Improved Objective Fluorodensitometric Determination of Aflatoxins in Cottonseed Products[1]

WALTER A. PONS, JR., ALVA F. CUCULLU, ANTHONY O. FRANZ, JR. and LEO A. GOLDBLATT, Southern Regional Research Laboratory,[2] New Orleans, Louisiana 70119

Abstract

Modifications in the extraction solvent, lead acetate, and silica gel extract purification steps, and the TLC development conditions of the aqueous acetone procedure of Pons et al. [JAOAC *49,* 554–562 (1966)] for the estimation of aflatoxins in cottonseed products, provides an improved method with essentially quantitative recovery of aflatoxins added to typical cottonseed materials. Both the accuracy and precision of aflatoxin estimates are significantly improved by the incorporation of an objective fluorodensitometric measurement of aflatoxins on TLC plates.

Introduction

EQUILIBRIUM EXTRACTION of cottonseed products with aqueous acetone, followed by lead acetate treatment to remove interfering gossypol pigments, and silica gel column cleanup to remove interfering fluorescent pigments, has provided the basis of a sensitive method for the estimation of aflatoxins in cottonseed products (1,2). In common with most present aflatoxin methods, this procedure incorporated a visual estimation of aflatoxins based on a comparison of the fluorescence intensities of aflatoxins in unknowns and appropriate standards, after their separation by thin-layer chromatography (TLC) on silica gel-coated plates. Large errors inherent in visual aflatoxin estimations, ±20–30% of the amounts present on a TLC plate (3–6), have heretofore prevented an objective evaluation of the accuracy and precision of this method.

The introduction of fluorodensitometric measurements of aflatoxins directly on TLC plates offers a much more accurate and precise analytical measurement technique (3,4,6,7,8). Experiments with an aflatoxin standard containing B_1 and G_1 resolved on TLC plates indicated that the use of fluorodensitometric measurements provided a measurement precision of ±2–4% respectively (3). With an aflatoxin standard containing B_1, B_2, G_1, and G_2, the precision of individual aflatoxin measurements was found to range from ±4–10%, while total aflatoxins could be estimated to about ±6% (3). Essentially similar results have been reported in a collaborative study of fluorodensitometric measurements of aflatoxins B_1, B_2, G_1, and G_2 added to a purified cottonseed extract, where the precision estimates ranged from ±4–9% for individual aflatoxins, ±5% for total aflatoxins, and average recovery of the amounts added ranged from 93–99% (8).

This communication is a report of a critical study of the aqueous acetone procedure of Pons et al. (1,2) for the estimation of aflatoxins in cottonseed products, using fluorodensitometric measurements for the evaluation of method parameters. Based on the results of the studies, suitable modifications have been made in the extraction, extract purification, TLC development, and aflatoxin measurement phases to provide an improved, more accurate, and more precise method.

Method

Reagents

Solvents. Chloroform, ACS (0.75% or less ethanol). For TLC, chloroform (0.1% ethanol), Burdick and Jackson, Muskegon, Michigan, is preferred. Diethyl ether, ACS anhydrous (0.01% ethanol); hexane, reagent, B.R. 68–69C; acetone, 2-propanol, acetic acid, ACS grade.

Extraction Solvent. Mix 850 ml acetone, 150 ml distilled water, and 8 ml glacial acetic acid.

Column Cleanup. (a) Silica gel: Mallinckrodt CC-7, 100–200 mesh, for column chromatography. Dry in ca. 1-in. thick layer for 2 hr at 110C, store in a screw-capped bottle. (b) Wash solvent: Mix 900 ml anhydrous ether and 300 ml hexane, store in a brown bottle. (c) Elution solvent: Mix 800 ml chloroform, and 200 ml acetone, store in a brown bottle.

Thin-Layer Chromatography. (a) Silica gel: Adsorbosil-1 (Applied Science Labs, State College, Pa). Other gels yielding adequate resolution of aflatoxins are satisfactory. (b) Development solvent: Mix 850 ml chloroform, 125 ml acetone, and 25 ml 2-propanol, store in a brown bottle.

Lead Acetate Solution. Dissolve 200 g ACS neutral lead acetate in distilled water, add 3 ml glacial acetic acid, dilute to 1 liter.

Filter Aid. Celite analytical filter aid (Johns Manville Corp.), or equivalent.

Sodium Sulfate. ACS anhydrous powder.

Aflatoxins standard. Prepare from accurately weighed high-purity crystalline aflatoxins B_1, B_2, G_1, and G_2 (9), dissolved in chloroform and suitably diluted to contain 1.0 μg B_1, 0.3 μg B_2, 1.0 μg G_1, and 0.3 μg of G_2 per milliliter. If desired, any appropriate combination of aflatoxins, or individual aflatoxins, of the above concentrations may be used. Store standards in a freezer (0 F) and suitably protect from evaporation loss. Primary standards containing B_1, B_2, G_1, G_2, or B_1–G_1, in chloroform solution may be obtained from the So. Utiliz. Res. Dev. Div., Box 19687, New Orleans, Louisiana 70119.

