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Enzyme-Assisted Extraction of Moniliformin from Extruded Corn Grits

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Water has been known to be the ideal solvent for moniliformin but is not suitable to extract this toxin from cooked matrices due to instant swelling upon addition of the solvent. In this study, an improved method to extract moniliformin from extruded corn grits using α-amylase was developed. In an effort to optimize the method, the efficacy of using a protease was also studied. Treatment with α-amylase resulted in a clear solution with decreased suspended solid content as measured by transmittance (%T), which improved from 0 to 96% in 10 min. The detected level of moniliformin from extruded corn grits was increased to 4.02 μg/g when extracted with 1% tetrabutylammonium hydrogen sulfate following α-amylase treatment compared to 2.56 μg/g when it was extracted with 90% acetonitrile without enzyme treatment. The average recovery of moniliformin from extruded corn grits was 96% when α-amylase was used in the extraction procedure. Overall, the amounts of moniliformin detected in extruded corn grits increased significantly by using enzyme hydrolysis. Chromatographic separation was also benefited by lesser interference and improved peaks.

KEYWORDS: Moniliformin; enzyme; extraction; extrusion; corn grits

INTRODUCTION

Moniliformin is a highly toxic mycotoxin, structurally characterized as the sodium or potassium salt of 3-hydroxycyclobut-3-ene-1,2-dione (*1*) (**Figure 1**). It is produced by several *Fusarium* species, of which *F. subglutinans* and *F. proliferatum* are, together with *F. verticilliodes* (*F. moniliforme*), important pathogens of corn and other cereal crops (*2*). Moniliformin has been reported to occur naturally in corn, wheat, rye, triticale, oats, and rice from different parts of the world (*3*−*6*). Moniliformin caused pathological changes including myocardial degeneration and necrosis in experiments with animals (*7*). The predominant mechanism of its acute toxicity is believed to be the inhibition of pyruvate dehydrogenase, by binding, preventing its entrance into the tricarboxylic acid cycle, therefore decreasing mitochondrial respiration (*8*). Dietary exposure indicated that moniliformin is more toxic to chickens than fumonisin B_1 (FB_1), another *Fusarium* mycotoxin (*9*). The toxin is also more cytotoxic than FB_1 on cultured mammalian cell lines (*10*, *11*) but is not genotoxic. Toxicity studies with animal species have also shown moniliformin to be a potent cardiotoxin (*12*).

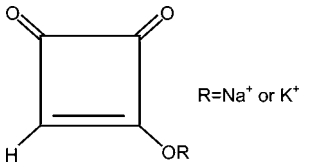

Figure 1. Structure of moniliformin.

A number of methods, including thin-layer chromatography (TLC), gas chromatography−mass spectrometry (GC-MS), liquid chromatography (LC), ion chromatography, and capillary zone electrophoresis, have been reported for the detection of moniliformin (*13*−*17*). In general, LC is preferred over TLC or GC-MS for the determination of moniliformin in corn and other cereal grains. Ion-paired reversed-phase LC has been used for determining moniliformin after extraction with water or a mixture of acetonitrile/water (*5*, *15*). Munimbazi and Bullerman (*18*) described a sensitive LC method for the detection of moniliformin in corn using 1% tetrabutylammonium hydrogen sulfate (TBAHS) as an ion-pairing agent. Extraction phases were separated with dichloromethane and cleaned up on strong anion exchange (SAX) columns. Extracts were analyzed by ion-pair reversed-phase LC with UV detection. Recoveries ranged from 96.5 to 97.8% with a detection limit of 0.025 μg/g of corn.

The ionic and polar nature of moniliformin makes extraction difficult, particularly from heat-processed corn matrices, as they absorb the aqueous extraction solvent and swell immediately.

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Extrusion cooking, which is used to manufacture various types of corn-based snack foods and breakfast cereals, subjects these matrices to high temperatures, high pressures, and severe mechanical shear. These forces may cause binding of moniliformin to the food matrix, resulting in low extraction efficiency and poor recovery. In addition, there is emerging evidence that chemical analysis of processed food products for fumonisins, another mycotoxin produced by *Fusarium* species, that involves extraction of the product does not account for all fumonisins in the food matrix because some are bound to food matrix components and are not extracted (*19*−*21*). Thus, a reliable method is needed for the extraction and analysis of moniliformin from extrusion-processed corn products. This study was undertaken to develop and optimize an extraction procedure using α-amylase and protease for the detection and quantification of moniliformin in extruded corn grits.

