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Simple, Rapid, and Inexpensive Cleanup Method for Quantitation of Aflatoxins in Important Agricultural Products by HPLC

VICTOR S. SOBOLEV[†]

National Peanut Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 509, Dawson, Georgia 39842

A chemical cleanup procedure for low-level quantitative determination of aflatoxins in major economically important agricultural commodities using HPLC has been developed. Aflatoxins were extracted from a ground sample with $MeOH/H_2O$ (80:20, v/v), and after a cleanup step on a minicolumn packed with Florisil, aflatoxins were quantified by HPLC equipped with a C_{18} column, a photochemical reactor, and a fluorescence detector. Water/MeOH (63:37, v/v) served as the mobile phase. Recoveries of aflatoxins B_1, B_2, G_1, and G_2 from peanuts spiked at 5, 1.7, 5, and 1.7 ng/g were 89.5 ± 2.2, 94.7 ± 2.5, 90.4 ± 1.0, and 98.2 ± 1.1, respectively (mean ± SD, %, $n = 3$). Similar recoveries, precision, and accuracy were achieved for corn, brown and white rice, cottonseed, almonds, Brazil nuts, pistachios, walnuts, and hazelnuts. The quantitation limits for aflatoxins in peanuts were 50 pg/g for aflatoxin B_1 and 17 pg/g for aflatoxin B_2. The minimal cost of the minicolumn allows for substantial savings compared with available commercial aflatoxin cleanup devices.

KEYWORDS: Aflatoxin analysis; cleanup minicolumn; Florisil; HPLC; agricultural commodities

INTRODUCTION

Aflatoxins are naturally occurring fungal secondary metabolites that are produced by some species of *Aspergillus*, most notably *A. flavus* and *A. parasiticus*. Aflatoxins are toxic and carcinogenic to animals and humans and often contaminate a wide variety of agricultural products, including corn, peanuts, cottonseed, and tree nuts. Considering the extremely high carcinogenicity of aflatoxins, most developed nations regulate limits of aflatoxins as low as reasonably achievable (*1*, *2*). The European Union (EU) has legislated maximum permitted levels of 2 ng/g for aflatoxin B_1 and 4 ng/g for total aflatoxins (B_1, B_2, G_1, and G_2) in various products (*1*). Such low limits require adequate methods of detection and quantitation of aflatoxins in agricultural commodities and food.

Since the discovery of aflatoxins in early 1960, many methods for aflatoxin analysis in food and feed have been developed. Modern methods, compared with earlier time-, labor-, and material-consuming methods, must be analytically superior; they also must satisfy high safety and environmental standards. To satisfy the standards, researchers use environmentally friendly extraction solvents (*3*), selective adsorbents to reduce solvent consumption (*4*, *5*), safe derivatization (*6*−*8*), nondestructive fluorescence enhancement (*9*) techniques, and miniature cleanup columns (*5*, *10*, *11*). Despite significant improvement in aflatoxin analytical methodology, the cost of existing methods for aflatoxin analysis remains high. Many of the methods currently used in the food industry, agriculture, and research institutions use antibody-based cleanup columns (*12*) or immunoassays (*13*−*15*). The advantages of these methods are that they are selective and sensitive, but they cost substantially more than chemical minicolumn methods, which lacked the required selectivity, sensitivity, and accuracy (*16*, *17*). Of particular interest is a Florisil-packed minicolumn because it can almost selectively adsorb aflatoxins and, therefore, can be considered an effective element in method development. Florisil was first used in aflatoxin analysis in 1968 (*18*). A Florisil minicolumn for the adsorption, detection, and estimation of aflatoxin concentrations was introduced by Velasco (*19*) in 1972; a modified version of the minicolumn was accepted as an official AOAC method (*20*). Since then, several cleanup methods using Florisil were offered and tested (*4*, *21*−*29*); however, all of them had significant disadvantages. The major drawback was that the methods were time-consuming and used significant quantities of toxic organic solvents both for extraction and for column cleanup.