Equipment

Mechanical Shaker. Burrell "Wrist Action," or equivalent, fitted to hold ₮ 500-ml Erlenmeyer flasks.

Chromatographic Tubes. Corning 3845, or Kimble 28570, 400 mm long by 20 mm id, with coarse-porosity fritted disc. If desired, the tip can be fitted with a Teflon stopcock.

TLC Spotting. Hamilton 701–SN, 10-μl syringe with 2-cm, 26-gauge tip, 22° point. Use template to hold syringe upright with point just touching gel layer. Significant errors are introduced if the gel layer is damaged during spotting. A stream of dry room temperature air directed along the spotting

---

[1] Presented at Joint AOCS-AACC Meeting, Washington, D.C., March 31–April 4, 1968.
[2] So. Utiliz. Res. Dev. Div., ARS, USDA.

line aids in solvent evaporation, and in obtaining tight origin spots.

TLC Tank. Glass tank, 8.5 in. wide by 8.5 in. high by 4.5 in. deep, with ground glass cover (Brinkmann Instruments 25–10–20). Cover outside with foil for development in subdued light. Use without interior liner.

TLC Plates. Shake 50 g TLC silica gel and 75 ml distilled water in stoppered 250-ml Erlenmeyer flask for ca. 1 min. Using a suitable applicator, coat five 20- by 20-cm plates with a 500-micron wet thickness layer of gel. Air dry for ca. 30 min, activate for 2 hr at 110C in a forced draft oven, and store in desiccator over drierite or silica gel.

Viewing Cabinet-Visual Analysis. Ultraviolet Products, Inc. Model C-4 or C-6, with four 15 w long-wave UV lamps, and contrast filter.

Densitometer. Basic Photovolt Model 530 TLC densitometer equipped with long-wave (320–390 mμ) UV lamp, and primary UV filter; 520-A multiplier-photometer with search unit containing 28-B phototube, 465 mμ secondary filter, and 0.1 by 15 mm, or 0.1 by 6 mm collimating slit. TLC stage with automatic drive (1 in./min); Varicord 42-B (3 in./min chart speed) or 43 (4 in./min chart speed) recorder; Integraph Model 49 automatic integrator. See reference (3) for stage modifications.

Analytical Procedure

Sample Preparation

Grind whole seed or kernels in a Wiley mill or equivalent, to pass a 2-mm screen. For seed containing lint, screen ground sample on a 4/64-in. screen to remove coarse lint. Grind meals to pass a 1-mm screen.

Sample Extraction

Weigh a 25-g sample into a ₸ 500-ml Erlenmeyer flask and cover with a layer of 6-mm solid glass beads. Add 250 ml of extraction solvent, stopper with a leakproof polyethylene or glass stopper, and shake vigorously for 30 min on a mechanical shaker. Filter the extract through a folded 18.5-cm circle of Whatman No. 4, or equivalent paper, collecting ca. 150 ml of filtrate.

Lead Acetate Treatment

Measure 125 ml of the crude extract into a 250-ml beaker, with a suitable calibration mark at 125-ml volume, add 20 ml of lead acetate solution, 25 ml distilled water, and several clean boiling chips. Stir, and boil on a steam bath until the volume is reduced to 125 ml. Cool to room temperature; transfer contents to a ₸ 250-ml graduated cylinder, washing beaker with distilled water; and dilute to 200 ml. Add 4–5 g of filter aid, mix well, filter through a folded 18.5-cm circle of Whatman 4 or equivalent paper. Collect ca. 170 ml of filtrate.

Chloroform Partition

Measure 160 ml of filtrate above into a 250-ml separatory funnel, add 50 ml chloroform, and shake vigorously for ca. 1 min. Drain the chloroform (lower) phase through ca. 2-in. column of anhydrous sodium sulfate in a Butt, or other suitable tube, collecting filtrate in a clean 250-ml beaker. Repeat extraction with a second 50-ml portion of chloroform. Wash the sodium sulfate tube with ca. 20 ml of chloroform, and evaporate combined chloroform extracts to near dryness on a steam bath, avoiding overheating of the dry extract. Dissolve extract in ca. 3 ml chloroform, and reserve for column cleanup below.

For screening purposes, the extract from the chloroform partition above is satisfactory for TLC analysis. For quantitative analysis, the column cleanup below provides cleaner extracts with a minimum of extraneous fluorescent materials.

Column Cleanup

Prepare column by placing a small pad of glass wool over the fritted disc of a chromatographic tube, cover with ca. 2 cm sodium sulfate as an adsorbent bed. Slurry 15 g (30-ml beaker filled to the lip) of CC-7, 100–200-mesh, silica gel with ca. 40 ml of ether:hexane wash solvent in a 100-ml beaker, pour into tube, and wash beaker with ca. 20 ml of wash solvent to aid transfer. When the gel settles, add ca. 2-cm layer of sodium sulfate to the top of the column.

