FOOD CHEMICAL CONTAMINANTS

High-Performance Liquid Chromatographic Method for the Determination of Moniliformin in Corn [1]

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A high-performance liquid chromatographic method using UV absorption was developed for determining moniliformin in corn. The toxin was extracted with water containing 1% tetrabutylammonium hydrogen sulfate (w/v). Paired moniliformin was partitioned into dichloromethane, which was evaporated to dryness at 50°C. The residue was dissolved in water and applied to a disposable strong-anion exchange solid-phase extraction tube. Adsorbed moniliformin was eluted from the tube with 0.05M sodium dihydrogen phosphate monohydrate (pH 5). It was determined by ion-pair reversed-phase chromatography and UV measurement at 229 nm. The minimum detectable amount of pure moniliformin was 0.25 ng/injection (signal-to-noise ratio = 3:1). The detector response was linear from 0.25 to at least 20 ng. The limit of determination was 0.025 μg/g corn. Recoveries of moniliformin from corn spiked at 0.025, 0.05, 0.25, and 1.0 μg/g averaged 96.5, 96.2, 97.2, and 97.8% respectively.

Moniliformin is a fungal metabolite structurally characterized as 3-hydroxycyclobut-3-ene-1,2-dione (Figure 1). It was first isolated in 1973 from corn culture that had been inoculated with *Fusarium proliferatum* but that had been misidentified as *F. moniliforme*, thus the name moniliformin (1). The metabolite is produced by at least 15 other *Fusarium* species (2–7). Of these, several species are particularly important pathogens of cereal grains throughout the world, *F. proliferatum* and *F. subglutinans* being the most important in corn. Moniliformin has been reported to occur naturally in corn, wheat, rye, triticale, oats, and rice from different parts of the world (8–15).

Moniliformin is a highly toxic metabolite. It is acutely toxic to many experimental animals including chickens, ducklings, and rats (1, 2, 16–18). The predominant mechanism of its acute toxicity is believed to be inhibition of pyruvate dehydrogenase. The toxin binds to pyruvate dehydrogenase, preventing entrance of pyruvate into the tricarboxylic acid cycle and therefore decreasing mitochondrial respiration (19–21). Dietary exposure indicated that moniliformin is more toxic to chickens than fumonisin B_1, another mycotoxin produced by *F. proliferatum* and *F. subglutinans* (22). It is also more cytotoxic than fumonisin B_1 on cultured chicken cells and other cultured mammalian cell lines (23, 24). Moniliformin is a potent cardiotoxic mycotoxin (1, 25–27). Although the acute and long-term toxicity of moniliformin for humans is not yet known, some Chinese scientists suggest that moniliformin is involved in the heart disease known as Keshan disease occurring in Chinese regions where inhabitants eat home-grown corn infected by *F. subglutinans* and contaminated with moniliformin (28). The lack of mutagenicity to *Salmonella typhimurium* (29) suggests that moniliformin is probably not carcinogenic.

Only a few analytical methods have been published for determination of moniliformin in agricultural products. These methods include thin-layer chromatography (TLC), gas chromatography/mass spectrometry (GC/MS), and high-performance liquid chromatography (HPLC). Most of the published methods are not entirely satisfactory for routine determination of moniliformin. They are either not sensitive and selective or not practical for routine analysis of moniliformin in a large number of samples. TLC methods allow detection limits usually in the range 0.1–1.0 μg/g sample depending on sample cleanup (2, 17, 30, 31). A GC/MS method with a detection limit of 5 pg of derivatized standard moniliformin was developed (32) but not applied to corn or other cereal grains. HPLC is generally preferred over TLC because of its improved sensitivity and resolution. Thiel et al. (8) used ion-pair reversed-phase and ion-exchange LC for determining moniliformin in corn. Recovery rates of the procedure were not given and were stated to be low and to vary considerably. The procedure included a 4 h sample cleanup. Shepherd and Gilbert (33) described an LC procedure that uses ion pairing for extraction and separation of moniliformin. The procedure was relatively sensitive (minimum detectable amount of standard moniliformin, 10 ng; determination limit, 0.1 μg/g corn), with recovery rates in the range 60–80% at spiking concentrations of 0.1–1.6 μg/g. However, the procedure suffered from coeluting interfering peaks that made interpretation of chromatograms difficult. The procedure, which required a 3-step cleanup, also was not practical for routine determination of moniliformin. Scott and Lawrence (10) developed an LC method with recovery rates of

