FOOD CHEMICAL CONTAMINANTS

Cleanup Procedure for Determination of Aflatoxins in Major Agricultural Commodities by Liquid Chromatography

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A simple, fast, reliable, and inexpensive chemical cleanup procedure was developed for quantitation of aflatoxins in major important agricultural commodities by liquid chromatography (LC). Aflatoxins were extracted from a ground sample with methanol–water (80 + 20, v/v), and after a single cleanup step on a minicolumn packed with basic aluminum oxide, they were quantitated by LC equipped with a C_{18} column, photochemical reactor, and fluorescence detector. Water–methanol–1-butanol (1400 + 720 + 25, v/v/v) served as the mobile phase. Recoveries of aflatoxins B_1, B_2, G_1, and G_2 from peanuts spiked at 5.0, 2.5, 7.5, and 2.5 μg/kg were 87.2 ± 2.3, 82.0 ± 0.8, 80.0 ± 1.8, and 80.4 ± 2.8%, respectively. Similar recoveries, precision, and accuracy were achieved for corn, cottonseed, almonds, Brazil nuts, pistachios, and walnuts. The quantitation limit for aflatoxin B_1 was 1 μg/kg. The minimal cost of the minicolumn allows for substantial savings compared with available commercial aflatoxin cleanup devices.

Aflatoxins are toxic, carcinogenic secondary metabolites produced by some species of *Aspergillus* fungi that often contaminate a wide variety of agricultural products. At present, production and storage techniques do not prevent the development of *Aspergillus* spp. Consequently, contamination of agricultural products with aflatoxins results. Because there is no threshold for aflatoxins below which no harmful effect is observed, no tolerable daily intake can be set (1). Therefore, legislators of most developed nations set limits as low as reasonably achievable (1–3). The European Union (EU) has legislated the maximum permitted level of 2 μg/kg for aflatoxin B_1 and 4 μg/kg for total aflatoxins (B_1, B_2, G_1, and G_2) in several products (1, 3).

Existing methods for aflatoxin analysis in food and feed are numerous and varied. Recent scientific advances, including optimization of aflatoxin extraction (4), use of selective adsorbents (5–7), various detection and derivatization techniques (8–10), and use of more sophisticated cleanup minicolumns (11, 12), allowed significant improvement in aflatoxin analytical methodology. However, the cost of existing methods for aflatoxin analysis used in industry and the aflatoxin research community remains high. Many of the methods currently use proprietary, antibody-based cleanup columns (13) or immunoassays (14, 15). Although these methods are selective and sensitive, and comply with legislated requirements, they cost substantially more than traditional minicolumn methods, which lack the desired selectivity, sensitivity, and reliability (16, 17).

The purpose of this work was to develop a simple, fast, reliable, inexpensive chemical cleanup procedure for quantitative determination of aflatoxins in major aflatoxin-important agricultural commodities by liquid chromatography (LC).

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Experimental

Apparatus

(**a**) *Liquid chromatograph*.—Pump, Model 515 LC (Waters Corp., Milford, MA); autosampler, Model 717 plus (Waters); fluorescence detector, Model RF-551 (Shimadzu, Kyoto, Japan); Class VP Chromatography Data System, version 4.2 (Shimadzu); photochemical reactor for postcolumn derivatization (PHRED; Aura Industries, New York, NY); column, Nova-Pak C_{18} (150 × 3.9 mm; 4 μm; Waters); operating conditions: column temperature 38°C; flow rate 0.8 mL/min; injection volume 20 μL; mobile phase water–methanol–1-butanol (1400 + 720 + 25, v/v/v); detector wavelength 365 nm (excitation) and 440 nm (emission).

(**b**) *Blender for extraction*.—High-speed (13 000 rpm) laboratory blender with 1 L glass jar and cover (Waring Products Div., Torrington, CT).

(**c**) *Vertical cutter-mixer for nuts*.—Model RSI6Y-1 (Robot Coupe, Ridgeland, MS).

