Rapid Analysis for Aflatoxins in Cottonseed Products with Silica Gel Cartridge Clean-Up

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ABSTRACT

A proposed rapid and versatile method of analysis for aflatoxins in cottonseed products using Waters Associates' Sep-Pak silica cartridge clean-up is presented. Samples are extracted with acetonitrile/water azeotrope and a filtered aliquot is evaporated to dryness. A toluene/acetonitrile (96:4) solution of the dry extract is applied to a Sep-Pak column. Gossypol derivatives and other artifacts are eluted with chloroform/ethyl ether (1:4). The Sep-Pak is then mated with either a Romer minicolumn for rapid minicolumn application or a small column of acid alumina deactivated with 4% water to produce a clean extract for quantitation by thin layer chromatography or high pressure liquid chromatography.

INTRODUCTION

Present sensitive, accurate and precise analytical methods (1-3) for determination of aflatoxins in cottonseed products require rather rigorous clean-up procedures for removal of the interferences resulting from gossypol derivatives present to the extent of 0.5-1.5% by wt of the cottonseed kernel. The need of a rapid, sensitive and simple method for screening purposes prompted the development of the proposed method for minicolumn application. Use of a small column of acid-washed alumina deactivated with 4% water and chloroform/acetone aflatoxin elution solvent provided a clean final extract for quantitative determination of the aflatoxins by thin layer chromatography (TLC) or high pressure liquid chromatography (HPLC).

Acetonitrile/water azeotrope (84:16, v/v) extraction solvent is a relatively low-temperature constant boiling solvent (76.5 C) that combines effective solubility of the aflatoxins with moderate solubility of gossypol derivatives and provides an extract that is essentially free of lipids.

Selective solvent systems for silica gel Sep-Pak clean-up and aflatoxin elution were designed for: (a) minimal chromatographic solvent polarity for removal of gossypol derivatives and other interferences; (b) maximal chromatographic solvent polarity for elution of the aflatoxins; (c) retention on the cartridge of highly polar interferences; and (d) analytical reproducibility for minor variations in adsorbent activity that may occur with different lots of Sep-Pak. The proposed method has advantages of simplicity, rapid sample preparation, reduced solvent and reagent consumption, and versatility of application while providing results comparable with those obtained using accepted methods of analysis for aflatoxins in cottonseed products.

EXPERIMENTAL PROCEDURES

Reagents

Solvents. ACS in glass: acetone, acetonitrile, chloroform (0.75% or less ethanol), diethyl ether (anhydrous, 0.01% ethanol) and toluene.

Column packings. Florisil: Fisher Scientific Co., 100-200 mesh; silica gel: E. Merck (Silica Gel 60) for column chromatography; alumina, neutral: Woelm Activity V, or E. Merck, Brockman Activity I, 100-200 mesh; alumina, acid: Fisher, Brockman Activity I, 80-200 mesh; calcium sulfate, anhydrous: Drierite, nonindicating, 20-40 mesh, or sea sand, washed, 20-40 mesh.

Equipment

We used a 10-mL syringe with luertip fitting and Sep-Pak silica cartridges (both available from Waters Associates, Inc., Milford, MA); Waring blender (explosion proof); 1-pt blender jars; 10-cm id funnels; Whatman No. 4 filter paper or equivalent, 20 cm; 100-mL beakers; 4-mL glass vials; 6-mm id glass tubing for clean-up columns or minicolumns; ultraviolet (UV) light (long-wave, with intensity of 430 μ/W/cm^2 at 15 cm at 365 μm) or Chromovue cabinet.

