The Rapid Separation of Aflatoxins on Silica Gel Coated Glass Cylinders[1]

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Abstract

A chromatographic method is described for the separation of aflatoxins on silica gel coated glass cylinders prepared with a chloroform-acetone slurry. This can be done in 35–40 min compared with more than 2 hr for thin layer chromatography (TLC). This method is more rapid, economical and its sensitivity is comparable to TLC.

Introduction

Owing to the susceptibility of a wide variety of agricultural crops to contamination with aflatoxin, a metabolite of the common mold *Aspergillus flavus,* the need exists for a rapid but sensitive method for the quantitation of aflatoxin in a variety of commodities.

Thin layer chromatography (TLC) is the most common method for obtaining presumptive evidence of aflatoxin contamination. Officially accepted analytical methods (1–4) require more than 2 hr. Holaday (5) and Whitten (6) developed rapid screening tests for aflatoxin in peanuts and cottonseed respectively. Neither method is as sensitive as TLC and quantitation is not possible with these techniques. Eppley (7) suggested a rapid screening test with microslides. Peifer described a method in which lantern slides were coated by dipping in a slurry of Silica Gel H in 2 + 1, $CHCl_3$ + MeOH and the plates were used immediately after air drying (8).

The present study was undertaken to develop a rapid and versatile method which could be used for the quantitation of aflatoxin and other fluorescent mycotoxins in peanuts, rice and broth.

Experimental Procedures

An aflatoxin standard was obtained from the Southern Utilization Research and Development Division, USDA, New Orleans.

Aflatoxin was extracted from peanuts, rice and yeast extract plus sucrose (YES) (9) medium in which *A. flavus* ATTC 15517 had grown by blending in a Waring blendor either 50 g or 50 ml of sample with 200 ml chloroform and filtering through two layers of Whatman No. 1 filter paper.

Preparation of Cylindrical Tubing

Glass tubing of various lengths (7–33 cm, 0.1–2.8 cm i.d.), bacteriological pipettes (5 and 10 ml) and test tubes (16 × 150 mm) were cleaned with acetone and then coated with a thin layer of Silica Gel G-HR. Owing to the length of time required for drying and activating TLC plates, a new method of preparing adsorbent was investigated. Slurries were prepared in two different ways: (a) with water (as for TLC); (b) with chloroform-acetone (2:1 v/v) as a substitute for water.

The following ratios of Silica Gel G-HR to water, or to chloroform-acetone, were used (w/v): 50:100, 50:110, 50:115, 50:120 and 50:125. Corn starch varying from 1–6% was added as a binder to prevent flaking of the coating.

The coating was prepared by mixing the Silica Gel G-HR (Macherey, Nagel and Co., Germany), cornstarch and chloroform-acetone in a 250 ml Erlenmeyer flask. The slurry was transferred to a 100 ml graduated cylinder and the cylindrical tubing was dipped into the slurry once, withdrawn slowly, and allowed to air-dry for 10–15 min at room temperature. This procedure resulted in a uniform coating of the outer surface of the glass cylinder with a thin layer (<1 mm) of silica gel. Thickness of coatings was varied by repeated dipping of the cylindrical tubing in the silica gel slurry. Activation of the silica gel was not necessary to obtain good resolution.

Test tubes were found more convenient than cylindrical tubing and were used throughout this study for the evaluation of materials and for comparison of methods.

Coated test tubes were supported at one end with 16 mm Bacti Capall stoppers (Sherwood Medical Industries, Inc., St. Louis). After developing, flanged stoppers were used at both ends so that test tubes could be placed on a flat surface without smearing the coating (Fig. 1).

Developing Solvents

The following solvent systems were used in developing glass cylinder chromatographic (GCC) tubes: chloroform-acetone (9:1 v/v); chloroform-acetone (85:15 v/v); benzene-ethanol-water (2:2:1 v/v); chloroform-benzene-methanol (1:2:2 v/v); benzene-ethanol-water (46:35:19 v/v); benzene-acetone-acetic acid (8:1:1 v/v).