(**d**) *Mill for cottonseed*.—Grinding/subsampling, Model 2A (Romer Labs, Inc., Union, MO).

(**e**) *Spectrophotometer*.—UV-visible, CARY 50 Conc (Varian, Palo Alto, CA).

(**f**) *Vortex*.—Touch mixer, Model 231 (Fisher Scientific, Pittsburgh, PA).

(**g**) *UV lamp*.—Ultra-Violet Products, Inc. (San Gabriel, CA).

(**h**) *Pipet*.—Eppendorf Series 2000 reference adjustable-volume (100–1000 μL) with matching 1000 μL Eppendorf tips (Brinkman Instruments, Inc., Westbury, NY).

(**i**) *Cleanup column*.—1.5 mL polypropylene with 2 matching polyethylene porous (20 μm) frits (Alltech, Deerfield, IL) packed with 200 mg basic aluminum oxide (40 μm flash; Scientific Adsorbents, Inc., Atlanta, GA). The frits were inserted with a 150 mm long rod (5.5 mm diameter glass rod or capillary tubing with working tip cut at 90° angle; Fisher). Aluminum oxide was placed into reservoir with preinstalled lower frit with a custom-made glass scoop calibrated to hold 200 mg adsorbent. The column was gently tapped 3–4 times against a hard surface followed by firm insertion of the upper frit.

(**j**) *Autosampler vial*.—700 μL polypropylene conical with cap (Waters, Cat. No. 22476).

(**k**) *Filter paper*.—No. 4 (Whatman, Inc., Clifton, NJ).

(**l**) *Dilution vial*.—4 mL (15 × 45 mm) clear glass (Waters).

(**m**) *Balances*.—Electronic, Type PM 4600 (Mettler, Hightstown, NJ).

Reagents and Materials

(**a**) *Solvents for LC*.—LC grade methanol, 1-butanol (Fisher). LC grade water was prepared with 4-bowl Milli-Q Water System, Model ZD20 (Millipore, Bedford, MA).

(**b**) *Solvent for extraction*.—ACS grade methanol (Fisher)–distilled water (80 + 20, v/v).

(**c**) *Solvent for dilution of the extracts*.—LC grade acetonitrile (Fisher).

(**d**) *Solvents for preparation of aflatoxin standards*.—ACS grade benzene, LC grade acetonitrile (Fisher).

(**e**) *Stock solutions of aflatoxins*.—Crystalline aflatoxins B_1, B_2, G_1, and G_2 (1 mg each, Sigma Chemical Co., St. Louis, MO) were used to prepare stock solutions of individual aflatoxins by dissolving each aflatoxin in 100 mL benzene–acetonitrile (98 + 2, v/v) for concentrations ca 10 ng/μL. The final concentrations were determined according to the official procedure (18).

(**f**) *Spike solution of aflatoxins*.—Appropriate portions of the stock solutions of aflatoxins were mixed and diluted with benzene–acetonitrile (98 + 2, v/v) to give the following concentrations: B_1, 200; B_2, 100; G_1, 300; and G_2, 100 μg/100 mL, respectively.

(**g**) *Standard solutions of aflatoxins for LC*.—Prepared daily by dissolving appropriate amount (0.125–2.5 mL) of spike solution in 100 mL methanol–water (80 + 20, v/v), and diluting 0.5 mL portion of it with 0.5 mL acetonitrile.

(**h**) *Commodities*.—Raw almonds, Brazil nuts, pistachios, and English walnuts were purchased at Sunnyland Farms (Albany, GA). Corn meal was purchased at a local department store. Raw (green) peanuts were obtained locally (Dawson, GA). Delinted untreated cottonseed was provided by K. Howard (Delta and Pine Land Co., Scott, MS) and T. Cleveland (U.S. Department of Agriculture, New Orleans, LA). Deltapine cottonseed treated with fungicides and insecticides (code 2) was purchased from Delta and Pine Land Co.

(**i**) *Silver nitrate*.—ACS grade (Fisher).

