$ ranged from 80 to 150 mM. Boiling times ranged from 7 min to 2.5 h, but steeping times were similar at 16−18 h (**Table 1**). There were important differences in the tortilla baking temperatures. The corn used at the time of the site visits came from the same provider and had between 0.6 and 1.6 μg of FB_1/g (**Table 2**). There are no

Table 2. FB_1 Reductions and Masa Production Variations at Four Tortilla Producers from Texas

tortilla producer	corn-to-lime ratio	cooking time (min)	raw corn[a] (ng/g)	cooked corn[a] (ng/g)	steeped corn[a] (ng/g)	masa[a] (ng/g)	tortillas[a] (ng/g)
factory A	120:1	7	1000.8 ± 0.3	440.8 ± 0.2 (56%)[b]	166.9 ± 0.03 (83%)	196.9 ± 0.1 (80%)	175.0 ± 0.1 (83%)
factory B	65:1	150	680.8 ± 0.1	181.9 ± 0.2 (73%)	138.8 ± 0.16 (80%)	103.9 ± 0.2 (85%)	112.9 ± 0.3 (89%)
factory C	100:1	10	1440.9 ± 0.2	250.2 ± 0.1 (83%)	167.9 ± 0.03 (88%)	ND[c]	ND
factory D	130:1	120	1652.9 ± 0.2	371.0 ± 0.1 (78%)	195.6 ± 0.02 (88%)	ND	ND

[a] Values indicate the mean ± and standard deviation of triplicate analyses. [b] Values in parentheses are percentage FB_1 reductions. [c] Not detected (<10 ng/g).

reports of HFB_1 being detected in nixtimalized tortillas made from corn containing <4 μg of FB_1/g (*10*).

Reductions in FB_1 concentration after the different processing steps are shown in **Table 2**. Tortillas from two of the four companies visited contained measurable amounts of FB_1. In FB_1-positive tortilla samples, concentrations ranged from 113 to 175 ng/g, and in masa from 104 to 197 ng/g. These are similar to values from Mexico and to data reported by the U.S. Food and Drug Administration from studies in the 1990s (*11*, *25*). A reduction of ∼80% was achieved by all four factories after the nixtamalization process was completed (**Table 2**). This shows that good reductions in FB_1 concentration were attained with differing recipes and procedures. However, residual FB_1 in tortillas needs to be considered in estimating exposure in this group of Mexican−American women. Tortilla consumption in this region is much greater than for the average American at 60−90 g/day. In Mexico and Latin America, annual consumption of tortillas is ∼186 kg or ∼500 g/day. In rural areas, tortillas are estimated to provide ∼70% of the daily caloric intake (*26*).

The factors investigated here that most affected residual FB_1 in masa and tortillas related to the amount of lime added (less) and the amount of fumonisins (more) in the corn. The effect of boiling time was modest (data not shown), an effect reported by Sydenham et al. (*27*) for nixtamal and by Shephard et al. (*7*) in studies of maize porridge. We used experimentally inoculated corn kernels containing 12 μg of FB_1/g that had been sterilized by irradiation rather than by autoclaving. This method closely simulates the use of poor-quality corn to make masa because it can be ground to a consistency similar to corn meal (*22*). We hypothesized that nixtamalization would be more effective in reducing FB_1 levels in poor-quality corn than in intact corn. However, higher initial FB_1 concentrations in damaged corn unexpectedly resulted in disproportionately lower FB_1 concentrations in tortillas as a function of increasing additions of damaged/contaminated corn. For the 60 min boiling time at 3.4 mM lime and initial concentrations of 150, 530, 1240, 5100, and 11800 ng of FB_1/g, the concentrations in the masa were 80.4 ± 0.2 ng/g (46.4%), 84.3 ± 0.2 ng/g (84.1%), 58.3 ± 0.3 ng/g (95.3%), 137.7 ± 0.1 ng/g (97.3%), and 106.2 ± 0.2 ng/g (99.1%), respectively (reductions). For the tortillas, FB_1 concentrations were 111.0 ± 0.1 ng/g (26%), 84.8 ± 0.2 ng/g (84%), 124 ± 0.2 ng/g (90%), 142.8 ± 0.4 ng/g (97.2%), and 188.8 ± 0.1 ng/g (98.4%). For the remaining treatments, the effect of doubling the initial FB_1 concentration on tortilla FB_1 content was approximately twice that of doubling the lime concentration (data not shown).