When the wash solvent level reaches the top sodium sulfate layer, add the sample, dissolved in ca. 3 ml of chloroform. A small funnel with an elongated stem reaching just above the top of the column ensures uniform sample adsorption. Wash the sample beaker twice with ca. 2 ml of chloroform, and transfer washings to the column. Wash the funnel with ca. 1 ml of chloroform.

Measure 150 ml of ether:hexane, wash solvent into the sample beaker, and add to the column when the chloroform level reaches the top sodium sulfate layer. Add the remainder of the wash solvent in increments, keeping the column about 80% filled. Discard the wash eluate.

Measure 200 ml of the chloroform:acetone elution solvent into the sample beaker, and add to the column when the wash solvent level reaches the top sodium sulfate layer. Collect the eluate in a clean 250-ml beaker, add several clean boiling chips, and evaporate to near dryness on a steam bath, avoiding overheating. Dissolve in chloroform, transfer quantitatively to a 2-dram vial, and evaporate to dryness on a warm surface under a stream of nitrogen. Cap, and reserve for TLC analysis.

Preliminary TLC

Dissolve the dry extract in exactly 0.5 ml of chloroform, stopper, and swirl gently. Spot 2-, 5-, and 10-μliter aliquots of the extract, and 1-, 3-, and 5-μliter aliquots of the aflatoxin standard (containing B_1, B_2, G_1, and G_2) on a TLC plate, placing the spots 1 cm apart along an imaginary line ca. 4 cm from the bottom of the plate. Four samples can be spotted on one plate. Remove ca. 0.5 cm of gel coating from the side edges of the plate, and scribe a line across the top ca. 12–13 cm beyond the origin as a solvent stop.

Develop the plate, in an upright position, in an unlined and unequilibrated tank containing 150 ml of the TLC development solvent, in subdued light, until the solvent front reaches the scribed line. Remove the plate, and air dry in the dark for ca. 15 min.

Observe the plate under long-wave UV in a suitable viewing cabinet, to confirm resolution of aflatoxins in the standards. Each standard should exhibit four clearly separated fluorescent spots, which in order of decreasing R_f are B_1, B_2, G_1, and G_2. Aflatoxins B_1 and B_2 are bluish fluorescent, while G_1 and G_2 are greenish fluorescent. If adequate

TABLE I

Sample Dilution and Aliquots for Visual and Densitometric Analysis

Approx. B_1 content from prelim. TLC ppb	Visual analysis[a]		Densitometric analysis[b]	
	Dilution of extract ml	Aliquots on plate µliter	Dilution of extract ml	Aliquots on plate µliter
0–10	0.25	3–5–7	0.25	10–10
10–25	0.50	3–5–7	0.25	5–5
25–50	1.0	3–5–7	0.50	6–6
50–75	1.5	3–5–7	1.0	6–6
75–125	2.0	3–5–7	1.0	5–5
125–150	2.5	3–5–7	1.5	5–5
150–175	3.0	3–5–7	2.0	6–6
175–200	4.0	3–5–7	2.5	6–6

[a] For standard containing 0.5 µg B_1 and G_1 and 0.15 µg B_2 and G_2 per ml.

[b] For standard containing 1.0 µg B_1 and G_1 and 0.30 µg B_2 and G_2 per ml.

standard resolution is not obtained, repeat the TLC development.

Examine the chromatograms of the sample aliquots, and compare with that of the standards to confirm the presence or absence of aflatoxins with R_f and fluorescence patterns similar to those of the standards. Aflatoxins G_1 and G_2 are seldom found in cottonseed extracts.

Some cottonseed extracts contain a bluish fluorescent nonaflatoxin component with R_f at or near that of aflatoxin G_2 (11). If positive identification of this nonaflatoxin component is desired, re-spot two 5-µliter sample aliquots on another plate, place 5 µl of the aflatoxin standard on top of one of the sample aliquots, and develop the plate in chloroform: methanol (95:5). Although aflatoxins are not well resolved with this TLC solvent, the bluish fluorescent nonaflatoxin component will be resolved from aflatoxin G_2, as indicated by the internal standard.

If aflatoxins are detected in sample aliquots from Preliminary TLC, compare with the fluorescence intensity of the standards, and select B_1 and B_2 (G_1 and G_2 if present) spots which most nearly match those of one of the standard aliquots. If necessary, interpolate when the intensity of the sample spot is judged to be between two standards.

Calculate the approximate B_1 content from the equation below:

$$B_1 \text{ (ppb)} = (V_s)(C_s)(SD)/(V_x)(W)$$

where (V_s) = µliter standard equal to unknown spot; (C_s) = B_1 concentration in standard, µg/ml; (SD) = sample extract dilution, in µliter; (V_x) = µliter of sample extract matching the B_1 standard; (W) = g of original sample represented by the sample extract, 10 g, if a 25-g original sample was used for analysis.