Spiking Technique

Aflatoxin-free ground samples (100 g) were evenly spiked with 50, 250, 1000, or 5000 μL aflatoxin spike solution, and left under running exhaust hood for 16–20 h.

Extraction and Cleanup

A 100 g amount of ground sample was extracted with 200 mL methanol–water (80 + 20, v/v) in a high-speed blender for 1 min 15 s followed by filtration through filter paper. A 0.5 mL aliquot of filtrate was mixed with 0.5 mL acetonitrile, and 0.5 mL of the mixture was pipetted into an Alltech 1.5 mL Extract-Clean reservoir packed with 200 mg basic aluminum oxide (9 mm high-layer adsorbent). Aflatoxins were eluted by gravity into a conical plastic vial. A plastic cap was placed on top of the vial, and was vortexed for 1–2 s. A 20 μL volume of purified extract was injected into the LC system.

Cottonseed and pistachio extracts were diluted, if required, with 0.5 mL of 0.2% solution of silver nitrate in acetonitrile to break the suspension that may be formed during extraction.

Results and Discussion

The proposed method is a 1-step minicolumn cleanup procedure that uses the property of aluminum oxide to adsorb a variety of compounds of high to medium polarity even from highly polar solvents like methanol, acetonitrile, and water. It eliminates time-consuming and costly steps such as redistribution of aflatoxin fraction into a nonpolar solvent and evaporation. The methanol–water mixture was chosen for extraction as one of the most effective, inexpensive, and environment-friendly solvents (4). However, the methanol–water extract could not be used for direct purification on the aluminum oxide column without preliminary dilution with an appropriate organic solvent to adjust the polarity and solubility of aflatoxins and impurities to the aluminum oxide adsorptive properties. The use of acetonitrile as solvent gave higher recoveries and purity of aflatoxins than did methanol and dimethyl sulfoxide (DMSO). The combination of methanol, water, and acetonitrile for extraction with a basic aluminum oxide column cleanup step allowed significant removal of polar and nonpolar (both neutral and acidic) interferences from the extract matrix, thus dramatically purifying the aflatoxin fraction. The use of a minicolumn packed with the mixture of aluminum oxide and ODS (C_{18}) reversed-phase (19) compared with the basic aluminum oxide column did not significantly improve extract purity.

Although optimum performance of the cleanup column was attained when 0.5 mL diluted extract was applied to the column, similar purity, recoveries, accuracy, and precision were achieved by applying 0.4–0.9 mL extract to the suggested column. When 0.5 mL extract was applied, 0.25 mL purified extract was recovered from the column, which was sufficient for analysis and dilutions if needed.

The suggested minicolumn was effective for purification of extracts of major aflatoxin-important commodities, such as peanuts, corn, cottonseed, almonds, Brazil nuts, pistachios, and walnuts without any significant modifications. No peaks

were present in chromatograms within aflatoxin retention times, which allowed reliable detection and quantitation of aflatoxins at the 1 μg/kg level. Although some purified extracts of corn and pistachios showed slight yellowish off-white color, this did not represent a problem in terms of purity of extract or longevity of the analytical column.

Table 1 shows that accuracy and precision of the method were sufficiently high for major aflatoxin-important commodities spiked within the range of 2.5–150 μg/kg, which represents the most common levels of contamination. Recoveries of aflatoxin B_1 in this range varied from 73.9 (cottonseed) to 90.3% (almonds). Recoveries of other aflatoxins ranged from 72.3 to 93.5%. The coefficient of variation (CV) for all tested commodities within the above range averaged 1.97% (from 0.0 to 5.9%; $n = 63$). Average CVs for corn meal, cottonseed, peanuts, almonds, English walnuts, Brazil nuts, and pistachios were 2.55, 1.47, 1.23, 3.05, 2.68, 1.61, and 1.20%, respectively. The highest recoveries of aflatoxins were obtained for corn meal (about 90%). Cottonseed gave the lowest recoveries, but they were satisfactory (about 75%). Recoveries at different levels for individual commodities were uniform, and standard deviations (and corresponding CVs) were essentially low. However, CVs for the 0.5–1.5 μg/kg spike level were higher, and averaged 9.41% ($n = 21$). This can be explained mainly by less than optimal integrating algorithm at decreased signal-to-noise (S/N) ratio. Increasing S/N ratio by injecting larger volumes of the extracts (diluted with water) significantly improved statistical parameters and increased sensitivity of the method at the lowest levels of contamination.