For the 150 ng of FB_1/g concentration treatment, FB_1 remaining after treatment corresponded to the results of other researchers who reported reductions ranging from 70 to 80% (*5*, *6*). The absolute amounts of FB_1 added and reductions observed in relation to the lime concentration for the 150 ng of FB_1/g treatment for masa and tortillas are shown in **Figure 1**. For this treatment, the loss of FB_1 at temperatures <150 °C followed expected thermal decomposition kinetics (*28*). The next higher addition of FB_1-containing kernels (530 ng/g) resulted in an apparently different rate of change (**Figure 2**). To examine this more closely, the half-life of FB_1 was calculated for each treatment. As expected, there was a significant effect of initial lime concentration on half-life independent of FB_1 concentration (**Table 3**). However, as implied by **Figure 2**, there was also a significant independent affect of initial FB_1 concentration on half-life (**Table 3**).

Calcium hydroxide solution degrades and solubilizes the cell wall components of maize kernels. This results in the removal of the pericarp and softening of the endosperm structure (*23*). At higher $Ca(OH)_2$ concentrations, more pericarp was removed from the kernels. At lower concentrations, the kernels remained more intact. This probably reduced the potential for the alkaline water to extract and hydrolyze FB_1. When the pericarp was removed during masa preparation, FB_1 concentration was reduced by ≥95%. It was observed during the experiments that pericarp removal increased with increasing $Ca(OH)_2$ added. This is in agreement with Sydenham et al. (*27*), who reported that maize kernels retained 31% of the original FB_1 when only partial removal of the pericarp occurred.

The identification of the reaction products, either true decomposition products or FB-matrix products, was not done in this study, but earlier work has shown a number of possibilities. Several studies have examined the effect of thermal processing on the fumonisin content in food. Boiling temperatures have little effect on FB_1 at neutral pH values (*29*, *30*). However, FB_1 may decompose by different mechanisms in dry versus aqueous environments with decomposition dependent on the pH. Studies to determine the effect of temperature and pH on FB_1 stability in water have indicated that no significant losses occurred at neutral pH during processing at 110−125 °C. However, at pH 10 the formation of HFB_1 was favored (*29*).

Howard et al. studied the conditions favoring the formation of *N*-(carboxymethyl)-fumonisin B_1, the principal product formed upon reaction with some plant reducing sugars (*31*). They showed that the formation of *N*-(carboxymethyl)-FB_1 required alkaline conditions. In the presence of glucose, no loss of FB_1 was detected at pH 4.8−6; however, loss of FB_1 occurred when the pH was increased to 7−8.9. This pH-dependent decrease in FB_1 was proportional to the increase in the formation of *N*-(carboxymethyl)-FB_1. Increases in *N*-(carboxymethyl)-FB_1 were also observed with increasing temperature or phosphate concentration. Formation was inhibited by chelators or oxygen radical scavengers (*30*). The reaction with reducing sugars could account for low FB_1 recoveries in the present study. These sugar products are not detectable by the OPA HPLC method because the amino group is no longer available for derivatization. However, Voss et al. did not find significant amounts of *N*-(carboxymethyl)-FB_1 or other FB_1−sugar adducts in nixtamalized products (*10*). This might be expected because sugar adducts are cleavable by alkaline hydrolysis and then further released as HFB_1. HFB_1 and partially hydrolyzed FB_1 were found but mostly in the boiling/steeping water fractions. Several