The purpose of this work was to develop a simple, rapid, cheap, reliable, and environmentally friendly chemical cleanup procedure for low-level quantitative determination of aflatoxins in major economically important agricultural commodities by HPLC.

MATERIALS AND METHODS

Reagents, Materials, and Basic Apparatus. HPLC grade solvents used in the sample extraction and in the preparation of mobile phases and aflatoxin standards as well as separations were obtained from Fisher

[†] Telephone (229) 995-7446; fax (229) 995-7416; e-mail vsobolev@nprl.usda.gov.

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(Suwanee, GA). HPLC grade H_2O was prepared with a ZD20 four-bowl Milli-Q water system (Millipore). A high-speed blender (13000 rpm) with a 0.5 L glass jar and cover (General Electric), a model 2A grinding/subsampling mill for cottonseed (Romer Labs, Inc., Union, MO), a commercial household coffee mill for nuts, a model Cary 50 Conc UV−visible spectrophotometer (Varian), and a model 231 touch mixer (Fisher) were used in the research.

Cleanup Column. A 1.5 mL polypropylene column with two matching polyethylene porous (20 μm) frits (Alltech Associates, Inc., Deerfield, IL) was packed with 130 mg of Florisil (60−100 mesh, Fisher). This amount of Florisil occupies 250 μL of volume, and the height of the adsorbent layer is 9 mm.

Stock Solutions of Aflatoxins. Crystalline aflatoxins B_1, B_2, G_1, and G_2 (1 mg each) (Sigma, St. Louis, MO) were used to prepare stock solutions of individual aflatoxins by dissolving each aflatoxin in 100 mL of MeOH to give approximate concentrations of 10 ng/μL. The final concentrations were determined according to the official procedure (*30*).

Spike Solution of Aflatoxins. The stock solutions of aflatoxins were mixed in the following proportions: $B_1/B_2/G_1/G_2$, (3:1:3:1, v/v), respectively.

Standard Solutions of Aflatoxins for HPLC. The solutions were prepared daily by dissolving the appropriate amount (6.7−666.7 μL) of the spike solution in 100 mL of H_2O/MeOH (63:37, v/v).

Commodity Varieties. Raw almonds, Brazil nuts, walnuts, hazelnuts, brown and white rice, corn meal, and dry-roasted pistachio nuts were purchased at a local department store (Albany, GA). Raw (green) peanuts were obtained locally (Dawson, GA). Delinted untreated cottonseed was provided by Dr. K. Howard (Delta and Pine Land Co., Scott, MS) and by Dr. T. E. Cleveland (USDA, ARS, Southern Regional Research Center, New Orleans, LA). A corn sample naturally contaminated with aflatoxins was available from Trilogy Analytical Laboratory (Washington, MO).

Spiking Technique. Aflatoxin-free ground samples (50 g) were evenly spiked with 6.7, 66.7, or 666.7 μL of the aflatoxin spike solution and kept for 1 h before extraction. Low-level spiking was performed with an appropriate amount of the spike solution diluted with MeOH (1:100, v/v).

Extraction and Cleanup. Fifty grams of ground sample was extracted with 100 mL of MeOH/H_2O (80:20, v/v) in a high-speed blender for 1 min and 15 s followed by filtration through a filter paper. Precisely measured 1−3 mL aliquots of the filtrate were transferred with a pipet into a 1.5 mL Extract-Clean reservoir (Alltech) packed with 130 mg of Florisil. The column was subsequently eluted with 0.5 mL of MeOH/H_2O (80:20, v/v), 0.5 mL of MeOH, and 0.5 mL of $CHCl_3$/MeOH (90:10, v/v). Aflatoxins were eluted by gravity into a 4 mL glass vial with 2 mL of an acetone/H_2O/formic acid mixture (96:3.7:0.3, v/v); then the solvent was removed in a stream of N_2 in a heated block at 45 °C. Dry residue was dissolved in precisely measured 0.3−1.0 mL of the HPLC mobile phase and vortexed for 2−3 s. The purified extract (20−100 μL) was injected into the LC system. Concentrations of aflatoxins were determined by reference to peak heights of corresponding pure standards.