MATERIALS AND METHODS

Samples. Food grade corn grits were obtained from Lauhoff Grain Co. (Crete, NE). Prior to extrusion, the moisture content of the grits was adjusted to 22% (dry weight basis, db) by adding distilled water and mixing for 30 min in a Hobart mixer (Hobart Corp., Troy, OH) followed by equilibration overnight in plastic jars. Moniliformin was added to the distilled water used to hydrate the grits to obtain moniliformin concentrations of 5 μg/g of grits (db). The grits were extruded at a screw speed of 142 rpm and a temperature of 140 °C at all three zone of the barrel using a model CTSE-V laboratory-scale twin-screw extruder (C. W. Brabender Instruments, Inc., Hackensack, NJ) with a 3 mm diameter cylindrical nozzle. Extruded samples were dried at 60 °C for 24 h, ground to a fine consistency using a household blender, and stored in plastic bags at −20 °C until analysis.

Relative Enzyme Activity. A subsample of 2 g of extruded corn grits was weighed into a 100 mL Erlenmeyer flask, and 20 mL of TBAHS solution at 0, 0.05, 0.1, 0.15, 0.2, 0.5, and 1.0% was added. Each mixture was shaken for 30 min at 37 °C, at 180 rpm in an INR-200 laboratory orbital incubator (Gallenkamp, U.K.). For measuring α-amylase activity at each TBAHS concentration, 200 μL of α-amylase solution was added to each flask and then incubated for 5 min. For measuring protease activity at each TBAHS concentration, 200 μL of protease solution was added to each flask and then incubated for 30 min. The enzyme reactions were stopped by the addition of 200 μL of 40% TBAHS solution. Relative α-amylase activity was determined by measuring the starch−iodine complex at 578 nm (*22*), and relative protease activity was measured according to a colorimetric method at 660 nm (*23*) using a Spectronic 1001 spectrophotometer (Bausch & Lomb, Rochester, NY).

Extraction and Cleanup. *Enzyme-Assisted Extraction.* A 10 g ground extruded sample was weighed into a 125 mL polyethylene sample bottle, and 100 mL of 0.1% TBAHS solution, prepared in LC grade water, was added. The sample was incubated for 30 min at 180 rpm and 37 °C, in a laboratory orbital incubator. This homogenate was then subjected to three types of enzyme treatments: (i) α-amylase only solution [1 mL of α-amylase (EC 3.2.1.1, Sigma Chemical Co., St. Louis, MO)] and shaking for 10, 20, 40, 60, and 120 min at 180 rpm and 37 °C; (ii) protease only solution [1 mL of protease (type XXIII, Sigma Chemical Co. and shaking for 20, 40, 60, 120, and 180 min at 180 rpm and 37 °C; and (iii) α-amylase followed by protease (1 mL of α-amylase treatment for 10 min and then addition of 1 mL of protease solution) and shaking for 10, 20, 40, 60, and 120 min at 180 rpm and 37 °C. At the end of each enzyme treatment, 2 mL of 40% TBAHS solution was added, mixed well, and centrifuged for 10 min at 10000*g* followed by phase separation and SAX cleanup as described previously (*18*).

Acetonitrile/Water Extraction. A 10 g ground extruded sample was extracted with 100 mL of acetonitrile/water (9:1, v/v) and cleaned up using SAX solid-phase extraction (SPE) columns according to the method of Fliek and Linder (*15*).

Ion-Pair Extraction. A 10 g ground extruded sample was weighed into a 125 mL polyethylene sample bottle. Then 100 mL of a 1% TBAHS solution, prepared in LC grade water, was added and shaken for 60 min at maximum speed on a wrist-action shaker (Burrell Corp., Pittsburgh, PA). The extract was centrifuged for 10 min at 10000*g* followed by phase separation and SAX cleanup as described by Munimbazi and Bullerman (*18*).

Recovery of Moniliformin. Moniliformin standard solutions were prepared in LC grade water (10, 50, 100, and 200 μg/mL), and then 0.1 mL standard solutions were added to 10 g of ground extruded corn grits. Samples were analyzed according to the α-amylase-assisted extraction for 10 min as described above, and percentage recoveries were calculated.