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74–83% at spiking concentrations of 0.05–1.0 μg/g corn and a detection limit of about 0.01 μg/g. However, chromatographic separations were very poor. Thiel (34) described 2 LC procedures using ion-exchange and ion-pair reversed-phase separation. The detection limit of standard moniliformin was 20 ng, and overall recovery rates were about 70%. The procedures depended upon a lengthy sample cleanup (4 h) that did not eliminate major interfering compounds and a lyophilization step. Sharman et al. (11) described a sensitive LC method for determining moniliformin in corn, wheat, rye, and triticale. Samples were extracted with 95% acetonitrile in water. Extracts were concentrated by evaporation at 40°C and cleaned up on a combination of reversed-phase and strong-anion-exchange (SAX) disposable cartridge columns. Extracts were analyzed by ion-pair reversed-phase HPLC with UV detection. Recoveries ranged from 81 to 96% for samples spiked at 0.25 and 0.5 μg/g and the limit of detection was 0.05 μg/g. Efforts to use this method in our laboratory were not successful. Recoveries of moniliformin extracted with mixtures of acetonitrile and water were very low when extracts were concentrated by evaporation. Recently, Filek and Lindner (35) reported a very sensitive and selective LC method. The procedure detected moniliformin at 0.02 μg/g, with overall recoveries of about 70% at spiking concentrations of 0.02–0.25 μg/g. The smallest detectable amount of moniliformin derivative was 0.5 ng. Unfortunately, the method required a time-consuming (2 h) fluorescence derivatization step.

We found the ion-pairing phenomenon very useful in a redesigned analytical procedure for determining moniliformin in corn. Compared with other published LC methods using UV detection, the primary advantages of this procedure are simple and efficient sample extraction and cleanup resulting in improved recoveries, chromatographic separation, and sensitivity.

METHOD

Moniliformin is a toxic substance and should be handled with caution. All apparatus and reagents may be replaced by equivalent substitutions.

Apparatus

(a) *Liquid chromatograph.*—Model 510 HPLC pump operated at 1 mL/min, Model 486 tunable absorbance detector set at 229 nm and 0.003 absorbance unit full scale (AUFS; Waters, Milford, MA), Valco EC6W injector valve (Vici Valco Instruments Co., Inc., Houston, TX) with a 20 μL injection loop, and Model HP3395 integrator with chart speed of 0.8 cm/min (Hewlett-Packard, Avondale, PA).

(b) *Analytical and guard columns.*—Ultremex C_{18} reversed-phase column (150 × 4.6 mm id, 5 μm) and Partisil 10 SAX guard column (30 × 4.6 mm id, 10 μm; Phenomenex, Torrance, CA).

(c) *Shaker.*—Wrist-action shaker (Burrell Corp., Pittsburgh, PA).

(d) *Solid-phase extraction (SPE) column.*—Disposable LC SAX tubes, 1 mL capacity containing 100 mg sorbent (Supelco, Inc., Bellefonte, PA).

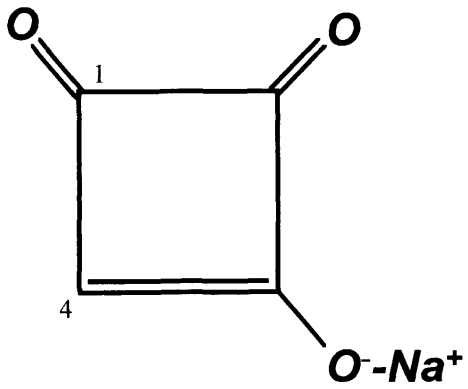

Figure 1. Structural formula of moniliformin (sodium salt).

Reagents

(a) *Solvents.*—Acetonitrile, dichloromethane, methanol, and water (all LC grade).

(b) *Chemicals.*—Tetrabutylammonium hydrogen sulfate (TBAHS) (98%; VWR Scientific products, Chicago, IL), potassium dihydrogen phosphate, sodium dihydrogen phosphate monohydrate (all LC grade), and *o*-phosphoric acid (85%).

(c) *Solution of ion-pair modifiers.*—Fifty milliliters of 40% tetrabutylammonium dihydrogen sulfate mixed with 100 mL 1.1M potassium dihydrogen phosphate (both solutions were prepared in LC grade water and filtered through 0.20 μm nylon membrane).