HPLC-MS Analyses. Analyses were performed using an HPLC system equipped with an LC-10AT*vp* pump (Shimadzu), a model DGU-14A degasser (Shimadzu), an SPD-M10A*vp* diode array detector (DAD) with Shimadzu Client/Server software, version 7.3, a photochemical reactor, "PHRED" (Aura Industries; New York, NY), a fluorescence RF-551 HPLC monitor (Shimadzu), and a model 717 plus autosampler (Waters). The separation was performed on a 50 × 4.8 mm i.d., 2.5 μm XTerra MS C_{18} analytical column (Waters). A water/MeOH (63:37, v/v) mixture served as the mobile phase. The flow rate was 0.9 mL/min. The column was maintained at 42 °C in a model TL-105-HT column heater (Timberline Instruments, Boulder, CO). Aflatoxins were detected and quantitated with the fluorescence detector at 365 nm (excitation) and 440 nm (emission) wavelengths. To obtain aflatoxin MS spectra, a mobile phase of H_2O/MeOH/formic acid (600:400:1, v/v) was used. The flow rate and column temperature were 0.9 mL/min and 42 °C, respectively. The eluate from the DAD was split with a T-unit (Upchurch Scientific, Oak Harbor, WA) for optimal MS performance. The flow rate through the ESI probe was set at 0.35 mL/min. ESI-MS/MS^2 data were obtained on a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with an ESI interface and operated with Xcalibur version 1.4 software (Thermo Electron Corp., San Jose, CA). Data were acquired in the full-scan mode (MS) from *m/z* 100 to 500. Heated capillary temperature was 250 °C, sheath gas flow was 40 units, capillary voltage was 9 V, and source voltage was 4.5 kV. In MS^2 analyses, the $[M + H]^+$ ions observed for each chromatographic peak in full-scan analyses were isolated and subjected to source collision-induced dissociation (CID) using He buffer gas. In all CID analyses, the isolation width, relative fragmentation energy, relative activation *Q*, and activation time were *m/z* 1.5, 35%, 0.25, and 30 ms, respectively.

RESULTS AND DISCUSSION

The proposed method is a one-step minicolumn cleanup procedure that uses the unique property of Florisil to adsorb aflatoxins and a limited number of other compounds from highly polar solvents such as MeOH and H_2O. Such a technique was overlooked in previous studies (*4*, *18*−*29*) that considered Florisil to be a traditional chromatographic adsorbent; conventional elution patterns used large quantities of toxic organic solvents.

This research demonstrated that Florisil is a unique solid-phase extraction adsorbent, which is highly selective to aflatoxins in polar solvents. Samples are extracted with a MeOH/H_2O mixture and directly applied to a column packed with commercial Florisil. This technique eliminates such time-consuming and costly steps as redistribution of the aflatoxin fraction into a nonpolar solvent and evaporation. The MeOH/H_2O mixture was chosen for the extraction as one of the most effective, inexpensive, and environmentally friendly solvents (*3*).

Florisil was reported (*31*) to vary from lot to lot in its ability to bind aflatoxins. Losses of up to 75% of added B_1 and as much as 100% of the G aflatoxins have been observed. In this research no discrimination in ability to retain aflatoxins was observed; the method uses high concentrations of H_2O at the moment of extract application that deactivates Florisil. Addition of just 0.3% of formic acid to the acetone/H_2O mixture allowed for near 100% recovery of standards of aflatoxins B_1, B_2, G_1, and G_2 at spike levels from 0.5 to 50 ng/g.