Liquid Chromatography. The concentrations of moniliformin in the extruded corn grits were determined by high-performance liquid chromatography (HPLC) as previously described (*18*). Briefly, a 10 g sample was extracted twice with 50 mL of 1.0% TBAHS in water for 30 min using a wrist-action shaker (Burrell Corp.). The extracts were centrifuged (5000*g*; 10 min) and filtered through Whatman no. 4 filter paper (Whatman International Ltd., Maidstone, U.K.). Then, 25 mL of the filtered extract was partitioned two times with 25 mL of dichloromethane. The two dichloromethane extracts were combined and evaporated to 5−10 mL 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. SPE columns (100 mg SAX) (Supelco, Inc.) were conditioned with 1 mL of methanol followed by 1 mL of 0.1 M orthophosphoric acid. The evaporated extract was redissolved in 1 mL of water and loaded into the SAX column. The column was washed with 1 mL of 50:50 methanol/water followed by 1 mL of water. The rinses were discarded, and air was forced through the columns to expel all rinse solutions. Moniliformin was eluted with 1 mL of 0.05 M sodium dihydrogen phosphate monohydrate, pH 5.0. A 20 μL aliquot of the extract was injected into an HPLC system consisting of a model 510 pump (Waters Corp., Milford, MA) equipped with an EC6W injector (Valco Instruments Co., Inc., Houston, TX), a 150 × 4.6 mm i.d., 5 μm, C_{18} reverse-phase column (Waters Corp.), and a model 486 absorbance detector set at 229 nm and 0.003 AUFS (Waters Corp.), controlled by Waters Millenium computer software (version 2.15). Moniliformin was eluted using isocratic conditions with a mobile phase of 8:92 acetonitrile/ion-pair solution at pH 6.5 and a flow rate of 1 mL/min. The pH of the mobile phase adjusted by adding 5 N KOH and the ion-pair modifiers were prepared by mixing 50 mL of 40% TBAHS in 100 mL of 1.1 M potassium dihydrogen phosphate.

RESULTS AND DISCUSSION

Water appeared to be the ideal solvent for extracting moniliformin because of the polarity and high solubility of the toxin in water. Thiel (*24*) used water to extract moniliformin from spiked corn and reported a 95% recovery rate for the extraction step. Also, ion-pairing of moniliformin with TBAHS in water was found to be useful for the extraction of the toxin from corn samples. In the case of extract-extruded corn grits, however, the use of water or aqueous TBAHS solution did not work well because the extruded grits absorbed all of the liquid and it was impossible to get a clear extract even with filtration and centrifugation. It was then decided to use α-amylase and protease to try to improve the extraction efficiency of moniliformin by reducing matrix-related problems through enzyme hydrolysis of starch and protein in corn grits.

To choose the optimum conditions for enzyme hydrolysis of extruded corn grits, relative enzyme activities were determined with TBAHS concentrations at 0−1.0%. The pH values of extracts were 6.4, 5.6, 4.7, 4.1, 3.6, 2.4, and 2.0 when the final concentrations of TBAHS were 0, 0.05, 0.1, 0.15, 0.2, 0.5, and 1.0%, respectively. Relative activities of α-amylase and protease were the highest at 0.1 and 0.05% TBAHS, respectively (**Figure 2**). At 0−0.2% TBAHS, α-amylase retained >95% of its activity, whereas protease activity decreased to 82 and 39% at 0.15 and 0.2% TBAHS, respectively. The relative activities of both enzymes were very low at 0.5 and 1% TBAHS. Thus, 0.1%

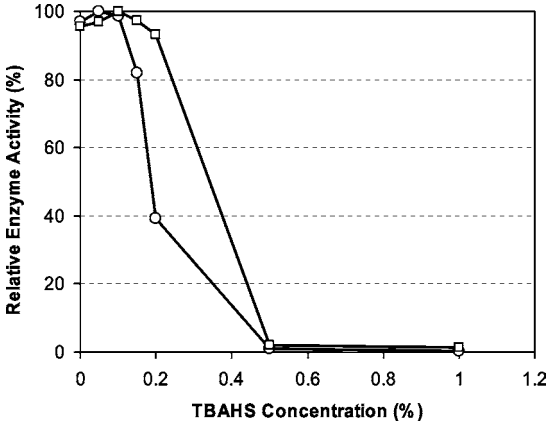

Figure 2. Relative activities of α-amylase and protease at various tetrabutylammonium hydrogen sulfate (TBAHS) concentrations: (○) amylase; (□) protease.

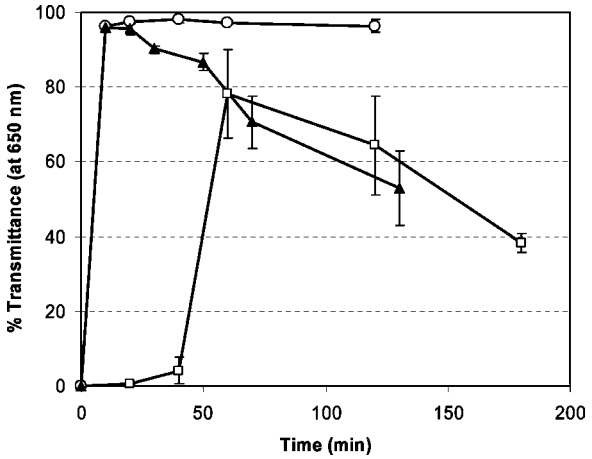

Figure 3. Changes of percent transmittance of extruded corn grit extracts after enzyme extraction: (○) amylase; (□) protease; (▲) amylase followed by protease.