The same procedure is used for aflatoxin B_2, and G_1 and G_2 (if present). Dry the remaining sample extract under a stream of nitrogen, and reserve for quantitative TLC analysis.

Quantitative TLC

Dissolve the dry sample extract from preliminary TLC in the appropriate volume of chloroform for either visual or densitometric analysis, as outlined in Table I.

Visual Analysis. Spot the sample aliquots shown in Table I, along with 2-, 3-, 4-, and 5-µliter aliquots of the aflatoxin standard diluted 1:1 to contain 0.5 µg B_1 and G_1, and 0.15 µg B_2 and G_2 per ml.

Observe the developed plate under long-wave UV, and select a sample aliquot where the B_1 fluorescence intensity matches one of the B_1 standard spots. Repeat for aflatoxin B_2 (G_1 and G_2 if present). Most accurate visual analysis is obtained by comparing sample and standard spots at the lowest matching fluorescence intensities.

Calculate the aflatoxin content using the equation given under Preliminary TLC, substituting the appropriate volumes and concentrations.

Densitometric Analysis. Dissolve the dry sample extract in the appropriate volume of chloroform shown in Table I. Spot the suggested duplicate sample aliquots, along with duplicate 5-µliter aliquots of the aflatoxin standard (Table I) on a TLC plate, placing the spots 2 cm apart along an imaginary line ca. 4 cm from the bottom of the plate. Develop as outlined under Preliminary TLC. If plate is visually inspected prior to measurement, use low-wattage UV lamp, and minimize exposure time.

Assemble the densitometer as previously described (3), allowing it to warm up for 20 min prior to use. With suitable channels or spacers on the top and bottom of the plate, place the plate on the stage, gel layer down, so that the direction of scan will be from just above the B_1 spot downwards through the G_2 spot.

Set the multiplier-photometer at suitable sensitivity (Position 3 for Model 520-A), and the recorder for millivolt operation at a suitable sensitivity (50 mv for Model 43). Locate the B_1 spot of one of the standards over the inlet UV aperture, lower and adjust the search unit to ca. 1 mm above the plate surface, and adjust the recorder to a convenient pen response (60% full scale) using the recorder sensitivity (Model 43), or full light control (Model 42-B). Using the manual stage rack, locate a blank zone on the plate just above the B_1 spot over the exit slit, and adjust the multiplier-photometer photometric scale to 0, and the recorder pen to a scale setting of 5 with the set zero (Model 43) or dark point (Model 42-B) recorder controls. Relocate the B_1 standard spot over the exit slit, and set the recorder pen at 75–85% full scale, racking the stage and sliding the plate laterally for maximum pen response. The latter adjustment is not critical if a 0.1- by 15-mm exit slit is used (7). Relocate a blank plate zone just above the B_1 spot over the exit slit, and readjust baseline, if necessary.

Activate the recorder chart drive, and set the automatic integrator for minimum baseline count (ca. 1 count/10 sec). Activate the automatic stage drive (1 in./min) and scan the plate from just above the B_1 spot downwards through the G_2 spot.

Repeat the scan for the second standard, and the sample aliquots. Any changes in baseline setting prior to these scans should be readjusted to a scale value of 5, with the set zero or dark point recorder controls. Recorder sensitivity should not be changed between scans on a plate.

If automatic integration is used, draw perpendicular lines from the beginning of the B_1 peak, the valleys of successive peaks, and the end of the G_2 peak downwards through the integrator trace. Determine the integrator area counts for each aflatoxin peak, averaging the counts of each aflatoxin in the duplicate standard and unknown aliquots. Integrator counts should agree to ±5% for corresponding peaks in duplicate aliquots. If triangulation is used instead of automatic integration, multiply peak height by width as a measure of peak area.

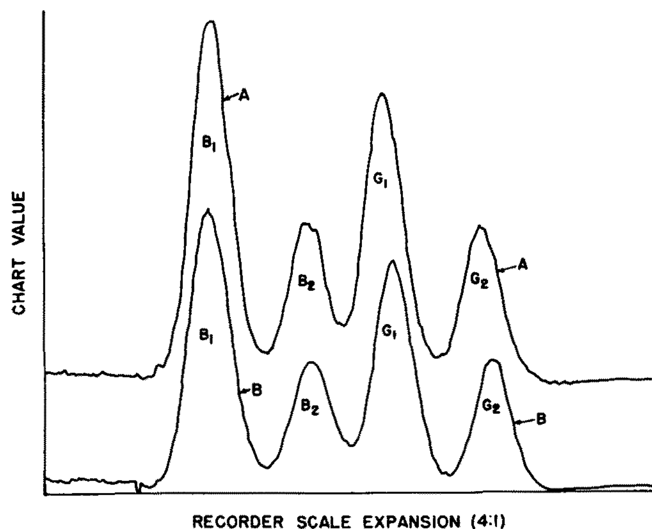

FIG. 1. A—Aflatoxin standard, B_1 (.005 μg); B_2 (.0015 μg); G_1 (.005 μg); G_2 (.0015 μg). B—Extract from meats spiked with 50 ppb B_1 and G_2, and 15 ppb B_2 and G_2 prior to analysis.