Aflatoxins can be eluted from the Florisil column only with acetone (*18*) and acetone/H_2O mixtures (*22*). Optimization of the elution solvent was required on the basis of the fact that aflatoxins can be eluted from the Florisil column only with large volumes of acetone (*18*), acetone/MeOH (*27*, *29*), and acetone/H_2O mixtures (*4*, *22*, *24*, *25*). The most reasonable proportions of acetone-based solvents were tested. The results are represented in **Figure 1**. The least effective solvents were acetone (I) and acetone/MeOH mixture (J), which eluted only about 7% of aflatoxins. Addition of H_2O (H) increased aflatoxin recovery to about 40%, but remained unsatisfactory. Significant changes in recovery occurred when a small amount of formic acid was added to acetone (A−G). However, acid alone, without the addition of H_2O, did not permit the elution of >80% of spiked aflatoxin (G). The best solvent combination of acetone/H_2O/HCOOH (96.0:3.7:0.3) (A) was chosen for further use, because higher concentrations of formic acid in the mixture (acetone/H_2O/HCOOH, 96.0:3.5:0.5 and 96.0:3.0:1.0, v/v) did not change the pattern of aflatoxin elution. On average, aflatoxin recovery was about 89% with the first 1 mL of the eluate. Two milliliters of the mixture chosen for the method eluted over 98% of aflatoxins from spiked peanut extract applied to the column. Further elution of the column with 4 mL of the mixture allowed recovery on average of 1.5% of aflatoxins. Overall satisfactory

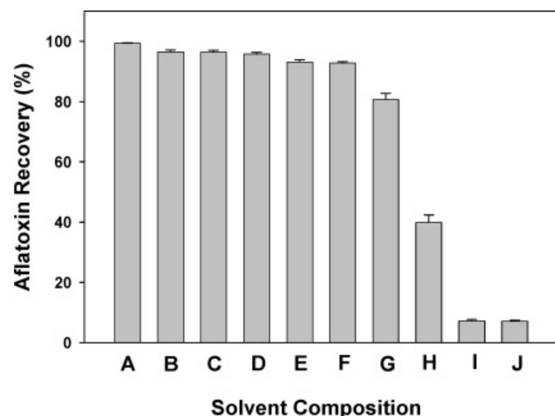

Figure 1. Recovery (percent, mean ± SD, $n = 3$) of aflatoxin standards (B_1, B_2, G_1, and G_2 combined) eluted from the Florisil column with 2 mL of acetone-based solvent mixtures: A, acetone/H_2O/HCOOH (96:3.7:0.3); B, acetone/H_2O/HCOOH (97:2.8:0.2); C, acetone/H_2O/HCOOH (97:2.7:0.3); D, acetone/H_2O/HCOOH (98:1.7:0.3); E, acetone/H_2O/HCOOH (97:2.9:0.1); F, acetone/H_2O/MeOH/HCOOH (97.7:1:1:0.3); G, acetone/HCOOH (99.7:0.3); H, acetone/H_2O (97:3); I, acetone (100%); J, acetone/MeOH (97:3). All solvent ratios are given as v/v.

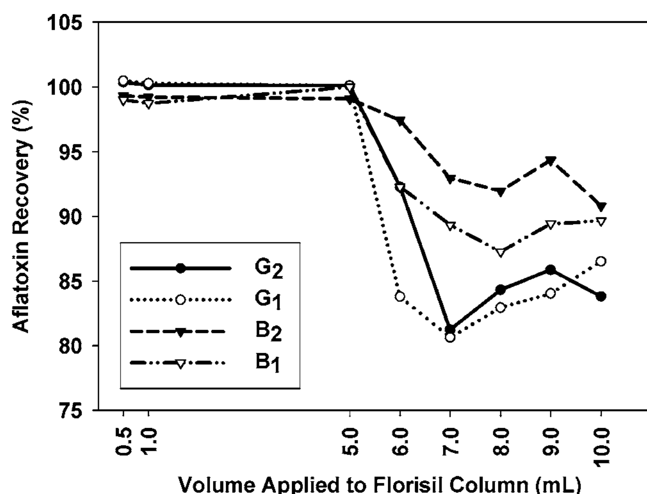

Figure 2. Recovery of aflatoxin standards from different volumes of the extracting solvent spiked at 5.0 ng/g level and applied to the Florisil column; in all cases aflatoxins were eluted with 2 mL of acetone/H_2O/HCOOH (96:3.7:0.3, v/v); average of two experiments.