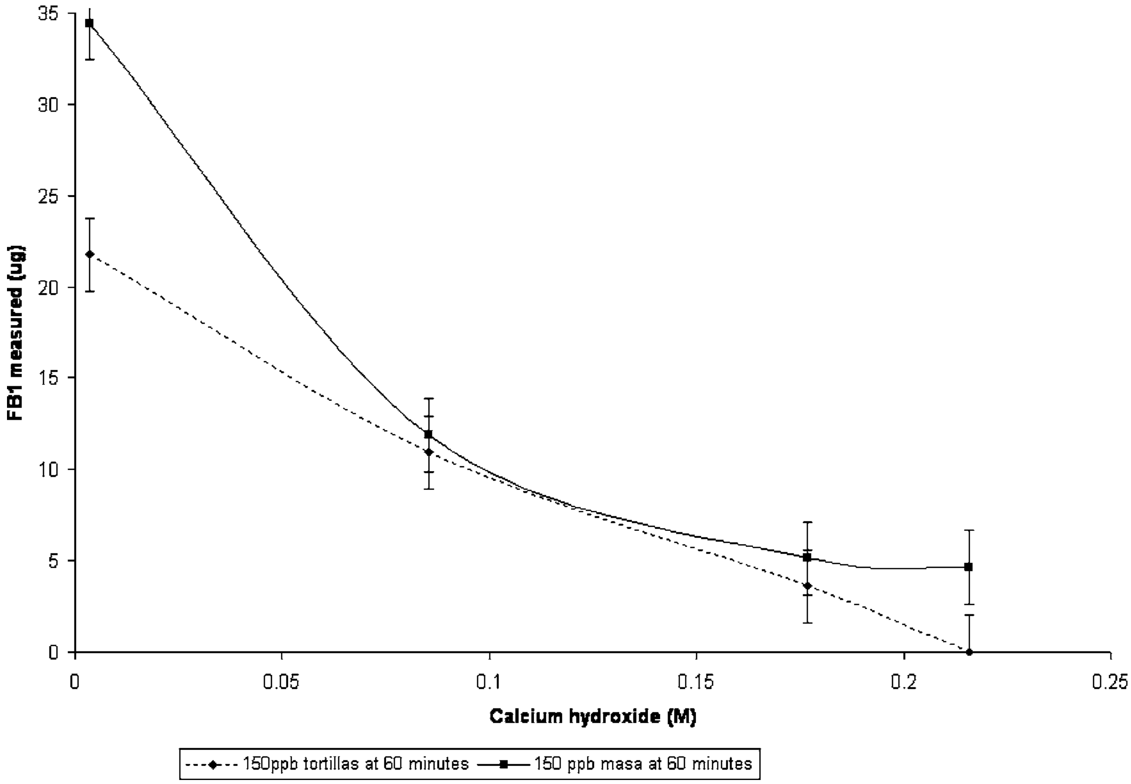

Figure 1. FB_1 present versus $Ca(OH)_2$ used in tortilla and masa samples at 150 ng/g.

