A Simplified Procedure for the Determination of Aflatoxin B_1 in Cottonseed Meals[1]

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Abstract

A simplified procedure for the detection of aflatoxin B_1 in cottonseed meals has been developed. The procedure substantially reduces the time and cost of aflatoxin analysis. A single chromatographic column of celite is used to concentrate and purify the aflatoxin fractions. A new solvent system of ether-methanol-water (96:3:1) improves separation of aflatoxin fractions on TLC plates. This improvement permits measurement of B_1 spots in one sweep of the plate with a densitometer using a 10–15 mm slit. The number of samples which can be screened for aflatoxin contamination on one TLC plate is doubled by re-use of the plate. The use of technical reagents and stainless steel beakers also helps reduce the time and cost of analysis. The procedure is sensitive to about 5 μg of B_1 per kg of meal.

Introduction

Our laboratory has been investigating the extent of aflatoxin contamination in cottonseed products for the past four years. More than 6,000 samples have been analyzed by the procedure of Pons et al. (1) for this toxic compound.

A field survey of the 1964, 1965 and 1966 cottonseed crops (2) revealed that less than 10% of the seed had a contamination of more than 30 μg of aflatoxin B_1 per kg. A maximum level of 30 μg of aflatoxin per kg of peanuts or other protein supplement has been recommended by the Protein Advisory Group of the United Nations Organization (3).

Since the extent of aflatoxin contamination of cottonseed and cottonseed meal was found to be so low, a simplified procedure, or better yet, a reliable screening method would certainly be welcomed. This would reduce time and material spent on cottonseed meal analyses for aflatoxin, in which 9 out of 10 samples may yield negative results.

This paper describes a procedure which simplified the detection of aflatoxin in cottonseed meals. It also includes techniques which reduce the cost of aflatoxin determinations.

Materials and Methods

Reagents

Chloroform, technical grade; methyl alcohol, ACS grade; Celite 545, acid washed, Johns-Mansville Company, used without further treatment; *n*-hexane, commercial Skellysolve B; sand; sodium sulfate, anhydrous, ACS grade; glass wool, pyrex; Silica Gel G-HR, Brinkmann Instruments, Inc.; aflatoxin standard B_1, Southern Utilization Research and Development Division, ARS, USDA.

Apparatus and Equipment

Chromatographic column, glass, 35 mm OD × 450 mm long with stopcock; TLC equipment; micro syringe 10, μl capacity; ultraviolet lamp, long wave, 3600 A; stainless steel beakers, 600 ml.

Preparation of Celite Column

Place a small flattened wad of glass wool in the bottom of glass column and add sand to form a layer about ¼ in. in depth. Add about 75 ml of hexane and level layer of sand by tapping glass column. Then add 7 g (±0.5 g) of Celite to the column and wash down sides of column with hexane from squeeze bottle. Open stopcock and allow Celite to settle into a compact column. Allow about 2 or 3 in. of hexane to remain above the Celite. Carefully add a protective layer of anhydrous sodium sulfate to the column, to form a layer about ½ in. in depth.

Extraction of Cottonseed Meal

Weigh 50 g of cottonseed meal into a 500 ml Erlenmeyer flask (℉); add 25 ml of water and blend into meal with stirring rod until all large lumps are broken. Add 250 ml of technical chloroform, stopper flask, and place on a shaker for 30 min. Filter extract through 24 cm fluted filter paper and transfer 150 ml of the filtrate into a 600 ml stainless steel beaker or into a 400 ml glass beaker. Place beaker on steam or water bath and evaporate the chloroform.

Transfer of Chloroform Extract Residue to Column

Wash down beaker containing extract residue with hexane until volume is diluted about 3 times. Transfer residue quantitatively to Celite column using hexane; rinse beaker twice with 10 ml portions of hexane and add to column. Open stopcock and allow column to drain until hexane level is almost flush with top of Celite column, close stopcock.

Add 100 ml of hexane and start column flow at a rate of 8–10 ml/min. Stop column flow when hexane level is again almost flush with top of Celite column; discard this portion.

Place a clean 250 ml beaker under column. Rinse beaker which contained extract residue with 10 ml of technical chloroform and transfer to column. Drain column until chloroform layer is flush with top of Celite column. Add 125 ml of technical chloroform to column using beaker, and elute the aflatoxin fraction at a rate of 8–10 ml/min. Evaporate the chloroform eluant and transfer the residue quantitatively to a 10 ml vial using technical chloroform. Evaporate the chloroform in the vial and store for spotting on TLC plate.