recovery of aflatoxins with 2 mL of the mixture allowed selection of this volume for the method; use of higher volumes of the mixture was beyond practicality. The fact that aflatoxins could not be completely eluted with <2 mL of acetone-based mixtures probably could be explained by the high affinity of aflatoxins to Florisil. A smaller Florisil fraction, 100−200 mesh, behaved like a 60−100 mesh fraction in every respect, except the column packed with 100−200 mesh Florisil was significantly slower. Preliminary deactivation of Florisil with MeOH/H_2O/HCOOH did not improve aflatoxin recovery or eluate purity; this fact, as well as the very high recovery of aflatoxins from the column, allowed the use of commercial Florisil without any preliminary treatment. The high affinity to aflatoxins allowed up to 5 mL of commodity extracts to be applied to the Florisil column that fully retained aflatoxins; however, application of >5 mL led to significant aflatoxin loss (**Figure 2**). Although the optimum performance of the cleanup column was attained when 0.5−3 mL of the extract was applied to the column, application of 1 mL of the extracts seems to be more practical for the majority of analyzed products.

Table 1. Recovery of Aflatoxins from Selected Agricultural Products [Mean (CV), %; $n = 3$]

spike level[a] (ng/g)	aflatoxin B_1	B_2	G_1	G_2
	Peanuts			
50	85.2 (1.0)	86.2 (1.0)	85.8 (0.5)	84.8 (0.4)
5	89.5 (2.5)	94.7 (2.6)	90.4 (1.1)	98.2 (1.2)
0.5	77.7 (3.4)	80.1 (2.0)	79.9 (6.3)	89.5 (8.0)
0.1	80.9 (6.0)	84.5 (5.4)	82.4 (4.9)	94.0 (7.8)
0.05	73.8 (13.7)	72.3 (10.1)	74.3 (20.0)	78.0 (21.7)
	Brazil Nuts			
5	87.0 (2.4)	91.4 (2.2)	89.1 (4.9)	97.3 (1.8)
0.5	84.0 (0.3)	88.3 (4.2)	81.9 (1.5)	89.8 (8.6)
	Corn Meal			
5	75.0 (0.8)	80.7 (0.7)	74.3 (3.0)	90.1 (4.0)
0.5	80.3 (2.9)	84.9 (3.6)	79.0 (4.7)	98.8 (6.5)
	Cottonseed			
5	75.6 (3.3)	80.1 (2.4)	75.8 (4.2)	84.5 (5.7)
0.5	76.2[b]	78.3[b]	75.8[b]	79.5[b]
	Hazelnuts			
5	76.5 (2.9)	78.6 (1.9)	81.0 (2.8)	91.6 (4.2)
0.5	68.1 (3.1)	77.2 (2.9)	75.4 (4.3)	84.8 (6.1)
	Almonds			
5	84.5 (1.5)	88.1 (2.1)	83.2 (2.6)	93.7 (1.6)
0.5	76.2 (2.1)	81.7 (3.7)	77.5 (2.4)	77.6 (5.7)
	Pistachios (Dry-Roasted)			
5	76.5 (4.0)	80.6 (3.4)	76.2 (4.3)	88.9 (6.7)
0.5	68.4 (5.0)	77.2 (6.1)	79.6 (7.5)	95.7 (12.9)
	Rice (Brown)			
5	89.0 (3.9)	92.0 (3.2)	90.2 (3.1)	99.0 (5.3)
0.5	71.0 (4.0)	82.0 (3.5)	72.1 (4.7)	92.7 (5.1)
	Rice (White)			
5	92.8 (5.1)	92.7 (5.0)	94.8 (3.6)	97.1 (5.6)
0.5	84.6 (5.5)	86.5 (4.8)	88.7 (2.7)	87.9 (4.6)
	Walnuts			
5	72.9 (3.4)	79.2 (3.9)	93.7 (7.4)	96.6 (11.8)
0.5	84.7 (8.2)	N/A[c]	N/A[c]	N/A[c]