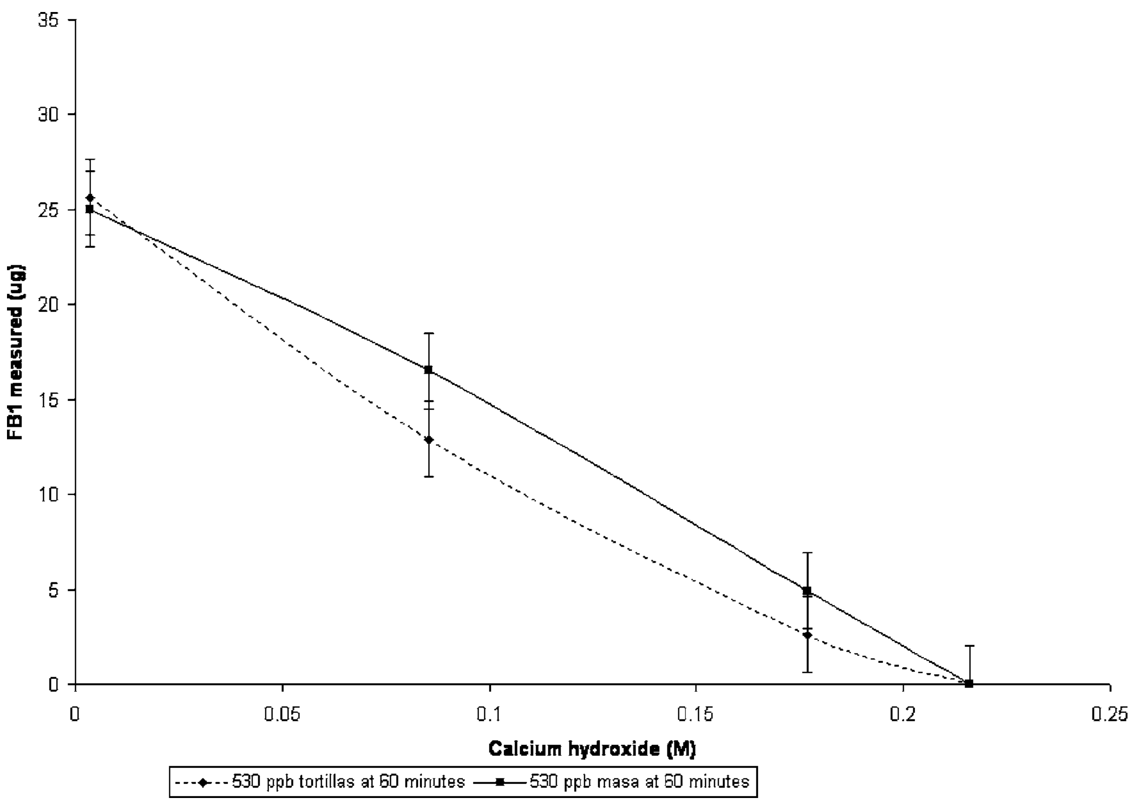

Figure 2. FB_1 present versus $Ca(OH)_2$ used in tortilla and masa samples at 530 ng/g.

surveys have shown that thermally processed food products generally contain lower FB_1 concentrations. Bordson et al. (*30*) and Scott and Lawrence (*4*) suggest that the observed loss of FB_1 in processed food may be due to matrix-related difficulties of recovery and detection, rather than actual FB_1 decomposition.

Poor-quality maize is commonly used even in advanced developing countries. An important consequence of this is that the amount of "disappeared" FB_1 from nixtamalized low-grade maize in such circumstances can apparently be quite high. Because the products of degradation are not fully known, their

Table 3. FB_1 Half-Life in Relation to Initial FB_1 Concentration and Lime Added in Tortillas

$Ca(OH)_2$ (mM)	FB_1 (ng/g)	mean ± SD[a] (min)
3.4	0.15	183.0 ± 6.6a
3.4	0.53	188.0 ± 2.6a
3.4	1.24	192.0 ± 7.2a
3.4	5.12	210.0 ± 7.5b
3.4	10.12	251.3 ± 7.1c

$Ca(OH)_2$ (mM)	FB_1 (ng/g)	mean ± SD[a] (min)
3.4	0.15	183.0 ± 6.6d
8.5	0.15	143.3 ± 5.0e
177.0	0.15	126.7 ± 4.0f
216.0	0.15	126.3 ± 4.2f
3.4	0.53	188.0 ± 2.6d
8.5	0.53	136.0 ± 4.4e
177.0	0.53	126.7 ± 4.5f
216.0	0.53	119.7 ± 5.0f
3.4	1.24	192.0 ± 7.2d
8.5	1.24	154.7 ± 6.1e
177.0	1.24	126.3 ± 3.8f
216.0	1.24	119.7 ± 5.5f
3.4	5.12	210.0 ± 7.5d
8.5	5.12	138.7 ± 4.7e
177.0	5.12	129.7 ± 3.8e
216.0	5.12	119.7 ± 5.7f
3.4	10.12	251.3 ± 7.1d
8.5	10.12	149.0 ± 5.2e
177.0	10.12	142.7 ± 6.0e
216.0	10.12	128.3 ± 2.1f

[a] Values followed by different letters are statistically different after ANOVA using the Tukey test. The statistical tests for the upper and lower sections of Table 3 were done independently.

identities must be determined. The cooking conditions that will reduce the concentration of FB_1, its reaction products, and potential residues need to be determined.

NOTE ADDED AFTER ASAP

Corrections have been made June 25, 2004, to **Table 3** after the initial ASAP posting of June 19, 2004.

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