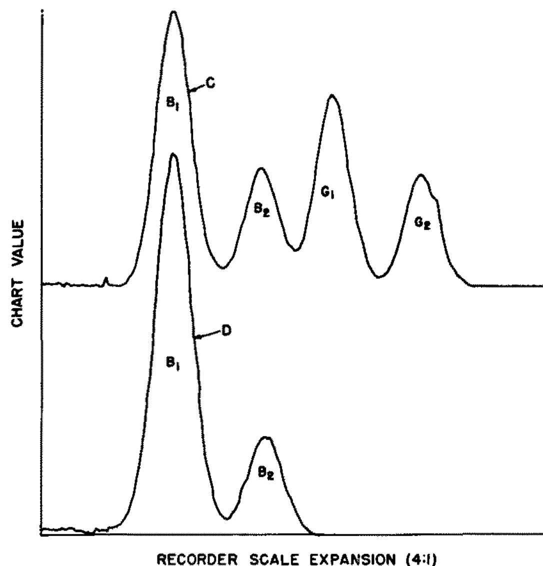

FIG. 2. C—Extract from prepress-solvent meal spiked with 100 ppb (B_1 and G_1), and 30 ppb (B_2 and G_2) prior to analysis. D—Extract from mold-damaged screw-press meal, 126 ppb B_1, 26 ppb B_2.

Calculate the aflatoxin B_1 content in the sample using the equation below.

$$\text{Aflatoxin } B_1 \text{ (ppb)} = [(A_x)(V_s)(C_s)(SD)/(A_s)(V_x)(W)] \times 1.04$$

where (A_x) is the area count of the B_1 sample spot; (A_s) is the area count of the B_1 standard spot; (V_s) is the μliter of standard spotted, and (V_x) is the μliter of sample extract spotted. (C_s), (SD), and (W) have been defined in the equation under Preliminary TLC. The multiplication factor 1.04 is a correction for the 3.4% of the sample extract used for Preliminary TLC, and should not be used if Preliminary TLC was not employed.

The same procedure is used for aflatoxin B_2 (G_1 and G_2 if present), substituting the appropriate area counts, aliquots, and standard concentration.

Experimental Section

All evaluations of method parameters were based on fluorodensitometric measurements of the aflatoxins on TLC plates as outlined under Densitometric Analysis.

TLC Conditions

In agreement with previous observations (3,10), improved TLC separation of aflatoxins in both standards and extracts were obtained using an unlined and unequilibrated tank. The best TLC results were obtained with Adsorbosil-1 gel which provided excellent resolution of aflatoxins, with a minimum of background fluorescence, and usually produced a drift-free baseline in the densitometric analysis. With this gel, the B_1 and B_2 peaks consistently returned essentially to baseline. In some instances separation between aflatoxins B_2 and G_1 were not so uniformly spaced as those between B_1–B_2, and G_1–G_2. Good analytical results were obtained in these instances, indicating that comparable resolutions were achieved between aflatoxins in unknowns and standards on a given TLC plate. Recorder traces representing satisfactory resolution of aflatoxins in standards and unknowns are shown in Fig. 1 and 2.

Plates coated with a 500-μ wet thickness layer of silica gel enabled tighter adsorption of sample extracts, and better resolution of aflatoxins than did 250-μ thick coatings. This factor is not critical, and inasmuch as TLC resolutions are markedly influenced by relative humidity and other factors, other gel thicknesses yielding adequate resolution of aflatoxins are equally satisfactory.

The TLC development solvent chloroform:acetone:2-propanol (850:125:25) was selected since it provided much better resolution of aflatoxins than chloroform:methanol, 97:3(1,2), and also allowed TLC separation of a bluish fluorescent nonaflatoxin material in some cottonseed extracts (11) from aflatoxin G_1. With this TLC development solvent, the interfering fluorescent material has an R_f either just below G_2, or at the R_f of G_2. Chloroform:acetone (85:15) is equally satisfactory (3) but the interfering material usually has an R_f value identical with that of aflatoxin G_1. Since G aflatoxins are rarely observed in cottonseed products (11), resolution of the interfering bluish fluorescent material from the G_1 area serves as a reliable index of the presence or absence of aflatoxin G_1. Although TLC development with chloroform:methanol (95:5) will effectively remove the interfering material from aflatoxin G_2 (11), the poor resolution of aflatoxins in this TLC solvent precludes its use in densitometric analysis.