(d) *LC mobile phase.*—Prepare by diluting 10 mL ion-pair modifiers with acetonitrile–water (8 + 92) to a final volume of 1 L. Adjust pH of mobile phase to 6.5 with 5N KOH and filter through a 47 mm × 0.45 μm nylon membrane. Degas before use. Run and allow the HPLC system to equilibrate for ca 1 h prior to use.

(e) *Moniliformin standard solution.*—Pure sodium salt of moniliformin (Sigma Chemical Co., St. Louis, MO) dissolved in 0.05M sodium dihydrogen phosphate monohydrate, pH 5.0 (200 μg/mL) and stored at 4°C.

(f) *Working standards.*—Prepare an intermediate solution of moniliformin standard (10 μg/mL) in 0.05M sodium dihydrogen phosphate monohydrate (pH 5). Use portions of the solution to prepare different concentrations of moniliformin in the range 0.01–1.0 μg/mL. Store solutions at 4°C. They are stable for at least 6 months.

Sample Extraction

Grind corn to pass U.S. No. 20 sieve. Place 10 g ground corn sample into 125 mL polyethylene sample bottle. Add 50 mL 1% tetrabutylammonium hydrogen sulfate (TBAHS) prepared in LC grade water and shake for 30 min at maximum speed on a wrist-action shaker. Filter extract by gravity through Whatman No. 4 filter paper, taking care to retain most solids in the sample bottle. Add 50 mL 1% TBAHS to solids in sample bottle and shake for additional 30 min. Filter extract through the same filter paper and combine the 2 extracts. Transfer 25 mL extract into a separatory funnel or 125 mL sample bottle and add 25 mL dichloromethane. Mix gently and avoid vigorous shaking. Let phases separate and drain lower phase into a 100 mL container. If an emulsion is formed, centrifuge at

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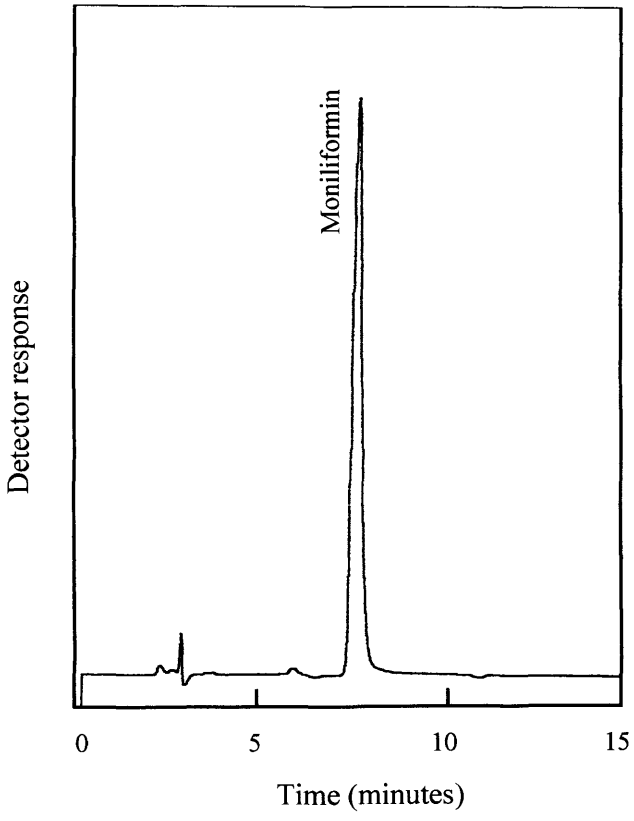

Figure 2. Chromatogram of standard moniliformin (20 ng injection).