Minicolumns and Clean-Up Columns

Minicolumns were constructed according to the method of Romer (3) from 6-mm id glass tubing of ca. 70-mm length with a tapered end. The column was prepared by tamping a small plug of glass wool into the tapered end and then packed in the following order: 10 mm anhydrous calcium sulfate or, if preferred, 10 mm of washed sea sand, 10 mm Florisil, 20 mm silica gel, 10 mm neutral alumina, and 10 mm calcium sulfate or washed sea sand with a small plug of glass wool tamped into the top of the minicolumn. The exact column length was determined so that the outlet of the Sep-Pak is near or in contact with the glass wool plug at the top of the minicolumn when mated with the Sep-Pak.

The extract clean-up columns were constructed from 6-mm id glass tubing, 50 mm in length with a tapered end. The columns were prepared by tamping a small plug of glass wool into the tapered end and the column was packed in the following order: 10 mm anhydrous calcium sulfate or 10 mm washed sea sand, 25 mm 4% water-deactivated, acid-washed alumina, 10 mm anhydrous calcium sulfate or washed sea sand, topped with a small plug of glass wool tamped into the top of the column.

Deactivated acid-washed alumina adsorbent was prepared by the addition of 4% (v/w) distilled water to acid-washed alumina, Brockman Activity I (100-200 mesh), with equilibration for a minimum of 24 hr. All other packing materials were dried for 1-2 hr at 110 C and the conditioned materials and columns were stored in airtight containers.

Sample and reference minicolumns, as well as 4% water-deactivated clean-up columns are available from Myco-Lab Co., Chesterfield, MO.

Sample Preparation and Extraction

The sample was prepared by grinding whole cottonseed or meats in a Wiley mill or equivalent to pass a no. 10 sieve. For seed containing lint, the ground sample was screened on a 4/64-in. screen to remove the lint. Meals were ground to pass a no. 18 sieve.

We weighed 25 g prepared analytical sample into a 1-pt blender jar, added 125 mL acetonitrile/water azeotrope (84:16) and blended for 3 min at high speed. The extract was filtered through Whatman No. 4 or equivalent filter paper into a 100-mL beaker and boiled just to dryness under a stream of dry nitrogen on a steam bath or hot plate adjusted to 100 C.

Sample Extract Clean-Up

We removed the plunger from the 10-mL syringe and

attached the short end of the Sep-Pak to the luertip of the syringe barrel. The dry extract was dissolved in 5 mL of toluene/acetonitrile (96:4) and poured into the syringe. The beaker was rinsed with an additional 5 mL of the solvent solution and this was added to the syringe. The plunger was inserted into the barrel and the sample solution gently pumped through the Sep-Pak with a dropwise flow of ca. 5 mL/min.

The Sep-Pak was removed from the syringe and the plunger withdrawn. We reinserted the cartridge in the luertip and added 3 mL of ethyl ether/chloroform (4:1) to the syringe, then inserted the plunger and gently pumped this solvent solution through the Sep-Pak. Again, the Sep-Pak and plunger were removed, reinserted into the luertip and residual solvent removed by pumping 10 mL of air through the cartridge. The 2 extract solutions were discarded.

Rapid Minicolumn Detection Method

For minicolumn detection, the Sep-Pak, at this point, was mated to a prepared minicolumn using a fabricated union or a 2-cm length of 3/16 in. id rubber tubing (Fig. 1). The combined Sep-Pak/minicolumn was attached to the luertip of the syringe. We then placed 5 mL of chloroform into the syringe and pumped it through slowly. The Sep-Pak/minicolumn was removed from the syringe, plunger withdrawn, the combined columns reattached and 5 mL chloroform/acetone (9:1) developing solvent was added through the combined columns with sufficient pressure on the plunger to insure that the solvent flow through the columns was no greater than 1.0 mL/min to prevent excessive spreading of the interference or aflatoxins bands. The minicolumn was observed under a long-wave UV lamp for presence and estimation of level of aflatoxin band at top of florisil layer (4).