Column Cleanup

Initial recovery experiments, using silica gel (E. Merck, 0.05–0.20 mm) for column chromatographic extract purification, and chloroform:methanol (97:3) for aflatoxin elution (2), indicated the loss or destruction of a significant portion of the aflatoxins during the chromatographic treatment. Accordingly this phase was systematically evaluated. A stock extract was prepared by combining 10 aqueous acetone extracts from 50-g portions of an aflatoxin-free cottonseed meal, each of which had been semi-purified by lead acetate treatment and chloroform partition. The combined extracts were concentrated, 40 μg each of pure aflatoxins B_1 and G_1 were added, and the stock extract was diluted to 100 ml. Aliquots of the stock extract, 5 ml, representing 10 g of

TABLE II

Recovery of Aflatoxins Added to Silica Gel Cleanup Column

Exp. No.	Column wash solvent		Column elution solvent		% Recovery aflatoxins[c]	
	ml	Composition	ml	Composition	B_1	G_1
1	100	ACS ether[a]	150	C:M (97:3)	37.0	36.9
2	100	Anhyd. ether[a]	150	C:M (97:3)	63.5	46.3
3	100	Anhyd. ether[b]	150	C:M (97:3)	66.2	64.7
4	100	Anhyd. ether[b]	150	C:AN (8:2)	85.3	72.0
5	100	Anhyd. ether[b]	150	C:A (8:2)	88.1	79.2
6	100	Hex:EtAc (3:1)[b]	150	C:A (8:2)	87.6	73.8
7	150	Ether:Hex (3:1)[b]	150	C:AN (8:2)	90.5	75.2
8	150	Ether:Hex (3:1)[b]	150	C:A (8:2)	96.8	86.2
9	150	Ether:Hex (3:1)[b]	200	C:A (8:2)	98.0	98.0

[a] 10 g silica gel, 0.05–0.20 mm (E. Merck) in slurry with 40 ml of wash solvent to prepare column.
[b] 15 g, silica gel CC-7, 100–200 mesh (Mallinckrodt) in 40 ml of wash solvent to prepare column.
[c] Avg of 2 experiments, 2.0 μg B_1 and 2.0 μg G_1 in 5 ml of cottonseed meal extract representing 1.0 g original sample added to column. C = $CHCl_3$; M = methanol; AN = acetonitrile; A = acetone; EtAc = Ethyl Acetate.

original sample and 2.0 μg each of aflatoxins B_1 and G_1 were subjected to chromatographic purification as outlined in Table II.

Silica gel (E. Merck, 0.05–0.20 mm) washed with diethyl ether, followed by aflatoxin elution with chloroform:methanol (97:3) as previously recommended (2), tended to yield low recoveries (experiments 1 and 2). Aflatoxins were not detected in the ether wash, and recovery was not significantly improved by increasing the chloroform:methanol elution volume to 200 ml, or by reducing the activity of the gel by equilibration with 2–10% of water prior to use (10). Significantly higher recovery values were found with Mallinckrodt silica gel for column chromatography (CC-7, 100–200 mesh). The somewhat lower surface activity of this gel required the use of 15 g for column treatment, and an ether:hexane (3:1) wash solvent to avoid loss of aflatoxins in the preliminary column wash. A number of wash and elution solvents were evaluated, a representative portion of which is shown in Table II. Optimum and essentially quantitative recoveries of added aflatoxins were obtained using 150 ml of ether:hexane (3:1) for preliminary column wash, followed by aflatoxin elution with 200 ml of chloroform:acetone (8:2). Ether:hexane removed extraneous fluorescent materials identical to those eluted with anhydrous ether (2), while the chloroform:acetone eluates were chromatographically cleaner than corresponding chloroform:methanol eluates in subsequent TLC analysis.

Acetone:Water Ratio

The amount of water in the acetone:water extraction solvent influenced the recovery of aflatoxins added to typical cottonseed materials prior to the extraction step. When the water content of the extraction solvent was varied from 30% to 10% by volume (Table III), improved recovery was obtained

TABLE III

Effect of Extraction Solvent on Recovery of Aflatoxin B_1 Added to Cottonseed Meal and Meats

Extraction solvent	Aflatoxin B_1 found, ppb					
	Cottonseed meal[a]			Cottonseed meats[b]		
Aflatoxin B_1 added, ppb[c]	25	50	100	25	50	100
70% Aceetone	17.9	38.4	77.8	18.0	30.0	61.1
85% Acetone	24.0	46.9	91.8	19.1	40.9	84.6
90% Acetone	23.4	47.8	92.8	......	......	......
85% Acetone + HAc[d]	23.0	46.0	95.0	23.8	45.5	91.0

[a] Prepress-solvent extracted meal.
[b] Hull-free kernels.
[c] Aflatoxin B_1 dissolved in chloroform added prior to extraction.
[d] Eight milliliter glacial acetic acid/liter.