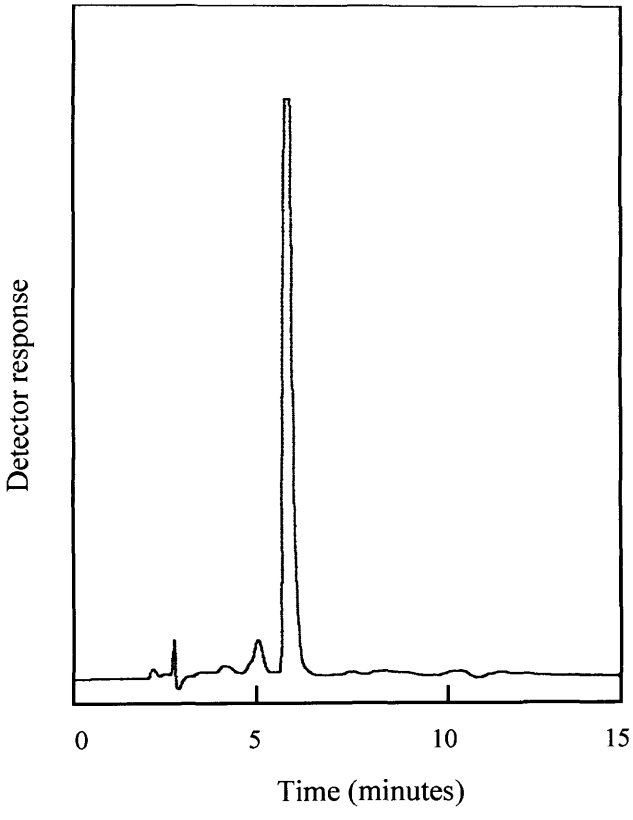

Figure 3. Chromatogram of moniliformin-free corn sample; 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

3000 rpm for 5 min to allow good phase separation. Repeat the partition with additional 25 mL dichloromethane and combine dichloromethane extracts. Evaporate dichloromethane to 5–10 mL at 50°C in a water bath under a stream of blowing air. Transfer the reduced volume of dichloromethane into a small vial and evaporate to dryness.

Extract Cleanup

Fit disposable SAX SPE tube on the end of a 10 mL syringe or port of vacuum manifold. Condition tube by washing successively with 1 mL methanol, 1 mL water, and 1 mL 0.1M *o*-phosphoric acid. Do not allow tube to dry. Dissolve extract residue into 1 mL LC-grade water and load onto the SPE tube. When all extract has passed through tube, wash tube with 1 mL water and force air through tube to expell all the wash solution. Elute adsorbed moniliformin with 1 mL 0.05M sodium dihydrogen phosphate monohydrate (pH 5.0). Filter eluate through a 0.2 μm nylon membrane and save eluate at 4°C before LC analysis.

Liquid Chromatography

Prepare a standard curve by injecting 20 μL moniliformin working standards. The retention time of moniliformin is ca 7.5 min. There is no need to prepare the standard curve daily, but injection of a moniliformin standard solution is required for each analysis. Inject 20 μL test solution. Identify peak and determine the quantity of moniliformin by comparing retention time and area with those of reference standard.

Spiking of Samples and Recovery

Prepare moniliformin standard solutions in LC grade water (2.5, 5.0, 25, and 100 μg/mL) and spike ground sample at 0.025, 0.05, 0.25, and 1.0 μg/g with a spiking volume of 0.1 mL for 10 g sample. Analyze samples according to the procedure described above and calculate percentage recoveries. Use 3 replicates of spiked samples at each concentration for each run and repeat analysis to determine recoveries and daily variation of the analytical procedure.

Results and Discussion

Extraction of Moniliformin

Water appeared to be the ideal solvent for extracting moniliformin because of the polarity and high solubility of the toxin in water. Thiel (34) used water (40 mL) to extract moniliformin from spiked ground corn (3 g) and reported a 95% recovery rate for the extraction step.The extraction procedure described here is based on the extraction procedure developed by Shepherd and Gilbert (33). However, conditions were profoundly modified to improve removal of interferences and recoveries. These investigators used water (150 mL) containing ion-pairing reagent (tetra-*n*-butylammonium hydroxide) to extract moniliformin in ground corn (30 g). The extraction was followed by