Sample Extract Column Clean-Up for TLC-HPLC Analysis

For quantitative aflatoxin analysis by TLC or HPLC, a prepared, deactivated acid-alumina clean-up column was attached to the Sep-Pak following initial clean-up using a fabricated union or a 2-cm length of 3/16 in. id rubber tubing; the combined Sep-Pak/clean-up column attached to the luertip of the syringe (Fig. 1). We placed 3 mL chloroform/acetone (4:1) aflatoxin elution solvent in the barrel of the syringe and slowly pumped at a rate of ca. 5 mL/min through the combined Sep-Pak/clean-up column into a 4-ml vial; the final extract was evaporated to dryness for aflatoxin analysis by TLC or HPLC. Figure 1 graphically illustrates the construction and mating of columns for use by either method.

RESULTS AND DISCUSSION

Acetonitrile/water azeotrope was selected as the extraction solvent of choice for the proposed method on the basis of lower solubility of gossypol derivatives and other pigmentation in comparison to most other solvents being used for extraction of aflatoxins. It also provides an extract that is essentially free of lipids and has a relatively low constant boiling point. Investigation of acetonitrile/water ratios providing maximal extraction of aflatoxins from typical cottonseed products was in accord with the findings of Pons et al. (5) with acetone/water ratios. Maximal aflatoxin recoveries were obtained with ca. 10-15% water in acetonitrile. As this water content is essentially the same as the azeotrope (84:16), this solvent ratio was adopted.

We found that a minimal solvent sample (v/w) ratio of 5:1 was necessary to obtain optimal extraction of the

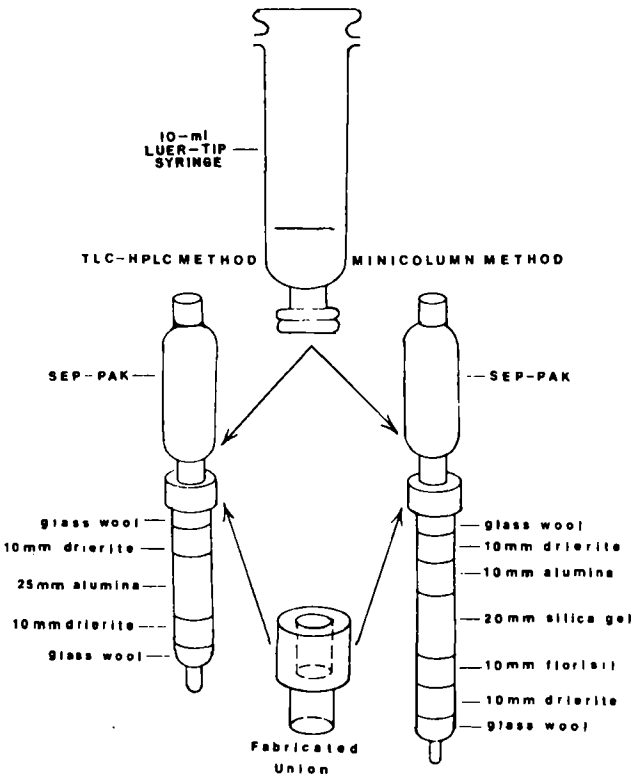

FIG. 1. Diagram illustrates Sep-Pak mating and composition of columns for TLC-HPLC eluate method or minicolumn method.

aflatoxins. Consequently, a 25-g sample was used in conjunction with 125 mL of extraction solvent. A 25-mL aliquot of extract, representing 5 g of cottonseed product, was taken for boil-down and cleaned-up for analysis.

To determine the optimal blender extraction time, tests were conducted with naturally contaminated and spiked samples of ground cottonseed meats and cottonseed meal. Analytical results obtained were compard to those obtained using the method of Pons (1). Tests were run with sample extraction times of 1, 2, 3, 4 and 5 min, blending at high speed. An extraction time of 3 min was found to be the optimal time necessary to provide maximal extraction of aflatoxins from cottonseed products.

