Conditions and Techniques for Thin Layer Chromatography of Aflatoxins

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Abstract

Satisfactory resolution of the four common aflatoxins, B_1, B_2, G_1 and G_2, on thin layer chromatograms has been a recurring problem. The most frequently observed cause of poor resolution and tailing of spots in the chromatograms was the variable properties of the commercial silica gel-calcium sulfate adsorbent preparations. Variations in quality were observed even from one container to the next within single lots produced by individual manufacturers. Other variables which affected the chromatography to some degree included adsorbent particle size, concentration and nature of the calcium sulfate binder, silica gel layer thickness and moisture content, vapor phase composition in the developing chamber and the solvent used for development.

Introduction

The four common aflatoxins, B_1, B_2, G_1 and G_2, are generally estimated by visual or densitometric comparison of the fluorescence intensity of spots appearing after thin layer chromatography (TLC). The individual toxins vary in potency (1) and fluorescence intensities (2), therefore they must be measured individually. Satisfactory resolution of the aflatoxins by TLC has been a recurring problem. Since 1963, when we started our aflatoxin investigations, we have sought optimum TLC conditions and investigated possible causes of poor resolution. An empirical approach was taken based on the techniques used at the time. Modifications were developed in the course of normal day-to-day operations and their effects on resolution and chromatogram quality were observed. The comparison of the resolution and quality of the various chromatograms was subjective. Polaroid pictures of chromatograms were used for comparison since the individual chromatograms were often prepared by different analysts at different times.

Experimental Procedures

In the TLC tests aflatoxins were superimposed on a spot of high roast, aflatoxin-free peanut butter extract that was prepared by the AOAC official first action method commonly called the Celite method (3). This procedure was followed in order to simulate the background and interference normally present in sample extracts, including the so-called peanut butter spot. The peanut butter spot can appear at the same R_f's as aflatoxins G_1 or G_2 with the acetone or methanol-chloroform developers and at an R_f close to that of B_1 with benzene-ethanol-water as the developer. The following series of three spots was applied in triplicate across each plate used to test the chromatographic variables: 5 μliters of peanut butter extract in chloroform, 5 μliters of peanut butter extract solution with 5 μliters of standard aflatoxin solution superimposed and 5 μliters of aflatoxin solution. The five μliters of peanut butter extract solution contained extract equivalent to 100 mg of peanut butter. Five μliters of standard aflatoxin solution contained 5, 1.3, 5 and 1.3 ng of aflatoxins B_1, B_2, G_1 and G_2 respectively. The TLC was performed according to published techniques (3,4) except where otherwise indicated. The developed chromatoplates were judged by visual inspection and scored on a scale ranging from one to eight on the basis shown in Figure 1.

Results

Twenty-three commercial silica gel preparations for TLC were tested with one or more developing solvent systems. The silica gels are listed in Table I according to the highest quality score achieved with any one of the developing solvents. Only the first eight silica

TABLE I
Performance and Properties of Various Commercial TLC Silica Gels

Trade name	Manufacturer	$CaSO_4$[a] (%)	Particle[a] size (μ)	Developing[b] solvent	Score[c]
Bio Sil A	Bio-Rad Lab.	5	2–10	1,2,3	8
SilicAR TLC-7G	Mallinckrodt	15	....	1,2,3	8
SilicAR TLC-4G	Mallinckrodt	15	....	1,2,3	8
Adsorbosil 1	Appl. Sci. Lab., Inc.	10	....	2	8
Adsorbosil 5	Appl. Sci. Lab., Inc.	0	....	2	8
Silica Gel D-5	Camag	5	....	2	8
MN G-HR	Macherey, Nagel & Co.	....	5–25	2,3	8
MN G-HR	Macherey, Nagel & Co.	....	5–40	2,3	8
Supelcosil 12B	Supelco, Inc.	....	....	2	6
Adsorbosil S1	Appl. Sci. Lab., Inc.	10	....	2	6
Aflasil 15E	Supelco, Inc.	....	....	2	5
Bio Sil A	Bio-Rad Lab.	0	2–10	2	5
SilicAR TLC-7GF	Mallinckrodt	14	....	2	4
Silica Gel Woelm TLC	M. Woelm	0	....	2	4
Bio Sil A[d]		5	10–30	1	4
Bio Sil A[d]		5	30–60	1	3
Silica Gel G	E. Merck, AG	....	5–20	2	2
Silica Gel G	Research Spec. Co.	....	....	1	2
Silica Gel H	E. Merck, AG	0	5–25	1	2
Silica Gel H-HR	E. Merck, AG	0	10–40	1	2
Silica Gel G Woelm TLC	M. Woelm	13	....	2	2
Chromedia SG 41	Whatman	0	....	2	1
SilicAR TLC-7	Mallinckrodt	0	....	1	1

[a] From manufacturers' label statement or literature.
[b] Developing solvent used with 0.25 mm, 8 × 8 in. chromatoplates; 1, methanol-chloroform (7:93); 2, acetone-chloroform (10:90); 3, upper phase of benzene-ethanol-water (46:35:19).
[c] Score is judgment of resolution and chromatogram quality made by visual inspection on a scale from 1 to 8 based on criteria shown in Figure 1, where 1 is the poorest and 8 is the best score.
[d] No longer manufactured.