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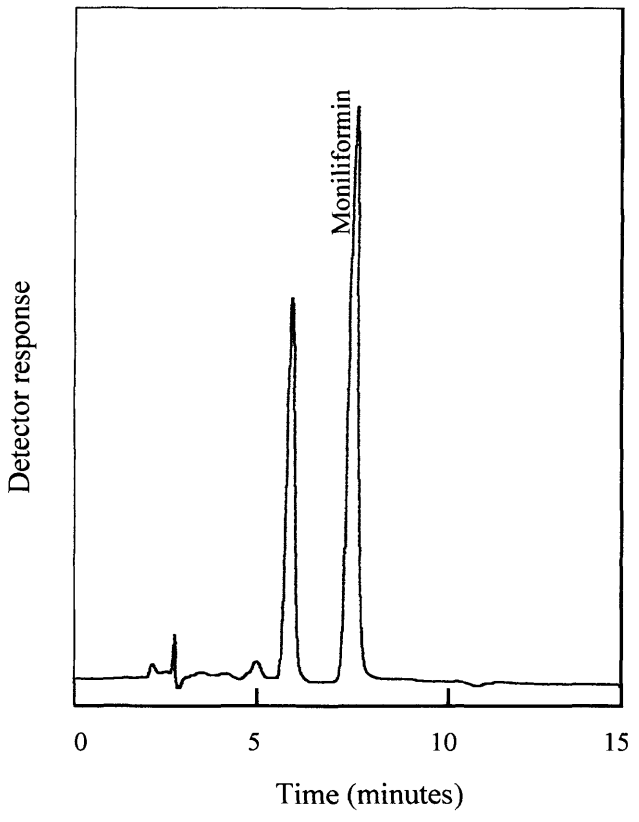

Figure 4. Chromatogram of corn sample spiked with moniliformin (1 μg/g); 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

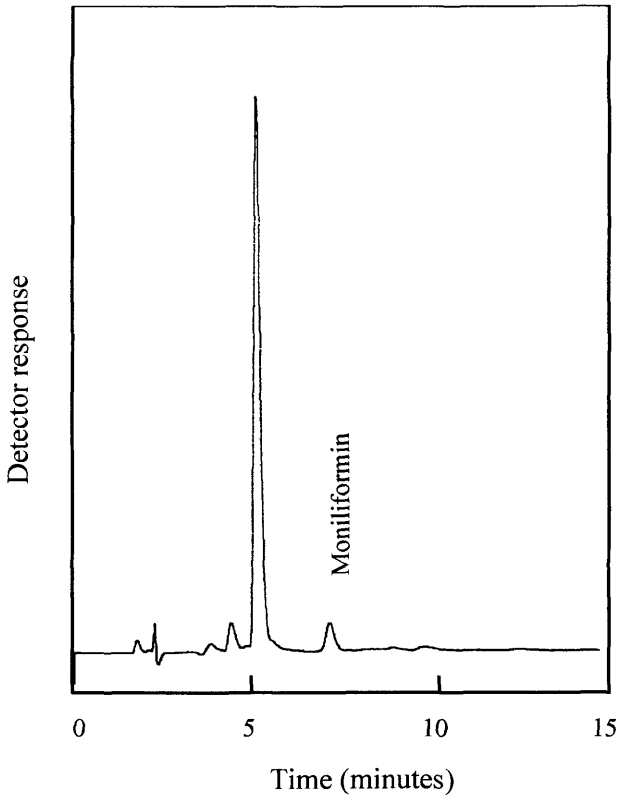

Figure 5. Chromatogram of corn sample spiked with moniliformin (0.025 μg/g); 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

cleanup on Amberlite IRC-50 resin and C_{18} Sep-Pak cartridge before another ion-pairing and final cleanup on "Chem Tube." Compared with extraction, ion-pairing, and cleanup procedures described by these investigators, conditions in our procedure were simplified very much, therefore making the procedure easier and more practical and reducing handling time. Extraction and ion-pairing of moniliformin were accomplished in a single step by using 1% TBAHS in water. No pre-cleanup on Amberlite IRC-50 resin or C_{18} Sep-Pak cartridge was required for successful ion pairing and subsequent partition of moniliformin. Pre-cleanup on C_{18} Sep-Pak cartridge was particularly omitted in our procedure because C_{18} Sep-Pak cartridges or columns bound up to 35% of free moniliformin and 100% of paired moniliformin in water solution. Two 50 mL volumes of 1% TBAHS were used to extract moniliformin. In general, the first volume extracted 70–75% of recovered moniliformin.

Moniliformin is frequently extracted with mixtures of water and organic solvents (10–12, 35). Initial efforts in our laboratory to extract moniliformin with various ratios of acetonitrile–water or methanol–water and concentrate extracts by evaporation gave overall recoveries not exceeding 40% and sometimes as low as 10% depending on the spiking level. Low or zero recoveries of moniliformin from water or mixed organic-aqueous extracts taken to dryness or near dryness have been reported (33). To prevent low recoveries of moniliformin, Rottinghaus recommended use of siliconized glassware and not overdrying when evaporating the extract of moniliformin (personal communication). However, this procedure did not improve recoveries of moniliformin from solutions taken to near dryness.