For boil-down of crude extracts, care must be exercised to obtain sufficient drying to remove essentially all azeotrope. Its presence could adversely affect activity of the Sep-Pak column, but drying should not be so excessive as to result in polymerization of extracted materials.

Recoveries of aflatoxins B_1 and B_2 from artificially contaminated cottonseed meats and cottonseed meals are presented in Table I. Recoveries from cottonseed meats averaged ca. 86% for aflatoxin B_1 and 78% for aflatoxin B_2 for a total of 18 samples containing 12-74 μg/kg total aflatoxin. Cottonseed meal sample recoveries averaged ca. 90% for aflatoxin B_1 and 80% for aflatoxin B_2 from a total of 18 samples containing 16-78 μg/kg total aflatoxins. Coefficients of variation for recoveries from cottonseed meats samples averaged ca. 11% for aflatoxin B_1 and 27% for aflatoxin B_2. Coefficients of variation for recoveries from cottonseed meals averaged ca. 6% for aflatoxin B_1 and 13% for aflatoxin B_2. These values would seem to be satisfactory for the range of aflatoxin levels of these samples.

Comparative analysis of naturally contaminated samples of cottonseed meats and cottonseed meals analyzed by both the proposed method and the Pons method (1) are

TABLE I

Recovery of Aflatoxins B_1 and B_2 from Artificially Contaminated Cottonseed Products[a]

Sample	No. samples	Added (μg/kg)		Recovered (μg/kg)		Recovery (%)		Range (μg/kg)		Coeff. var. (%)	
		B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2
Cottonseed meats	6	9	3	7	2	78	67	5- 9	1- 4	17.6	53.9
	6	25	6	22	5	88	83	20-26	4- 6	11.2	20.0
	6	56	18	51	15	91	83	48-54	14-16	4.1	7.3
Cottonseed meal	6	12	4	11	3	92	75	10-12	2- 3	9.7	10.9
	6	31	9	27	7	87	78	26-28	6- 9	4.1	16.3
	6	60	18	55	16	92	88	52-58	14-18	4.1	12.5

[a]All samples spiked with chloroform solutions of aflatoxins B_1 and B_2.

TABLE II

Determination of Aflatoxins B_1 and B_2 in Naturally Contaminated Cottonseed Products

Sample	No. samples	Sep-Pak means (μg/kg)		Range (μg/kg)		Coeff. var. (%)		Pons method[a] (μg/kg)	
		B_1	B_2	B_1	B_2	B_1	B_2	B_1	B_2
Cottonseed meats	8	11	3	10-14	3- 5	13.1	20.2	12	4
	8	28	7	26-31	6- 9	6.4	13.0	31	9
	8	72	12	70-74	12-16	2.2	31.4	78	12
Cottonseed meal	8	14	4	12-16	3- 6	23.1	28.9	18	4
	8	36	8	34-39	7- 9	6.4	13.2	39	10
	8	77	21	72-80	18-24	4.5	8.3	75	19

[a]Pons et al. (1).

presented in Table II. Average coefficients of variation for a total of 24 samples of cottonseed meats with results in a range of 13-90 μg/kg total aflatoxins were ca. 7% for aflatoxin B_1 and 21% for aflatoxin B_2. Average coefficients of variation for 24 samples of cottonseed meal with results in a range of 15-104 μg/kg total aflatoxins were ca. 11% for aflatoxin B_1 and 17% for aflatoxin B_2.

Analytical results by the Pons method (1) for 24 samples of the same naturally contaminated cottonseed meats and cottonseed meals were slightly higher in average results for both aflatoxins B_1 and B_2. Individual sample results were, in most cases, very close and we feel the 2 methods are comparable in both precision and sensitivity.

The proposed method is fast, versatile, simple, economical and reliable, whether the application is extract clean-up for analysis by TLC and HPLC or rapid minicolumn detection. The method would seem to be a very good one for the analyst who routinely analyzes large numbers of cottonseed product samples for aflatoxins.

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