Thin-Layer Chromatographic Plates

Set adjustable applicator, if available, between 0.25 and 0.50 mm. This will produce thin layers of about 0.37 mm. Weigh 35 g of silica gel G-HR and add 70 ml of water. Shake vigorously for about 45 sec and pour into applicator. Apply to 20 × 20 cm plates or to 10 × 10 cm plates. Allow plates to dry overnight and activate at 100 C for 1 hr. Place plates in storage cabinet to cool and equilibrate.

Application of Sample Extract to TLC Plate

Pipette 0.5 ml of technical chloroform into vials containing sample extracts. Spot 5 μl of each sample

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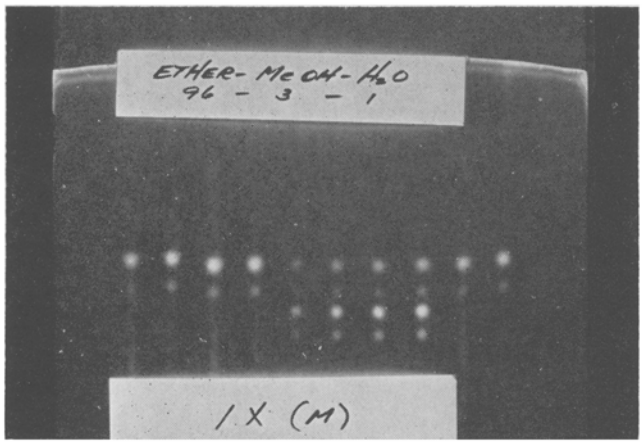

FIG. 1. TLC separation of aflatoxin using solvent system ether-methanol-water (96:3:1); development time 55 min in unlined chamber; 5 μliter of cottonseed meal extracts (1–4) and (9–10); standard aflatoxin fractions (5–8) in increasing concentration, 0.003–0.012 μg B_1; standard fractions in descending order B_1, B_2, G_1, G_2.

and appropriate volumes of standard B_1 solution containing 1 μg/ml to plate. Place plate in an unlined chromatographic tank containing solvent system of Ether-methanol-water (96:3:1). Allow 55 min for plate development.

Detection and Estimation of Aflatoxin Fractions

Place plate in a dark viewing cabinet or box and expose to long-wave ultraviolet light. Estimate the aflatoxin contents of the sample spots by matching their fluorescent intensity with standard spots.

Sample volume may be increased or decreased to bring it to the level of the B_1 standard solution.

Calculation of Aflatoxin B_1 in Meal

$$\mu g/kg = \frac{\text{Std Spot } (\mu l)}{\text{Smpl Spot } (\mu l)} \times \text{SV(ml)} \times \text{Std } B_1 \text{ Soln } (\mu g/ml) \times \frac{1000 \text{ g}}{50 \text{ g} \times 0.6}$$

Std Spot, μl standard spot equivalent to sample spot; Smpl Spot, μl of sample spotted; SV, final sample volume of extract; Std B_1 Soln, concentration of B_1 in standard solution (μg/ml).

Discussion and Results

The present methods for aflatoxin determination are modifications of the procedure developed jointly in England by Sargeant et al. (4) at the Tropical Products Institute in London and by Allcroft and Carnaghan (5) at the Central Veterinary Laboratory in Weybridge. Their investigation of peanut meals established aflatoxin as the compound responsible for severe outbreak of poisoning in young turkeys and ducks.

TABLE I

Aflatoxin Content (B_1) Found in Replications of Selected Samples of Cottonseed Meal by the P-A and the Proposed Column Methods of Analysis

Composite Meal Sample	Aflatoxin content B_1 repilcate, ug/kg					
	P-A Method			Column Method		
	A	B	Av	C	D	Av
1	435	420	428	407	425	416
2	190	185	188	184	201	193
3	89	78	84	73	81	77
4	38	20	28	23	26	24

In cottonseed the detection and estimation of aflatoxin are hindered by the presence of pigments derived from gossypol. The procedure of Pons et al. (P-A) removes most of these pigments from solution by precipitation with lead salts followed by filtration, solvent extraction and column clean-up of the aflatoxin fractions.

Since chromatographic columns adsorb gossypol more strongly than aflatoxin this procedure appeared suitable for simplification of the aflatoxin procedure for cottonseed meals. A single chromatographic column could be used in place of the more elaborate P-A procedure to clean, concentrate and separate the aflatoxin fraction from the gossypol pigments.

The chromatographic materials investigated for this column set-up had been used in the purification of the various aflatoxin fractions. These were alumina in the acid and neutral form (4); Silica gel, Kieselgel G and Celite (6).

The best results were obtained with Celite columns. Aflatoxin was found to be adsorbed by Celite columns from solutions of hexane or benzene and to be eluted from these columns by chloroform or diethyl ether.