[a] Spike levels are given for aflatoxins B_1 and G_1; for aflatoxins B_2 and G_2 the multiplication factor of 0.33 should be used. [b] Represented data are from one analysis only due to lack of sufficient quantities of aflatoxin-free cottonseed samples. [c] Aflatoxins detectable, but not calculable, by the software due to abnormal peak shapes.

The in-house method characteristics were determined by using spiked products. Lack of natural aflatoxin contamination of tested commodities was assured by preliminary analyses using a published procedure (*11*). Identity of aflatoxins eluted from the Florisil column was confirmed by matching their retention times, UV, and mass spectra with those of authentic standards. The ratios of aflatoxins in the eluates were similar to those in the mixture of spiking aflatoxin standards; this served as an additional indication of aflatoxin-free substrates. Mass spectrometric data of aflatoxins eluted from the Florisil column were consistent with the published data (*32*).

The suggested minicolumn was effective for purification of the extracts of major aflatoxin-important commodities, such as peanuts, corn, hazelnuts, brown and white rice, cottonseed, almonds, Brazil nuts, pistachios, and walnuts, without any modifications. No significant peaks were present in the chromatograms within aflatoxin retention times, which allowed reliable detection and quantitation of aflatoxins at the 0.1−0.5 ng/g levels.

As seen in **Table 1**, the accuracy and precision of the method was sufficiently high for tested agricultural commodities spiked within the range of 0.5−5 ng/g, which represents the most critical levels of contamination in terms of aflatoxin control.