TABLE IV

Effect of Extraction Solvent on Aflatoxins Found in Mold-Damaged Screw-Pressed Meal

Extraction solvent	Sample weight g	Aflatoxins found	
		B_1 ppb	B_2 ppb
Acetone:$CHCl_3$:water (48.9:39.1:4.0)[a]	50	107.5	18.9
Acetone:water (70:30)	50	100.6	18.8
Acetone:water (80:20)	50	108.0	18.7
Acetone:water (85:15)	50	106.0	17.6
Acetone:water (85:15)	25	113.3	16.3
Acetone:water:HAC (85:15:0.8)[b]	25	126.0	26.0

[a] Solvent system of Purchase and Steyn (13) for extraction of aflatoxin M_1 from milk. Shaker extraction used.
[b] Proposed method, 850 ml acetone + 150 ml H_2O + 8.0 ml glacial HAC.

with 10–15% water in acetone. The highest recovery values were found with acetone:water (85:15) containing 8 ml of glacial acetic acid per liter. Numerous recovery experiments with aflatoxins B_1 and G_1 added to prepress-solvent, screw-press, and direct solvent meals, as well as ground meats, consistently yielded higher and essentially quantitative recoveries when acetic acid was incorporated in the extraction solvent. This trend was also noted with a mold-damaged meal, as shown in Table IV. High water content in the extraction solvent apparently influences the distribution of aflatoxins between the solvent and the sample, while acetic acid serves as a desorbing agent during the equilibrium extraction.

Sample Weight

The sample size also influenced the recovery of aflatoxins, probably due to alteration in the distribution of aflatoxins between the solvent and sample. Recovery experiments with sample sizes ranging from 10–50 g indicated that a 25-g sample allowed essentially quantitative recovery of added aflatoxins. Reduction of sample size below 25 g did not have any detectable effect on recovery. Data outlined in Table V for the analysis of several mold-damaged cottonseed meats and meals indicate a consistent trend to slightly higher aflatoxin B_1 and B_2 values for a 25-g analytical sample.

Visual vs. Densitometric Analysis

Extracts from 25-g samples of cottonseed meats and meals were analyzed by the densitometric and visual analysis techniques previously outlined under Quantitative Analysis. The results, recorded in Table V, indicate that although visual analysis is in essential agreement with densitometric analysis, the

TABLE V

Comparison of Densitometric and Visual Analysis

Mold-damaged products	Sample wt g	Densitometer analysis		Visual analysis[a] two observers	
		B_1 ppb	B_2 ppb	B_1 ppb	B_2 ppb
Meats—A	25	56.9	12.5	50; 50	12; 7
Meats—A	50	50.6	10.8		
Meats—B	25	134.3	26.0	100; 100	20; 20
Meats—B	50	131.3	21.9		
Meal —A	25	31.8	2.5	33; 50	7; Tr.
Meal —A	50	27.5	2.5		
Meal —B	25	143.8	16.3	133; 100	20; 30
Meal —B	50	135.5	14.3		
Spiked products	wt	B_1	G_1	B_1	G_1
Meats[b]	50	33.7	10.6	50; 37	12; 12
Meal[c]	50	45.5	18.4	37; 50	8; 12

[a] Experienced observers.
[b] 33.4 ppb B_1 and 12.0 ppb G_1 added.
[c] 47.4 ppb B_1 and 18.8 ppb G_1 added.

TABLE VI

Recovery of Aflatoxins B_1, B_2, G_1, and G_2 Added to Typical Cottonseed Products

Aflatoxins added, ppb	Average per cent recovery of added aflatoxins[a]				
	Hulls	Meats	P.P.-Solv. meal	Solvent meal	Screw-press meal
		Aflatoxin—B_1			
50	......	91	92	93	92
100	100	91	90	95	94
200	......	87	95	87	98
		Aflatoxin—B_2			
15	......	94	100	96	100
30	87	95	97	97	100
60	......	88	97	100	93
		Aflatoxin—G_1			
50	......	97	92	93	90
100	100	94	86	88	94
200	......	88	87	84	100
		Aflatoxin—G_2			
15	......	99	98	100+[b]	100+[b]
30	92	98	96	100+[b]	100+[b]
60	......	96	98	95	96

[a] Avg values from 2 experiments, densitometric measurement.
[b] High values due to interfering fluorescent material at R_f of G_2.

differences between trained observers emphasize the errors involved in visual aflatoxin analysis. Similar general agreement between visual and densitometric analysis has also been reported by Ayres et al. (6) for the estimation of B_1 in cottonseed meals. Inasmuch as the densitometric technique is a relatively new development, and many laboratories have developed a background and experience in visual analysis, the proposed method incorporates both densitometric and visual estimation for widest application.