In order to have Celite columns with good flow characteristics, glass columns of about 30 mm in diameter are used. Smaller columns give flow rates that are too slow for routine use. The optimum amount of Celite used with these columns is about 7 g; when more than 8 g of Celite are used the column flow rate becomes too slow.

With standard amounts of B_1 added to columns of 7 g Celite, recoveries of 100% were obtained with the solvent system of hexane and chloroform. Recovery was tested with only the B_1 fraction since it is the principal and most toxic component of aflatoxin (7) and also because, to this date, no fractions of aflatoxin G have been detected in field samples of cottonseed or meal by this laboratory.

Test extractions of cottonseed meal were made with acetone and chloroform, solvents most commonly used to extract aflatoxin, to determine which was best suited for use with Celite columns.

Chloroform results were the best. The meal was first moistened with half its weight of water to more fully extract the aflatoxin (8).

In the interest of economy, technical grade chloroform was compared with reagent grade in the extraction and thin-layer development steps. In tests conducted over a period of two years, and using chloroform lots from different manufacturers, we found no significant difference in B_1 results between these two grades of chloroform at levels down to 5 μg/kg.

We also found that we can reduce the evaporation time of crude chloroform extracts from about 30 min to less than 10 min by use of stainless steel beakers. In one year of testing we found no significant difference in B_1 results by use of stainless steel rather than glass beakers.

This specified procedure was compared with the P-A procedure at various levels of aflatoxin contamination. These levels were obtained by dilution of a composite mixture of field samples, of high aflatoxin contents, with uncontaminated meals. Samples were analyzed in duplicate and measurements of B_1 fluorescence were taken with a densitometer (9).

At the levels indicated in Table I aflatoxin contents by the column method are comparable to those of the P-A procedure.

Attempts to determine aflatoxin B_1 at the 1 μg/kg level by meal dilution were not successful because

of the interference of background streaking. This was true for both procedures.

Recently we found that addition of water to the TLC solvent system greatly improves the separation of the aflatoxin fractions. Not only is separation greater between B_1 and B_2 but equally as important is the increased uniformity in the upward migration of B_1 spots all across the TLC plate. Separation between B_1 and B_2 is about 10 mm when plates are developed in a solvent system of ether-methanol-water (96:3:1) for 55 min in an unlined tank.

Figure 1 shows a plate developed in this new solvent system. Fluorescent measurement of all B_1 spots can be made with one sweep of the densitometer, using a 15 mm slit, along a line parallel to the solvent front and origin line. There is no overlapping of B_2 during this sweep if the plate is positioned so that the lower edge of the slit is about 3 mm above the highest B_2 spot.

Another technique which reduces the time and cost of aflatoxin analysis is to reuse the once-developed TLC plates. Samples can be screened for aflatoxin by spotting a second set of samples, opposite the first set, on used plates and developing in the usual manner.

Although used plates do not have to be reactivated for this second development, they should be protected from lint and dust. The plates are handled carefully so as not to damage the layers where the samples will be spotted. The edges of the plate are not scraped clean as recommended.

Figure 2 shows the development of two sets of samples on one plate. The second set (2X) was spotted four days after the first set (1X). In order to use a plate for two developments it is best to use a silica gel which leaves a firm layer. Silica Gel G-HR from Macherey & Nagel and Silica Gel 7G from Mallinckrodt were found to produce the firmest layers.

An observation made in TLC development of aflatoxin is that R_f's for B and G fractions are reversed when they are developed in a second direction using the same solvent system thus $B_2 > B_1$, $G_2 > G_1$.

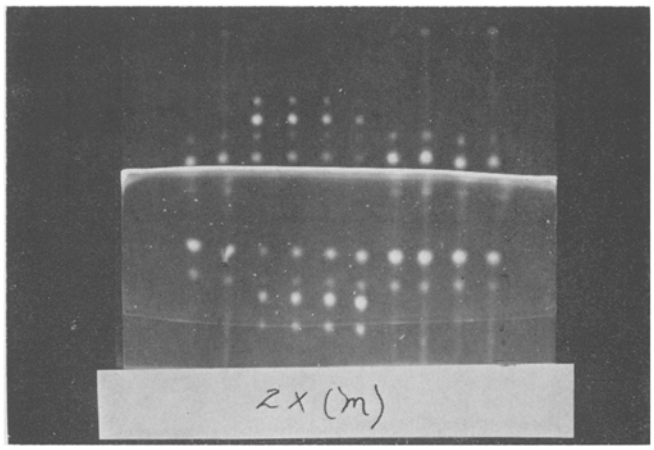

FIG. 2. TLC plate with two sets of samples; second set (2X) spotted and developed four days after first set (1X); plate not reactivated for second development; conditions and materials same as in Figure 1; unsymetrical sample spot second from left caused by damage to thin layer.

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