

Reduction of Moniliformin During Alkaline Cooking of Corn¹

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ABSTRACT

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The incidence of moniliformin (MON) producing *Fusarium* spp. in selected corn (*Zea mays* L.) samples from Mexico and the United States and the effects of alkaline cooking and the tortilla manufacturing processes on the reduction of MON were determined. The percentage of infected kernels with *Fusarium* spp. ranged from 0 to 22% in eight food-grade corn samples, including six from Mexico and two from the United States. Complete (100%) reduction of MON was observed when a naturally

contaminated corn sample containing 1.4 µg of MON/g of corn was used in a pilot-scale alkaline cooking and tortilla manufacturing process. In a companion laboratory-scale study, using a cultured corn sample containing 17.6 µg of MON/g of corn, a 71% reduction of the toxin was observed during the process. Alkaline cooking appeared to be an effective method for reduction of MON in corn.

Fusarium proliferatum and *F. subglutinans* are known to be the main producers of the mycotoxin moniliformin (MON) in corn (Marasas et al 1979; Farber et al 1988; Lew et al 1996). MON is regarded as cytotoxic but not genotoxic, based on animal studies (Norred et al 1992; Knasmüller et al 1997). Toxicity studies of MON have shown it to be cardiotoxic. Symptoms in animal species include a progressive muscular weakness, lack of muscular coordination, respiratory distress, cyanosis, coma, and death (Cole et al 1973; Kriek et al 1977; Allen et al 1981). Intestinal and kidney hemorrhages in ducklings have also been reported (Vesonder and Wu 1998).

The natural occurrence of moniliformin was reported at 16 µg/g of corn in samples from the Transkei in South Africa (Thiel et al 1982). Surveys done in numerous countries have found that MON contents ranged from 0.015 to 530 µg/g of corn in cereal grains and finished food products (Sharman et al 1991; Lew et al 1993, 1996; Logrieco 1993; Adler et al 1995; Golinski et al 1997; MAFF-UK 1998). In the United States, 2.92 µg of MON/g of corn was reported from corn screenings associated with a field outbreak of leukoencephalomalacia in horses in Pennsylvania (Thiel et al 1986). MON contents of up to 0.87 µg/g of corn were also found in corn and corn-based retail products destined for human consumption (Sharman et al 1991; Gutema et al 2000). Currently, there are no Food and Drug Administration guidelines for MON in food in the United States.

The traditional method of processing corn into tortillas (nixtamalization) was developed by Latin American Indians many centuries ago (Cravioto et al 1952). In this process, corn is cooked in boiling or near-boiling lime solution for a relatively short time and steeped overnight. The cooked steeped corn (nixtamal) is washed to remove excess alkali and loose pericarp tissue. The nixtamal is then ground into masa (corn flour) and flattened into thin disks that are baked on a hot surface (Bedolla and Rooney 1982).

The nixtamalization process has been reported to reduce or eliminate the presence of mycotoxins in corn including aflatoxin, fumonisin, deoxynivalenol, and zearalenone (Price and Jorgensen 1985; Abbas et al 1988; Sydenham et al 1995; Dombrink-Kurtzman et al

2000). To date, there are no reports of the effects of processing corn under alkaline conditions on the reduction of MON, though we have shown that MON was reduced when heated at high temperatures under alkaline conditions in aqueous solutions (Pineda-Valdes and Bullerman 2000). The experiments described here were designed to determine the incidence of MON-producing fungi in eight selected corn samples and to investigate the effects of the nixtamalization process and tortilla manufacture on the reduction of MON in corn.

MATERIALS AND METHODS

Corn Samples

Corn samples used for analysis of MON and for making tortillas in the pilot plant, were obtained from the Cereal Technology Laboratory, University of Nebraska-Lincoln. A total of eight food grade corn samples designated M1–M8 were available. Samples M1–M6 were white corn samples from Mexico, whereas M7 and M8 were yellow corn samples from the United States, but which had been exported to Mexico. The bulk corn samples were cleaned at a commercial corn cleaning facility (Flatwater Milling Inc., Hastings, NE). Clean corn was used to determine the percent of kernels infected with total mold and *Fusarium*. Bulk corn, clean corn, and corn screenings were collected and analyzed for MON content. The samples were ground to a fine meal (except the portions used to determine internal infection) using a grinding/subsampling mill (model 2A, Romer Labs, Union, MO) and frozen until analyzed for MON.