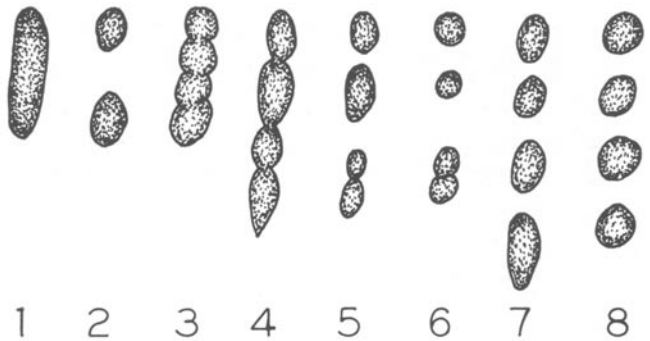

FIG. 1. Key to quality judgment of thin layer chromatograms. The drawings are representative of increasing degrees of quality in resolution of the four aflatoxins B_1, B_2, G_1 and G_2, listed in decreasing order of R_f. The values are assigned as follows: 1, smear or spot of all four toxins; 2, separation of B_1 and B_2 from G_1 and G_2; 3, indication of four spots, but not clearly separated; 4, good separation, but with extensive smearing; 5, two pairs of spots close together and some tailing in one or more; 6, four round spots, one pair not completely separated; 7, four separate spots, some tailing; 8, four well-separated round spots.

gels listed were judged to be satisfactory or better. This listing is applicable only to the particular lots tested; the chromatogram quality, i.e., the resolution and absence of tailing, varied from lot to lot and even within lots. Illustrative of the difference between lots are photographs of the chromatograms prepared from the two lots of SilicAR TLC-7G (Fig. 2). This variation was observed in chromatograms prepared from two different lots of SilicAR TLC-7G developed with methanol-chloroform (7:93) and in chromatograms prepared from two different lots of silica gel MN G-HR developed with acetone-chloroform (10:90). Chromatograms from one lot of each brand yielded four well separated aflatoxin spots but chromatograms from different lots of the same brand yielded smeared G_1 and G_2 spots.

To confirm that these observed differences were due to the nature of the silica gel and not to some other factor, plates were prepared in such a way that the

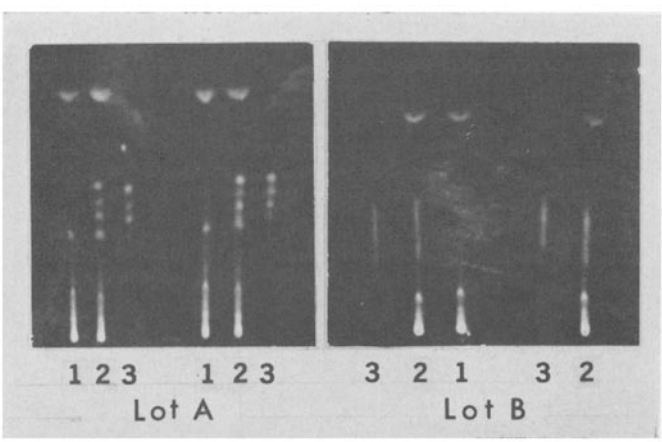

FIG. 2. TLC separation of aflatoxins on different lots of SilicAR TLC-7G silica gel. Developing solvent, methanol-chloroform (7:93); layer thickness 0.25 mm on 8 × 8 in. plate. Test mixtures of compounds: 1, extract from 100 mg aflatoxin free peanut butter prepared according to reference 3; 2, extract as in spot 1 and superimposed with a mixture of four standard aflatoxins, 5 ng each of B_1 and G_1 and 1.3 ng each of B_2 and G_2; 3, mixture of the standard aflatoxins as in 2. The individual spots in order of decreasing R_f are B_1, B_2, G_1 and G_2. The fluorescent spot appearing at an R_f similar to that of G_2 in pattern 1 is an interferenee present in some peanut products and is not G_2.