Densitometric Measurements

The fluorescence intensity and aflatoxin concentration relationship is linear over a wide concentration range on TLC plates (3); hence the use of multiple standard aliquots and the plotting of calibration curves for each plate are not required. Based on preliminary TLC screening analysis, the sample extract is suitably diluted so that the aliquot spotted provides a B_1 concentration on the plate comparable to that of the standard. This further insures a linear relationship, provides comparable area measurements for both unknown and standard, and yields the highest degree of measurement precision (3). The use of duplicate sample and standard aliquots also increases the accuracy and precision of the measurement, and serves to detect errors in transfer of aliquots to the plate.

Numerous experiments in which pure aflatoxins B_1 or B_2 were spotted as internal standards along with aliquots of purified extracts from aflatoxin-free meats or meals failed to disclose the presence of either fluorescence quenching or intensifying materials at the R_f of B_1 or B_2 in cottonseed extracts. The essentially quantitative recovery of aflatoxins added to cottonseed materials (Table VI) is further confirmation of the absence of interfering materials in extracts purified by the proposed procedure.

Recovery

Known amounts of aflatoxins B_1, B_2, G_1, and G_2 dissolved in chloroform were added to 25-g samples of representative products, prior to extraction, and analyzed by the previously outlined analytical procedure, using fluorescence densitometry for evaluation of the TLC plates. Although aflatoxins G_1 and G_2 are rarely found in cottonseed products (11,12), adequate recovery of all four aflatoxins was obtained, as shown by the results outlined in Table VI. In some instances high values were obtained for aflatoxin G_2 due to incomplete TLC resolution of an interfering nonaflatoxin bluish fluorescent material with R_f similar to that of G_2. When present, this interfering material may be distinguished from authentic G_2 by application of the supplementary TLC development system outlined under Preliminary TLC.

TABLE VII

Replicate Analyses of Mold-Damaged Cottonseed Meal

Day[a]	Aflatoxins, ppb	
	B_1	B_2
1	132.5	27.8
2	127.2	26.7
3	127.2	26.3
4	121.4	25.6
5	122.3	25.6
6	124.3	25.6
7	127.2	24.1
Mean	126.0	26.0
SD	±3.8	±1.2
CV	±3.0%	±4.4%

[a] Single 25-g samples analyzed on each of 7 days by proposed method, using densitometry for TLC evaluation.

Precision

The precision of the method, using densitometric analysis of the extracts, was evaluated by analysis of single samples of a mold-damaged meal over a 7-day period (Table VII). The results indicate a precision of measurement of about ±3–4%, as measured by the coefficient of variation. These precision estimates are similar to those obtained by interlaboratory collaborative study using a spiked cottonseed meal extract (8).

The improved analytical procedure outlined here has also been applied to peanuts, peanut butter, mixed feeds, and a number of other agricultural products (2), with satisfactory results.

ACKNOWLEDGMENT

J. A. Robertson of this laboratory provided the purified aflatoxins; Garlon A. Harper, National Cottonseed Products Association, provided some of the cottonseed samples.

REFERENCES

1. Pons, W. A., Jr., and L. A. Goldblatt, JAOCS, *42*, 471–475 (1965).
2. Pons, W. A., Jr., A. F. Cucullu, L. S. Lee, J. A. Robertson and L. A. Goldblatt, J. Ass. Offic. Anal. Chemists, *49*, 554–562 (1966).
3. Pons, W. A., Jr., J. A. Robertson and L. A. Goldblatt, JAOCS, *43*, 665–669 (1966).
4. Beckwith, A. C., and L. Stoloff, Report at 81st Meeting Assoc. Offic. Anal. Chemists, Washington, D.C., Oct. 9–12, 1967.
5. Nesheim, S., J. Ass. Offic. Anal. Chemists, *47*, 1010–1017 (1964).
6. Ayres, J. L., and R. O. Sinnhuber, JAOCS, *43*, 423–424 (1966).
7. Stubblefield, R. D., O. L. Shotwell, C. W. Hesseltine, M. L. Smith and H. H. Hall, Appl. Microbiol., *15*, 186–190 (1967).
8. Pons, W. A., Jr., Report at 81st Meeting of Ass. Offic. Anal. Chemists, Washington, D.C., Oct. 9–12, 1967.
9. Robertson, J. A., W. A. Pons, Jr. and L. A. Goldblatt, Agr. and Food Chem. *15*, 798–801 (1967).
10. Eppley, R. M., J. Ass. Offic. Anal. Chemists, *49*, 473–474 (1966).
11. McMeans, J. L., L. J. Ashworth and W. A. Pons, Jr., JAOCS, in press.
12. Whitten, M. E., Cotton Gin and Oil Mill Press, *67* (No. 26), 7–8 (1966).
13. Purchase, I. F. H., and M. Steyn, J. Ass. Offic. Anal. Chemists, *50*, 363–366 (1966).

[Received April 2, 1968]