Determination of Incidence of Molds

Dichloran 18% glycerol agar (DG18) was used to determine total mold infection (Hocking and Pitt 1980). Czapek's iprodione agar (CZID) was prepared as described by Abildgren et al (1987) and used for the selective determination and isolation of *Fusarium* species. CZID is a selective medium for *Fusarium* species and allows the formation of large and easily recognizable colonies. Fifty kernels of corn from each sample were analyzed by the direct plating technique for internal mold infection. Samples were surface sanitized by soaking in full strength household bleach (5.25% NaOCl) for 1 min and rinsed three times with sterile distilled water. The kernels were dried on sterile paper towels and aseptically placed on the surface of pre-poured DG18 and CZID agar in petri dishes. All the plates were incubated at 25°C for 10–14 days. The *Fusarium* species that grew from inside the kernels on CZID were isolated by the single spore technique using water agar (1.8% agar) as described by Nelson et al (1983). Single spores that germinated on the water agar were transferred to carnation leaf agar (CLA) (Fisher et al 1982) plates and incubated under natural lighting for 14 days at room temperature. The *Fusarium* species were identified by spore morphology and culture characteristics (Nelson et al 1983).

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Pilot-Plant Scale Nixtamalization and Tortilla Manufacturing Processes

Water (80 kg) was pumped into a near-commercial size pilot-scale, gas-fired cooking tank (model OCT002-01, Lawrence Equipment, So El Monte, CA). Food grade lime [Ca(OH)₂] was added to the cooking tank (1%, corn basis) and mixed until dispersed. The lime solution was heated to 88°C then 20 kg of corn was added to the tank. Additional heating with gentle agitation was needed to compensate for the temperature drop resulting from the addition of the corn. The corn was cooked for 20 min once the temperature of the corn-lime solution reached 88°C. At the end of heating process, the tank was uncovered and the corn was steeped for 16 hr. The steeped corn (nixtamal) was then rinsed twice with 50 kg of water. Water was poured from a measured cylinder into the cooking tank and the nixtamal was vigorously stirred. After the wash water was drained, masa was prepared by grinding the clean nixtamal in a commercial-scale stone grinder (model OCG2000-01, Lawrence Equipment). Masa was sheeted and cut into tortillas in an automated tortilla rolling/sheeting machine (model OFD1002-02, Lawrence Equipment). The tortillas were then baked in a gas-fired automated tortilla baking oven, preheated to ≈365°C. The tortillas were baked for ≈3 min and collected at the end of a continuous cooling conveyor (model OCC1208-03, Lawrence Equipment). Samples of corn and water were collected after each step. All corn, nixtamal, masa, and tortilla samples were dried overnight at room temperature, ground, and frozen until MON analysis.

Laboratory-Scale Nixtamalization and Tortilla Manufacturing Processes

A culture of *Fusarium fujikuroi* M-1214 (*Fusarium* Research Center, Pennsylvania State University) was grown on carnation leaf agar plates for 14 days at room temperature. Spores were harvested with 0.05% Tween 80 solution and used to inoculate 500 g of yellow dent corn in 1-gallon glass jars. The moisture content of the corn was adjusted to ≈26% (dry wt basis) by adding distilled water containing 10 mL of the spore suspension. The corn was incubated in a dark chamber at 25°C for four days. Jars were agitated at least twice a day to allow even distribution of the spores and hyphal growth and to prevent clumping. After four days of incubation, the corn was washed three times with distilled water, one time with 0.01% Tween 80, and two more times with distilled water to remove excess surface mold hyphae. The corn was then washed as previously described, dried on paper towels, and further dried at 50°C overnight. The corn was then ground and analyzed by HPLC for MON content as described below.

For the laboratory-scale nixtamalization and tortilla-making procedures, the conditions used at the pilot-plant level were duplicated. Briefly, 4 g of food-grade lime were dissolved in 1,600 mL of cold water, and heated to 88°C in a Kitchen Kettle multi-cooker/steamer (National Presto Industries, Eau Claire, WI). Cultured corn (400 g) was cooked in the lime solution for 20 min. The mixture was then transferred to a 1L beaker and steeped for 16 hr with the beaker

inside a polyfoam container to allow a slower cooling rate and simulate pilot-plant conditions. The corn was then rinsed twice with 500 mL of water, and masa dough was made using a grinding/subsampling mill (model 2A, Romer Labs). Tortillas of 13 cm diameter and 2 mm thickness were made with a tortilla press, and cooked for 3 min on each side at ≈250°C. Samples of corn and water were taken after each step. All samples were dried overnight at room temperature and frozen until analyzed for remaining MON.