Application and Development of Sample

A 10 μl Hamilton microsyringe was used to apply 10–12 discrete spots varying from 1 to 5 μl around the circumference of the tube at a distance of 2 cm from the bottom. Aflatoxin samples and standard were dissolved in chloroform and spotted as for TLC (1,2); each spot was allowed to dry before superimposing another. The tube was then developed for 15–20 min in an unequilibrated Brinkman developing chamber filled with developing solvent to a depth of 0.8–1.5 cm. The developing solvent consisted of chloroform-acetone (9:1 v/v) (4). The test tube was then allowed to air-dry for 30–60 sec after developing. R_f values of fluorescent metabolites were then compared to R_f values of standard aflatoxin under long wave ultraviolet light in a Chromato-Vue viewing cabinet (Ultra-violet Products, Inc., San Gabriel, Calif.). Samples could be quantitated by comparing to standard aflatoxin spots developed either on the same or on another test tube, as described by Eppley et al. (10) for 20 × 20 cm thin layer chromatographic plates.

Results and Discussion

A total of 536 tubes were prepared, spotted and developed. Very good resolution of aflatoxins B_1, B_2,

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TABLE I

Comparison of Three Chromatographic Methods for the Separation of Aflatoxins

Treatment or test	GCC[a]	TLC[b]	Millicolumn[c]
Solvent for adsorbent slurry	Chloroform-acetone (2:1 v/v)	Water	None
Treatment of adsorbent	None	2 hr minimum activation at 110 C	24 hr adjustment to 80% R.H.
Capacity of chromatogram or column	12 spots	14–15 spots	1 sample
Developing time	15–20 min	40 min	10–15 min
No. aflatoxins separated	4	4	2
Quantitation	Feasible	Feasible	Not feasible

[a] Glass cylinder chromatography.
[b] Thin layer chromatography (1–4).
[c] Millicolumn chromatography (5).

G_1 and G_2 was obtained when test tubes were uniformly coated by dipping once in a slurry formed by using 50 g silica gel with 3% binder and 115–125 ml of either water or chloroform-acetone (2:1 v/v) (Fig. 1).

When the adsorbent slurry was prepared with water, under ordinary conditions of room temperature and relative humidity (i.e., RH, 38–48%), a drying time of 3–4 hr was required or 1½ hr in a drying oven at 110 C was needed before the test tubes could be spotted without flaking. However, when chloroform-acetone (2:1) was used, the solvent mixture evaporated in 30–60 sec at room temperature and the test tubes could be spotted immediately.

Satisfactory separation of the four aflatoxins (B_1, B_2, G_1, G_2) was obtained with chloroform-acetone (9:1 v/v) in an unequilibrated tank. Aflatoxin B was separated from G with benzene-ethanol-water (2:2:1 v/v); B_2 and G_2 were also separated when the ratios were changed to 46:35:19 benzene-ethanol-water. Aflatoxins were not resolved by these two solvent systems: chloroform-benzene-methanol (1:2:2 v/v) and benzene-acetone-acetic acid (8:1:1 v/v). Glass cylinders of various sizes were coated, spotted and developed under identical conditions. Good resolution of the four aflatoxins was obtained with all sizes after the solvent front moved 7.5 to 9.0 cm from the origin. With test tubes and tubing of about 16 mm diameter, the solvent moved this distance in 15–20 min. The time increased for larger tubing and decreased for smaller tubing. However, fewer samples could be spotted on small tubing and application of the spots was more difficult.

Duncan (11) used a Silica Gel slurry in methanol-water (1:1 v/v) to resolve terpene derivatives after activation of the plates. Hoerhammer et al. (12) used either ethyl acetate or acetone in preparing adsorbent slurries for TLC plates. Methanol-water (1:1 v/v), ethyl acetate, acetone and chloroform were tried