Moniliformin spiked into water or an aqueous extract of corn (0.1 μg/mL) could not be partitioned into dichloromethane, chloroform, or ethyl acetate without prior pairing of the toxin with tetrabutylammonium counter ion. When a moniliformin-free corn sample was extracted with 1% TBAHS and the extract spiked with moniliformin, about 100% of moniliformin was partitioned into dichloromethane, 53% into ethyl acetate, and less than 5% into chloroform. Paired moniliformin in aqueous extract was partitioned into 2 volumes of dichloromethane. Approximately 85% of paired moniliformin partitioned in the first volume and 15% in the second volume. Compared with previously published methods, pairing moniliformin with TBAHS followed directly by partition into dichloromethane before cleanup was a new step in moniliformin analysis. The described pairing and partition procedure enhanced overall recoveries of moniliformin from spiked corn.

Shepherd and Gilbert (33) indicated that exposure of moniliformin to low pH leads to loss of the toxin. Therefore the stability of moniliformin in 1% TBAHS aqueous extract of corn (pH of about 2.2) was studied by holding extracts at room temperature for up to 24 h before cleanup and analysis. Results indicated that paired moniliformin was very stable in 1% TBAHS extracts with no loss at all. Additional studies also in-

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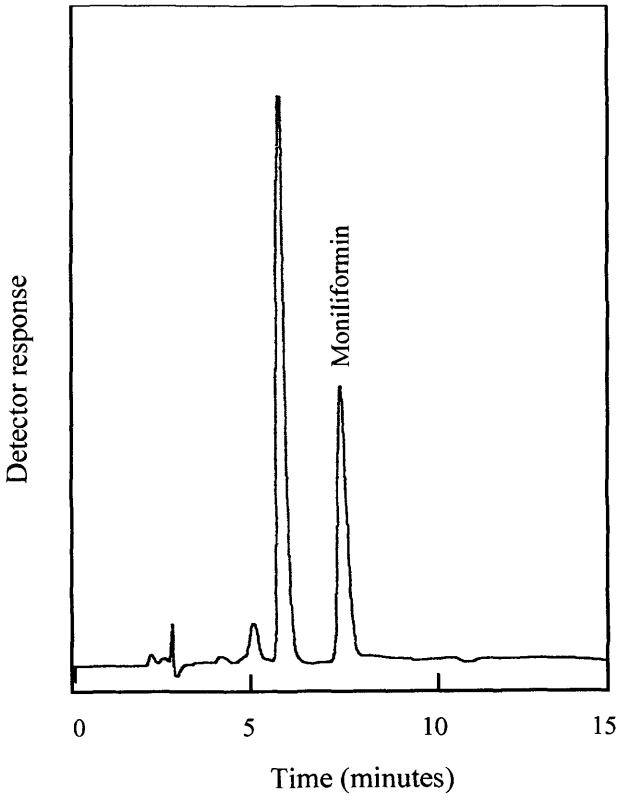

Figure 6. Chromatogram of corn sample naturally contaminated with moniliformin (0.2 μg/g); 0.05 g corn equivalent injected; absorbance wavelength, 229 nm; AUFS, 0.003.

dicated that the toxin was very stable in dichloromethane after partition and in water after evaporation of dichloromethane (data not shown). This high stability of moniliformin is a real advantage of the proposed analytical procedure.

Cleanup Procedure

Cleanup on disposable SAX SPE tubes was adapted from procedures described by other investigators (11, 35). However, the C_{18} SPE column used in combination with SAX SPE column (11) was omitted in our procedure because of adverse effects of the C_{18} cleanup column on recovery. A 1 mL SAX tube was preferred over the 3 mL tube used by other investigators. It required lower amounts of solvents and appeared to be more efficient than a 3 mL tube at retaining interference compounds. Sodium phosphate buffer (pH 5) was preferred over solvents used by Sharman et al. (11) or Filek and Lindner (35) for eluting moniliformin from the SAX tube. The buffered water was more efficient at eluting adsorbed moniliformin, and it eluted fewer interferences than other solvents.