Figure 1a shows the liquid chromatography of an extract of peanuts spiked with aflatoxin B_1, B_2, G_1, and G_2 at 5.0, 2.5, 7.5, and 2.5 μg/kg, respectively. It also shows liquid chromatograms of extracts of Brazil nuts (Figure 1b) and cottonseed (Figure 1c) naturally contaminated with aflatoxins B_1 and B_2 at 8.5 and 1.3, and 2.6 and 0.5 μg/kg, respectively. After elution of solvent (with some impurities) only aflatoxins can be seen in the chromatograms, which demonstrates high selectivity of the method. Coupling the suggested cleanup procedure with postcolumn chemical derivatization (PHRED) to increase fluorescence response of aflatoxins B_1 and G_1 (10) permitted sensitive detection and quantitation of all 4 common aflatoxins with advantages of simplicity and high reproducibility without requiring chemical reagents, additional pumps, or electrochemical cells.

The total analysis time for a ground sample (including weighing, extraction, purification, and LC determination) did not exceed 15–17 min (multiple samples can be processed in <10 min). The method did not require wash solvent or vacuum or pumping devices. However, some pistachio and untreated cottonseed samples formed fine suspensions that required a significantly longer time for column cleanup by gravity. To break the suspension, the methanol–water extracts were diluted with a 0.2% silver nitrate solution in acetonitrile. The suspended particles agglutinated within seconds, which allowed application of the diluted extract to the cleanup column without delay. The use of silver nitrate did not noticeably change aflatoxin recovery, precision, or accuracy of the method.

Table 1. Recovery of aflatoxins from major commodities, mean CV, % ($n = 3$)

Spike level, μg/kg[a]	Aflatoxins			
	B_1	B_2	G_1	G_2
Corn meal				
100	89.2 (5.0)	90.5 (2.8)	86.4 (5.9)	89.9 (3.8)
20	90.0 (1.9)	91.4 (0.9)	87.3 (1.8)	89.2 (1.1)
5	89.5 (0.1)	93.5 (3.3)	89.2 (0.9)	92.5 (3.1)
1	87.1 (6.4)	100.4 (3.4)	91.1 (3.3)	105.3 (12.2)
Cottonseed[b]				
100	74.5 (0.9)	75.9 (0.9)	75.6 (2.2)	77.4 (0.6)
20	73.9 (0.5)	75.6 (0.0)	74.7 (0.3)	83.4 (2.0)
5	74.4 (1.7)	73.7 (3.8)	77.9 (2.3)	87.7 (2.4)
1	80.4 (15.8)	75.4 (7.6)	107.3 (7.1)	115.0 (13.5)
Peanuts				
100	87.1 (0.6)	86.4 (0.3)	82.7 (1.1)	84.3 (0.6)
20	89.3 (0.4)	86.9 (0.2)	85.6 (0.2)	84.2 (1.9)
5	87.2 (2.6)	82.0 (1.0)	80.0 (2.3)	80.4 (3.5)
1	103.7 (5.4)	80.1 (7.1)	69.9 (10.4)	75.8 (7.9)
Almonds				
100	90.3 (3.9)	91.1 (1.6)	87.7 (3.9)	90.2 (0.6)
20	82.2 (5.0)	85.0 (4.8)	80.4 (3.7)	83.3 (4.2)
5	81.2 (4.3)	84.9 (1.5)	82.7 (0.4)	80.5 (2.7)
1	79.8 (2.9)	84.7 (6.0)	71.9 (16.6)	68.4[c]
English walnuts				
100	82.1 (2.7)	81.8 (2.6)	82.5 (2.5)	83.6 (3.0)
20	78.5 (2.2)	80.1 (2.0)	81.1 (2.0)	80.0 (1.5)
5	80.1 (3.7)	80.1 (3.2)	86.1 (3.7)	72.3 (3.0)
1	72.3 (10.7)	88.9 (6.5)	97.2 (13.6)	NC[d]
Brazil nuts				
100	83.2 (1.6)	83.00 (1.7)	79.4 (1.6)	80.8 (2.0)
20	83.2 (0.8)	82.4 (0.8)	80.1 (0.7)	80.7 (0.7)
5	78.9 (1.4)	80.8 (1.0)	82.7 (2.3)	83.3 (4.7)
1	91.8 (2.5)	74.1 (13.5)	90.7 (17.9)	86.2[c]
Pistachios[e]				
100	76.4 (0.7)	76.5 (0.3)	79.3 (0.5)	78.2 (0.4)
20	75.2 (1.5)	75.1 (1.6)	77.4 (1.4)	76.0 (2.5)
5	78.2 (1.5)	78.2 (1.3)	79.5 (1.9)	79.8 (0.8)
1	86.3 (13.4)	78.2 (6.3)	93.7 (15.4)	81.8 (7.9)