Mycotoxin Standards

Purified MON was purchased from Sigma Chemical Co. (St. Louis, MO). Working standard solutions were prepared in 0.05M sodium dihydrogen phosphate monohydrate (pH 5) to concentrations up to 200 µg/mL. All reagents were of analytical grade, and solvents used for high performance liquid chromatography were of HPLC grade.

Analysis of MON in Corn, Masa, and Tortillas

MON content in corn samples was determined as described by Munimbazi and Bullerman (1998). A 10-g sample was extracted twice with 50 mL of 1.0% tetrabutyl ammonium hydrogen sulfate in water for 30 min using a wrist-action shaker (Burrel, Pittsburgh, PA). The extracts were centrifuged (10 min) and filtered through Whatman No. 4 filter paper. To improve the recovery, the MON in the initial masa and samples from each step in the process was extracted by the method developed by Munimbazi and Bullerman (2001). Briefly, 10-g of ground sample was extracted with three 100-mL portions of water heated each time in the microwave oven (1,000W for 60 sec at 100% power). The combined extracts were then centrifuged for 10 min at 5,000 × g and filtered through Whatman No. 4 filter paper. For corn samples, masa and samples from each step, 25 mL of the filtered extract was partitioned two times with 25 mL of dichloromethane. The two dichloromethane

TABLE II
Concentration of Moniliformin (µg/g) Remaining After Making Tortillas from Naturally Contaminated Corn (pilot-plant scale) or Cultured Corn (laboratory-scale)

Process Step	Pilot Plant	Laboratory
Raw corn (clean)	1.42 ± 0.15 ^a	17.64 ± 2.16 ^a
Cooked corn	0.04 ± 0.01 ^b	8.06 ± 1.07 ^b
Cooking water	nd	nd
Steeped corn	nd	6.28 ± 0.66 ^c
Steep water	nd	nd
Corn after rinse 1	nd	5.16 ± 0.45 ^d
Rinse water 1	nd	nd
Corn after rinse 2	nd	5.39 ± 0.71 ^d
Rinse water 2	nd	nd
Masa	nd	5.19 ± 0.28 ^d
Tortillas	nd	5.35 ± 0.43 ^d

^a Values in the same column with different letters are significantly different ($\alpha = 0.05$).

^b Not detected.

TABLE I
Internal Mold Infection Rate (%) and Occurrence of Moniliformin in Food-Grade Corn Samples^a

Sample	Total Mold (DG18)	<i>Fusarium</i>			Moniliformin (µg/g) ^b			
		(CZID)	<i>moniliforme</i>	<i>proliferatum</i>	<i>subglutinans</i>	Bulk	Clean	Screenings
M1	20	8	4	4	nd	nd	nd	0.12 ± 0.00
M2	8	8	2	6	nd	nd	nd	0.15 ± 0.01
M3	nd ^c	nd	nd	nd	nd	0.05 ± 0.00	nd	0.10 ± 0.01
M4	80	50	38	12	nd	nd	nd	0.07 ± 0.01
M5	10	6	6	nd	nd	0.09 ± 0.01	0.01 ± 0.00	0.38 ± 0.10
M6	40	40	14	22	4	0.08 ± 0.00	0.12 ± 0.01	0.91 ± 0.01
M7	26	10	4	4	2	0.85 ± 0.10	0.66 ± 0.06	1.64 ± 0.01
M8	52	16	4	8	4	1.50 ± 0.10	1.42 ± 0.15	1.85 ± 0.16

^a Percent of infection in 50 corn kernels.

^b Each value represents average of three replicates ± one standard deviation of the mean.

^c Not detected.

extracts were combined and evaporated to 5–10 mL at 50°C in a water bath under a stream of air. The reduced volume of dichloromethane extract was transferred into a small vial and evaporated to dryness.

Solid phase extraction columns (100 mg, SAX, Supelco, Inc., Bellefonte, PA) were conditioned by eluting 1 mL of methanol followed by 1 mL of 0.1M *o*-phosphoric acid. The evaporated extract was redissolved in 1 mL of water and loaded onto the SAX column. The column was washed with 1 mL of 50:50 methanol-to-water followed by 1 mL of water. The rinses were discarded and air was forced through the columns to expel all rinse solutions. MON was then eluted with 1 mL of 0.05M sodium dihydrogen phosphate monohydrate (pH 5.0).