TBAHS solution was used for hydrolysis of extruded samples. With 10 g of extruded sample, 100 mL of 0.1% TBAHS solution and a mixing time of ~30 min were needed to get a homogeneous mixture. This was followed by three types of enzyme hydrolysis treatments, which were then followed by the addition of 2 mL of 40% TBAHS to stop the enzyme hydrolysis and promote the ion-pairing extraction of moniliformin.

During enzyme hydrolysis, the extract became clear and solids decreased. These changes were measured as percent transmittance (%T), as shown in **Figure 3**. With α-amylase alone, %T of the extract became 96.3 in 10 min and remained constant after continued reaction for 120 min. Protease treatment alone caused only small changes of %T for 40 min but increased to 83.1%T at 60 min. Protease hydrolysis, however, produced a yellow pigment after 60 min, which caused the %T to decrease upon continued reaction. In the case of the combined treatment, with α-amylase added first, followed (after 10 min) by protease, %T of the extract rose to 99.0 at 10 min after protease addition. On continued reaction, a yellow pigment was again formed and %T decreased in the same manner as when protease was used alone. All three of the enzyme treatments improved phase separation and made the SAX SPE cleanup much easier.

The amounts of moniliformin detected in extruded corn grits using enzyme hydrolysis/extraction steps are shown in **Table 1**. The maximum amount of moniliformin detected was ~260 ng/g for each of the three enzyme treatments. The main difference was the amount of time needed to reach the maximum concentration of moniliformin, 10 and 60 min for the α-amylase and protease treatments, respectively, and similar levels were maintained during further enzyme reaction time. In the case of α-amylase followed by protease, additional protease treatment after 10 min of α-amylase did not increase the amount of moniliformin detected. Of the three types of enzyme treatments, use of α-amylase for 10 min was chosen for improving the extraction of moniliformin because of the ease of handling and shorter reaction time compared with those required by the other two enzyme treatments.

Table 1. Levels of Moniliformin in Extruded Corn Grits by Enzyme-Assisted Extraction

enzyme used	extraction time (min)	level of moniliformin[a] (ng/g)
none[b]	60	204.7 ± 25.6
α-amylase	10	262.6 ± 5.7
	20	264.6 ± 5.8
	40	256.0 ± 4.7
	60	258.0 ± 7.2
	120	255.3 ± 6.3
protease	20	237.7 ± 14.1
	40	246.8 ± 12.0
	60	264.4 ± 14.1
	120	254.1 ± 13.5
	180	261.1 ± 10.4
α-amylase (10 min) followed by protease	10	256.8 ± 4.3
	20	260.6 ± 14.7
	40	256.7 ± 14.5
	60	248.6 ± 10.6
	120	255.8 ± 15.7

[a] Mean ± SD of triplicate experiments. [b] Ion-pair extraction with 1% tetrabutylammonium hydrogen sulfate (TBAHS).

Table 2. Levels of Moniliformin Found in Unextruded and Extruded Samples by Different Extraction Procedures

		level of moniliformin[a] (μg/g)	
sample	extraction procedure	corn grits	spiked corn grits
unextruded	1% TBAHS[b]	0.32 ± 0.08	5.01 ± 0.16
extruded	90% CH_3CN[c]	0.15 ± 0.01	2.56 ± 0.40
	1% TBAHS	0.20 ± 0.03	3.25 ± 0.39
	0.1% TBAHS + α-amylase	0.26 ± 0.01	4.02 ± 0.12

[a] Mean ± SD of triplicate experiments. [b] Extracted with tetrabutylammonium hydrogen sulfate (TBAHS). [c] Extracted with acetonitrile/water (9:1, v/v).