[a] Spike levels are given for aflatoxin B_1; for aflatoxins B_2, G_1, and G_2, the following multiplication factors should be used: 0.5, 1.5, and 0.5, respectively.

[b] Treated cottonseed were used because aflatoxin-free untreated seeds were not available at the time of the research. Dilution with 0.2% silver nitrate solution in acetonitrile was used before column cleanup.

[c] Aflatoxins detectable, but calculable by integrator only for 1 replicate.

[d] Aflatoxins detectable, but not calculable by integrator.

[e] Dilution with 0.2% silver nitrate solution in acetonitrile was used before column cleanup.

Figure 1. Liquid chromatogram of extract of peanuts (a) spiked with aflatoxins B_1, B_2, G_1, and G_2 at 5.0, 2.5, 7.5, and 2.5 μg/kg, respectively; Brazil nuts (b) and cottonseed (c) naturally contaminated with aflatoxins B_1 and B_2 at 8.5 and 1.3, and 2.6 and 0.5 μg/kg, respectively.

The cost of the suggested minicolumn is several times lower than that of commercial proprietary cleanup columns (11, 12), which is a substantial saving. The LC column showed high longevity: no significant change in the column performance was detected after analysis of more than 1100 samples of peanuts and hundreds of samples of cottonseed, corn, and tree nuts combined. The column is still in use.

The cleanup procedure is solvent- and material-efficient. It requires only 0.5 mL methanol–water extract and the same amount of acetonitrile and would therefore be practical for analyses of small quantities of representative samples or individual kernels. Use of disposable plastic funnels for filtration is not required for the same reason; a folded filter paper, submersed into the extract in the extraction jar, provides the required amount of extract within seconds. A portion of the filtered extract can be collected by pipet inside the paper filter cone.

The method allows dilution of highly contaminated samples (>700 μg/kg equivalent to B_1, which is beyond the linear range of the detector) before LC analysis. The minicolumn used for extract purification can be used to estimate the dilution ratio for the purified extract by observing the fluorescence of the adsorbent and the eluate remaining in the tip when exposed to longwave UV light. The lower portion of the adsorbent and eluate is free of any fluorescent impurities when samples do not contain aflatoxins. When the level of contamination exceeds 250–300 μg/kg, aflatoxins produce distinctive fluorescence both on the adsorbent and in the eluate. An experienced technician can easily estimate the intensity of such fluorescence and decide on the dilution ratio (1:10, 1:100, and 1:1000 are adequate in most cases).

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 18 h of analysis.

The suggested minicolumn also can be used for fig extract purification, although a different extraction procedure is required. The research is in progress, and the results will be reported elsewhere.

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