HPLC Separation and Determination

The optimized extraction–cleanup procedure gave excellent chromatograms free of coextractive interferences. Typical chromatograms of standard moniliformin (20 ng injection), moniliformin-free corn, spiked corn samples (0.025 and 1 μg/g), and naturally contaminated corn (0.2 μg/g) are shown in Figures 2–6. In all cases, moniliformin eluted as a very sharp peak without tailing and well separated from other constituents. Reproducibilities of retention time, peak area, and width determined over many days were all excellent (Table 1). The limit of detection of pure moniliformin was 0.25 ng (signal-to-noise ratio = 3:1), which is lower than the 1 ng reported by Thiel (34) or 0.5 ng moniliformin-1,2-diamino-4,5-dichlorobenzene fluorescent derivative reported by Filek and Lindner (35). The chromatographic response was linear ($R^2 = 1.00$) between 0.25 and at least 20 ng moniliformin injected onto the column.

Different reversed-phase columns and guard columns were compared for chromatographic separation of moniliformin from other compounds in corn extracts. The combination of a Partisil 10 SAX guard column and Ultremex C_{18} column gave the best separation of moniliformin from interfering compounds, which could not be obtained by using either a Partisil 10 SAX column in combination with a similar guard column or an Ultremex C_{18} column and a similar guard column. The limit of determination of moniliformin spiked into ground corn was 0.025 μg/g corn, which is lower than the 0.1 μg/g corn reported by Shepherd and Gilbert (33) and comparable with the 0.01 and 0.02 μg/g corn reported by Scott and Lawrence (10) and Filek and Lindner (35), respectively. Chromatograms were better than any previously published. The peak corresponding to moniliformin was sharp and free of interfering compounds. Average recovery rates of moniliformin spiked into ground corn at 0.025–1 μg/g varied from 96 to 98% (Table 2), which are higher than the 70–80% recovery rates reported by other investigators (10, 33–35).

Extraction and recovery rates were compared when moniliformin spiked in ground yellow corn at 1 μg/g was extracted with water alone followed by ion pairing with TBAHS, 1% aqueous solution of TBAHS, and aqueous solutions of tetrabutylammonium hydroxide (TBAH) or tetrabutylammonium hydrogen phosphate (TBAHP) (Sigma Chemical Co.,

Table 1. Reproducibility of moniliformin determination by ion-pairing reversed-phase chromatography[a]

Parameter	Retention time, min	Peak area	Peak width
Mean (n = 15)	7.50	1839536	0.244
Standard deviation	0.01	15815	0.002
Coefficient of variation, %	0.16	0.9	1.0

[a] Column: Ultremex C_{18} reversed-phase column (150 × 4.6 mm; 5 μm) with Partisil 10 SAX guard column (30 × 4.6 mm, 10 μm). Mobile phase: 10 mL of modifiers (50 mL 40% TBAHS + 100 mL 1.1M potassium dihydrogen phosphate) diluted with acetonitrile–water (8 + 92); final volume, 1 L; pH, 6.5. Flow rate, 1 mL/min; detection wavelength, 229 nm (0.003 AUFS); injection, 20 ng standard moniliformin.

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Table 2. Recoveries of moniliformin added to ground corn

Moniliformin added, μg/g	Recovery		
	Average, %	Range, %	CV, %
0.025	96.5	86.3–109.9	8.1 (n = 9)
0.050	96.2	83.0–109.1	9.7 (n = 12)
0.250	97.2	88.3–102.2	4.4 (n = 12)
1.0	97.8	95.4–105.7	2.8 (n = 15)

St. Louis, MO) with molar concentration equal to that of 1% TBAHS solution. Extraction with 1% TBAHS was the prefered procedure; extracts were very clear and filtered faster than others. TBAHP solution extracted more solids than TBAHS or water alone, thus making filtration by gravity very difficult. Extracts formed a thick emulsion upon partition with dichloromethane. TBAH solution extracted yellow pigments and a large amount of solids. It is not possible to filter the extracts by simple gravity. Similar average recovery rates, about 98%, were obtained with TBAHS solution, water alone followed by pairing with TBAHS, and TBAP solution. Chromatograms were excellent in all 3 cases. Moniliformin was not detected in spiked samples extracted with TBAH solution.

Compared with previously published HPLC methods for determining moniliformin in corn, this analytical procedure is excellent in terms of efficient and easy sample extraction and cleanup, resulting in improved recovery rates and chromatographic separation. Handling time was reduced. The time required for complete analysis of 15 spiked samples was about 8–9 h. The method was reproducible, and its sensitivity was at least comparable with that reported for previous methods.

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