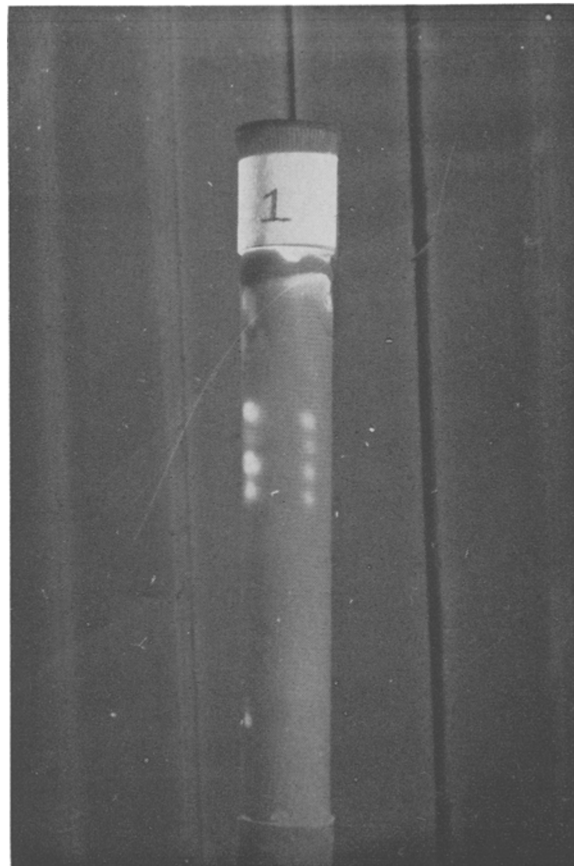

FIG. 1. Separation of four aflatoxins on GCC tube coated with a slurry of Silica Gel G-HR and chloroform-acetone, 2:1 (v/v). The GCC tube was developed in chloroform-acetone (9:1). From right to left are the 5 μl aflatoxin standard (0.005 μg B_1 and G_1; 0.0015 μg B_2 and G_2), and the 5 μl sample which was extracted from peanuts on which *A. flavus* was cultured. From top to bottom are aflatoxins B_1, B_2, G_1 and G_2.

with Silica Gel G-HR in this study with and without the addition of starch. Uniform coating of the glass tubing was not obtained with those solvents and drying of the adsorbent coat was delayed with ethyl acetate. Resolution of the four aflatoxins was not achieved under these experimental conditions.

Table I compares the GCC method with the TLC technique and Holaday's millicolumn technique. The millicolumn method, although rapid after columns are equilibrated for 24 hr at 78–80% relative humidity, provides a crude estimate of aflatoxin content and quantitation is not possible due to smearing of the millicolumn with fluorescent substances other than aflatoxins B and G.

Resolution of aflatoxins B_1, B_2, G_1 and G_2 on GCC is comparable to that obtained with TLC. Time re-

TABLE II

Visual Quantitation of Aflatoxins B_1, B_2, G_1 and G_2 on TLC plates and GCC Tubes

Sample No.	Standard aflatoxin[a]	TLC plates[b]						GCC tubes[c]					
		μl Estimated by analyst					No. incorrect comparisons	μl Estimated by analyst					No. incorrect comparisons
	μl Spotted	A	B	C	D	E		A	B	C	D	E	
1	2	2	2	2	4	2	1	2	2	2	2	2	0
2	1	1	1	1	2	1	1	1	1	1	1	1	0
3	3	2	3	3	4	2	3	3	2	3	3	4	2
4	5	3	5	4	5	4	3	4	5	5	5	5	1
5	3	2	2	2	5	2	5	4	3	4	4	4	4

[a] One microliter contains 0.001 μg B_1; 0.0003 μg B_2; 0.001 μg G_1; 0.0003 μg G_2.
[b] Thin layer chromatography (1–4).
[c] Glass cylinder chromatography.

quired for preparation and extraction of the sample is the same for both GCC and TLC methods and depends on the degree of purity required. However, preparation of GCC tubes requires less skill than is required to make uniform coatings on TLC plates, and resolution of the sample can be accomplished with the GCC method in a total of only 15–20 min compared to 40 min for 20 × 20 cm TLC plates. Activation of the silica gel, a time-consuming procedure in TLC (2,3), is not necessary with GCC, and the initial investment in equipment is smaller for the GCC method compared to TLC.

Quantitation by GCC and TLC

Five spots of standard aflatoxin solution (1–5 μl) were spotted in sequence on TLC plates and GCC tubes. In addition, one sample was spotted on the GCC tube with the standards for qualitative comparison of R_f values. Standard aflatoxin (1–5 μl) was randomly spotted on the other half of the TLC plates and on separate GCC tubes which were developed at the same time in chloroform-acetone (9:1 v/v). Spots were compared by rotating the tubes until a match was found. Table II shows the results of visual comparison made by five people who were unaware of the amounts of aflatoxin spotted.

As shown in Table II, perfect matching of all samples was not achieved by any of the analysts with either of the methods. With few exceptions, when a perfect match was not obtained, analysts estimated the amounts as 1 μl over or under the actual amounts spotted. For aflatoxins B_1 and G_1, this variation represents ± 0.001 μg and for aflatoxins B_2 and G_2, ± 0.0003 μg.

The five analysts called upon to give the estimates recorded in Table II had no experience with the GCC method and four had no experience with either the GCC or the TLC method. These analysts were asked to indicate by which of the two methods readings were more easily obtained. Four analysts indicated a preference for the GCC method because sample and standard could be placed nearer each other and were more easily compared. The remaining analyst preferred the TLC method because of his familiarity with that procedure and because he liked the larger spots for reading.

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