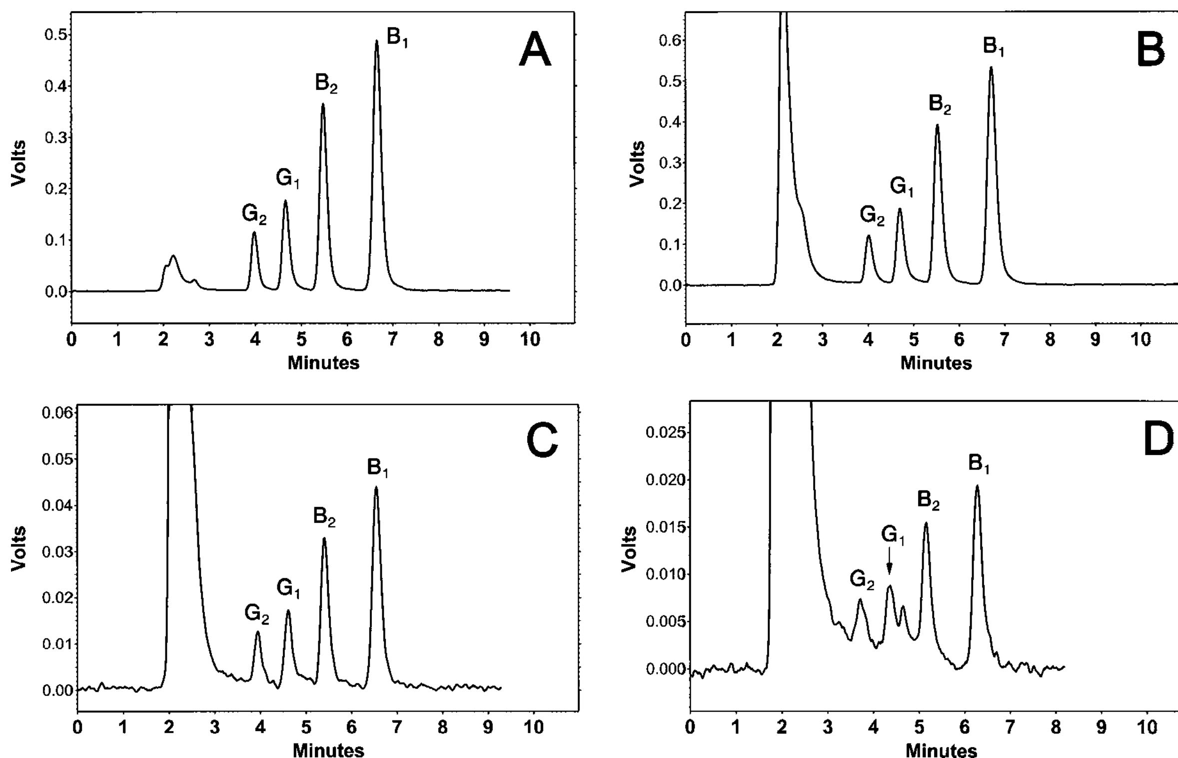

Figure 3. (**A**) HPLC of purified extract of peanuts spiked with aflatoxins B_1, B_2, G_1, and G_2 at 50.0, 16.7, 50.0, and 16.7 ng/g, respectively; 1 mL of the extract was applied to the Florisil column; dry eluate was redissolved in 1 mL of the mobile phase; 20 μL was injected into HPLC. (**B**) HPLC of purified extract of peanuts spiked with aflatoxins B_1, B_2, G_1, and G_2 at 5.0, 1.7, 5.0, and 1.7 ng/g, respectively; 3 mL of the extract were applied to the Florisil column; dry eluate was redissolved in 0.3 mL of the mobile phase; 20 μL was injected into HPLC. (**C**) HPLC of purified extract of peanuts spiked with aflatoxins B_1, B_2, G_1, and G_2 at 0.5, 0.17, 0.5, and 0.17 ng/g, respectively; 1 mL of the extract was applied to the Florisil column; dry eluate was redissolved in 0.3 mL of the mobile phase; 50 μL was injected into HPLC. (**D**) HPLC of purified extract of peanuts spiked with aflatoxins B_1, B_2, G_1, and G_2 at 0.05, 0.017, 0.05, and 0.017 ng/g, respectively; 3 mL of the extract was applied to the Florisil column; dry eluate was redissolved in 0.3 mL of the mobile phase; 100 μL was injected into HPLC.

The recoveries of aflatoxin B_1 in this range varied from 68.1% (hazelnuts) to 92.8% (white rice). Recoveries of other aflatoxins ranged from 72.1 to 99.0%. The highest average recoveries of total aflatoxins at the same two levels (0.5−5 ng/g) were obtained for brown rice, Brazil nuts, and peanuts (90.6, 88.6, and 87.5%, respectively). The lowest, but satisfactory, average recoveries were observed for cottonseed, hazelnuts, and pistachio nuts (79.0, 79.2, and 80.4%, respectively). The recoveries at different levels for individual commodities were uniform (**Table 1**), and the standard deviations (and corresponding CV values for three different extractions) were essentially low. However, CV values for the 0.05−0.1 ng/g spike levels in peanuts were higher and averaged 12.0%. This can be explained mainly by proportional losses of aflatoxins at lower concentrations and a less than optimal integrating algorithm at decreased signal/noise ratio. Increasing the signal/noise ratio by using larger extract volumes applied to the cleanup column and injecting larger volumes of the extracts significantly improved the statistical parameters and increased the sensitivity of the method at the lowest levels of contamination, as seen from **Figure 3A**,**B**. The chromatogram with a spiking level of 5 ng/g for aflatoxin B_1 that was obtained after increased application volume of the extract (**Figure 3B**) practically matched the chromatogram with a spiking level of 50 ng/g (**Figure 3**). Panels **A**−**C** of **Figure 3** represent chromatograms of the extracts of peanuts spiked with aflatoxins B_1, B_2, G_1, and G_2 at different levels ranging from 50 to 0.5 ng/g. In all of the cases aflatoxins can be reliably quantitated. **Figure 3D** shows satisfactory aflatoxin separation and high signal/noise ratios even at the 50 pg/g (aflatoxin B_1) and 17 pg/g (aflatoxin B_2) spiking levels in peanuts. The chromatogram itself allows for quantitation of aflatoxins at such low levels; however, overall low precision (average CV of 16.4% for all aflatoxins) of the method at these spiking levels should be taken into account (**Table 1**). **Figure 4** shows a chromatogram of the extract of pistachio nuts spiked with aflatoxins at 1.0 ng/g. Surprisingly, naturally colored pistachios and cottonseed (containing gossypol) with complex matrixes gave clean extracts. Very clean extracts were obtained from white and brown rice, almonds, Brazil nuts, and peanuts, which demonstrates high selectivity of the method. **Figure 5** shows a chromatogram of naturally contaminated corn. Reliable quantitation of aflatoxins B_1 and B_2 was possible at 0.89 and 0.06 ng/g, respectively.