A 20- μ L aliquot of the final extract was injected into an HPLC system which consisted of a HPLC pump (model 510, Waters Corp., Milford, MA), connected with an injector (EC6W, Valco Instruments Co., Houston, TX), a reverse-phase column (Nova-Pak C₁₈ 150 \times 4.6 mm, Waters) and a model 486 tunable absorbance detector set at 229 nm with 0.003 AUFS (Waters), controlled by Waters Millennium computer software (v. 2.15, Waters). MON was eluted using isocratic conditions with a mobile phase of 8:92 acetonitrile-to-ion pair solution (10 mL of ion-pair modifiers in 990 mL water, pH 6.5) at a flow rate of 1 mL/min. The ion pair modifiers were prepared by mixing 50 mL of 40% tetrabutyl ammonium hydrogen sulfate in 100 mL of 1.1M potassium dihydrogen phosphate.

Data of MON content obtained from HPLC were analyzed using analysis of variance (SAS Institute, Cary, NC). Least square means procedures were used to separate means when differences were statistically significant ($P \leq 0.05$). The entire experiment was replicated three times and all determinations were done in duplicate.

RESULTS AND DISCUSSION

Kernel Infection and MON Content

The total mold infection rates (% kernels infected) in corn samples were 0–80% when determined on DG18 plates, whereas infection rates of *Fusarium* spp. were 0–50% on CZID plates (Table I). Among the *Fusarium* species identified, *F. verticillioides* (formerly known as *F. moniliforme*) and *F. proliferatum* were more common contaminants than *F. subglutinans* in the corn. Previous reports also indicate these two *Fusarium* species are common pathogens and endophytes in corn around the world (Chu and Li 1994; Miller 1995; Marin et al 1996; Kosiak et al 1997). Although *F. verticillioides* was more common in some samples tested in this study, this species is not known to produce MON. In addition, the incidence of *Fusarium* spp. was not directly correlated with the MON content of a sample.

The eight samples each of bulk corn, clean corn, and corn screenings were analyzed for MON (Table I). Corn screenings showed the highest concentrations for MON, followed by bulk corn, and clean corn with concentrations of 0.1–1.85, 0–1.50, and 0–1.42 μ g/g, respectively. These results show that the concentration of MON in corn might be reduced to some extent by cleaning, and that the toxin might be more concentrated in certain fractions such as screenings. Other mycotoxins, including deoxynivalenol and zearalenone, have been reduced by washing with water (Trenholm et al 1992). In addition, mycotoxins tend to be in certain parts of the kernel, which results in varying concentrations in different fractions from processes such as milling (Katta et al 1997). In general, the MON content of the corn in this study was similar to concentrations found in other surveys of food-grade corn in the United States, Canada, and Europe (Scott and Lawrence 1987; Lew et al 1993; MAFF-UK 1998; Gutema et al 2000).

Pilot-Plant and Laboratory-Scale Nixtamalization

The fate of MON in a tortilla manufacturing process simulating a commercial procedure was studied with a cleaned corn sample

(M8) contaminated with the highest naturally occurring concentration of MON (1.4 μ g/g) found in this study. Tortillas were prepared using pilot-plant equipment and the results are shown in Table II. The concentration of MON was reduced by 97% during alkaline cooking of corn from the raw material. MON was not detected in any steps after alkaline-cooking, including the rinsing water. Because of the low initial concentration of MON, either complete loss of the toxin occurred during alkaline cooking or there was a partial loss combined with dilution below detectable levels during the process.

A parallel experiment performed in laboratory scale using cultured corn with an initial concentration of MON at 17.6 μ g/g resulted in a 71% reduction of MON from the initial concentration in the corn to the final product (Table II). The greatest reduction of MON (54%) resulted from the alkaline cooking step. Statistical analysis showed a significant difference ($P \leq 0.05$) in the concentration of MON between cooked corn (samples taken immediately after cooking) and steeped corn (samples taken after 16 hr of steeping). An additional 10% reduction of MON occurred during the steeping step following alkaline cooking. Though MON was not detected in the rinse water, toxin content in first-rinse corn was significantly ($P < 0.05$) reduced compared to that in steeped corn. No significant difference was found in MON contents among the samples of rinsed corn, masa, and tortillas. As shown in Table II, no MON was detected in any water sample analyzed, probably because the MON was too diluted to be detected with the method used.

In general, MON was reduced significantly by the nixtamalization process probably due to both heat and alkaline pH. These results agreed with the previous report that MON was not stable when heated in an aqueous solution at 150°C and pH 10 (Pineda-Valdes and Bullerman 2000). The nixtamalization procedure has also been effective for reduction of other mycotoxins. deArriola et al (1988) found nixtamalization to be effective in reducing aflatoxin in corn (\approx 89% reduction). Significant reductions of zearalenone (59–100%) and deoxynivalenol (72–82%) were also previously reported (Abbas et al 1988). This study has shown that the nixtamalization process is also an effective method for removing MON from corn.

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