Concentrations and chromatographic separation of moniliformin were compared when extruded samples were extracted with acetonitrile/water, 1% TBAHS, and α-amylase. Corn grits and spiked corn grits contained 0.32 and 5.01 μg/g of the toxin, respectively, before extrusion cooking. In the case of extruded samples, extraction with α-amylase gave the highest detection levels of moniliformin, 0.26 and 4.02 μg/g for corn grits and spiked corn grits, respectively (**Table 2**), and best separations on chromatograms (**Figure 4**). The lowest concentrations of the toxin detected, 0.15 and 2.56 μg/g for corn grits and spiked corn grits, respectively, were obtained using acetonitrile/water as extracting solvent, even though the extracts were clear and chromatograms showed good separation. The amount of toxin recovered increased by ~33% with 1% TBAHS extraction as compared to the value obtained with acetonitrile/water extraction. The TBAHS extract, however, contained more solids than extracts obtained with acetonitrile/water or α-amylase and formed a thick emulsion upon partition with dichloromethane.

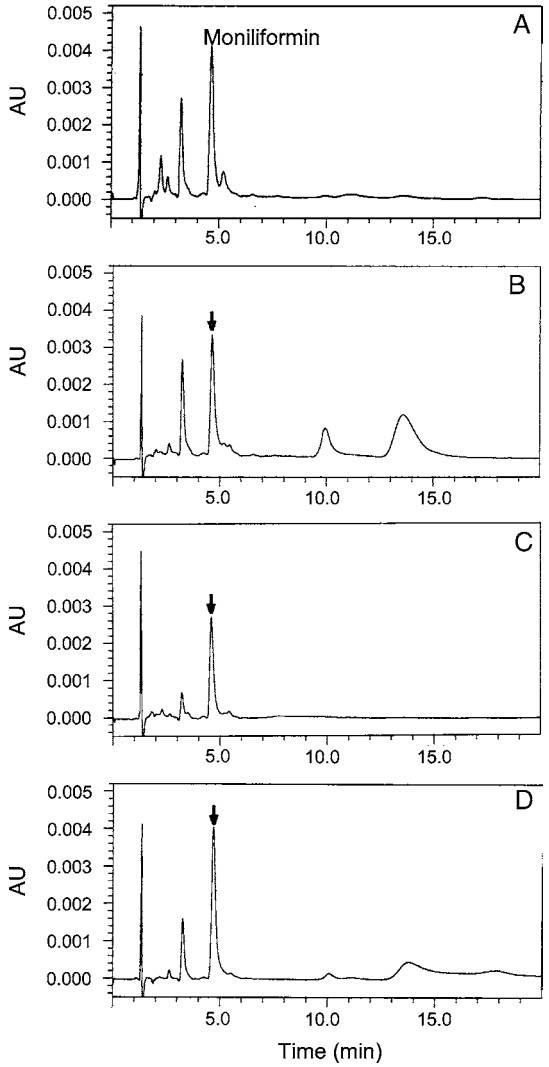

Figure 4. Chromatograms of corn grit samples naturally contaminated with moniliformin; 0.02 g of sample equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003: (**A**) unextruded corn grits, extraction with 1% TBAHS; (**B**) extruded corn grits, extraction with 1% TBAHS; (**C**) extruded corn grits, extraction with acetonitrile/water (9:1, v/v); (**D**) extruded corn grits, extraction with α-amylase.

Also with TBAHS, moniliformin peaks had tails and other broad peaks appeared in the chromatograms, thus making HPLC analysis longer than when extractions with either acetonitrile/water or α-amylase were used. When the samples were extracted with α-amylase, clear extracts and >95 mL were obtained, and the amounts of moniliformin detected increased by ca. 173 and 157% for corn grits and spiked corn grits, respectively, over the values obtained with acetonitrile/water extraction. These results were used to determine that only ~19% of moniliformin was degraded after extrusion cooking when α-amylase was employed in the extraction procedure, whereas degradations of the toxin were overestimated as ca. 51 and 36%, respectively, when the samples were extracted with acetonitrile/water and 1% TBAHS.

The average recovery of moniliformin from extruded corn grits for four concentrations tested was 96% when α-amylase was used in the extraction procedure. The results are shown in **Table 3**. Recoveries from samples spiked at 0.05 and 0.25 μg/g were higher than from samples spiked at 0.5 and 1.0 μg/g because the extruded sample used for the recovery tests contained 0.26 μg/g of moniliformin before spiking with the toxin. Compared with previously published extraction procedures, using acetonitrile/water and 1% TBAHS for the extraction of moniliformin in extruded corn grits with α-amylase was more efficient, gave cleaner extracts, resulted in improved detection levels and better chromatographic separations, and reduced the handling time.

Table 3. Recoveries of Moniliformin Added to Extruded Corn Grits by Extraction Using α-Amylase

moniliformin added (μg/g)	recovery[a] (%)
0.05	99.3 ± 27.5
0.25	102.8 ± 11.2
0.5	92.7 ± 1.5
1.0	90.5 ± 7.1

[a] Mean ± SD of triplicate experiments.

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