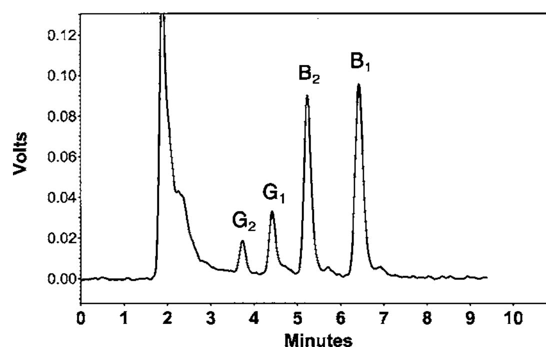

Figure 4. HPLC of purified extract of dry-roasted pistachio nuts spiked with aflatoxins B_1, B_2, G_1, and G_2 at 1.0, 0.3, 1.0, and 0.3 ng/g, respectively; 1 mL of the extract was applied to the Florisil column; dry eluate was redissolved in 0.3 mL of the mobile phase; 50 μL was injected into HPLC.

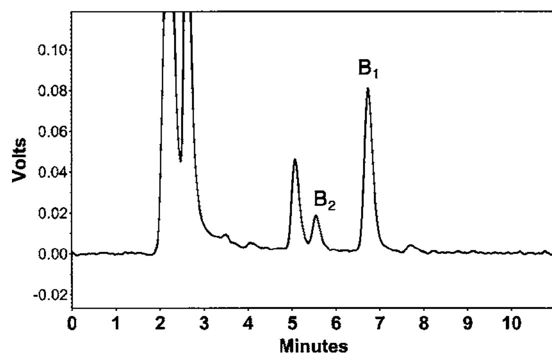

Figure 5. HPLC of purified extract of naturally contaminated yellow corn. Concentrations of detected aflatoxins B_1 and B_2 were 0.89 and 0.06 ng/g, respectively; 1 mL of the extract was applied to the Florisil column; dry eluate was redissolved in 0.3 mL of the mobile phase; 50 μL was injected into HPLC.

The total analysis time for a ground sample (including weighing, extraction, filtration, purification, and LC determination) did not exceed 20−25 min; multiple samples could be processed simultaneously, with little increase in overall time. The method did not require any vacuum or pumping devices. The cost of the suggested minicolumn is 10−15 times less than that of commercial proprietary cleanup columns, which is a substantial saving. The cleanup method is solvent- and material-efficient. In most cases it requires only 1 mL of the MeOH/H_2O extract and a total of 3.5 mL of common organic solvents. Therefore, the method would be practical for analyses of small quantities of representative samples or individual seeds. Use of disposable plastic funnels for filtration is not required for the same reason; a folded filter paper, submerged into the extract in the extraction jar, provides the required amount of the extract within seconds. A portion of the filtered extract can be collected inside the paper filter cone with a pipet. The stability of purified extracts was high enough to allow autosampler overnight injections at ambient temperature. No significant changes in concentration or purity of aflatoxins were detected within 16 h of analysis.

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