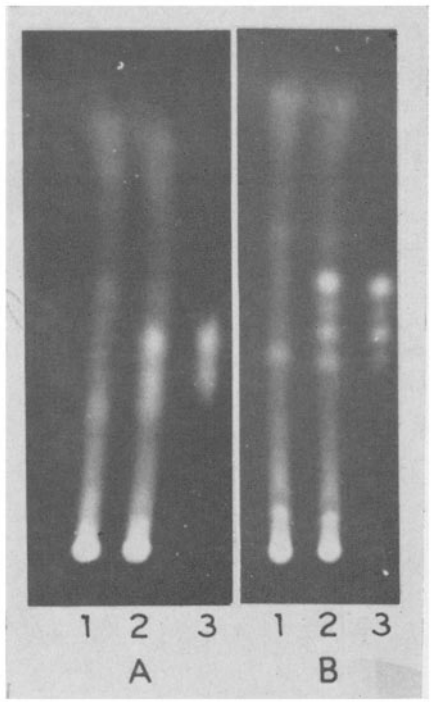

FIG. 3. Chromatogram quality and resolution of aflatoxins on silica gel chromatoplates prepared with and without $CaSO_4$ binder. Developing solvent, methanol-chloroform (7:93). A, SilicAR TLC-7 (no binder); B, SilicAR TLC-7 with 10% precipitated $CaSO_4$. For description of spots and other parameters, see Figure 2.

left half of a plate was coated with silica gel MN G-HR from one container and the right half was coated with the same brand from another container. It was not known if the materials in the two containers were from the same or different lots as no lot numbers were given by the manufacturer. Whether the plates were developed with methanol-chloroform (7:93) or acetone-chloroform (10:90), one half of a plate always had better resolution and less tailing than the other half.

The $CaSO_4$ binder is usually considered an inert ingredient and its concentration is not always stated on the label. However, both the concentration and

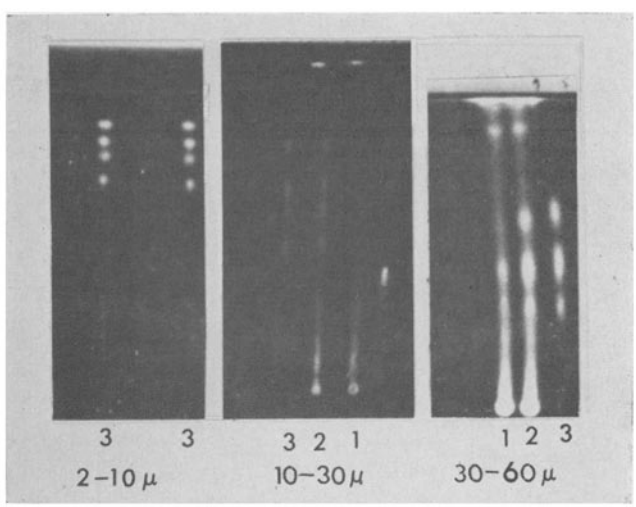

FIG. 4. Comparison of resolution of aflatoxins on chromatoplates prepared from Bio Sil A silica gel (5% $CaSO_4$ binder) of particle sizes indicated. For description of spots and other parameters, see Figure 2. Particle size according to label statement and binder as given in manufacturer's literature.

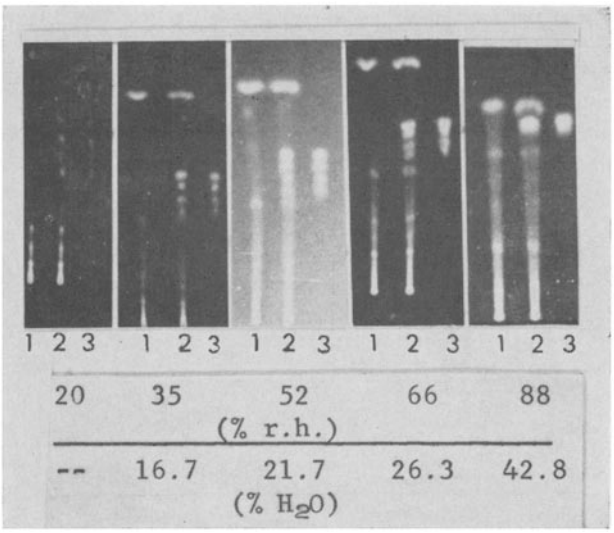

FIG. 5. Chromatogram quality and resolution of aflatoxins on Bio Sil A, 2–10 μ (5% $CaSO_4$ binder) equilibrated at the indicated relative humidities. The per cent H_2O shown is the weight loss at 105 C of the silica gel on the equilibrated plate as per cent of the dry silica gel weight. Developing solvent, methanol-chloroform (7:93). For description of spots and other parameters, see Figure 2. Particle size according to label statement and binder as given in manufacturer's literature.

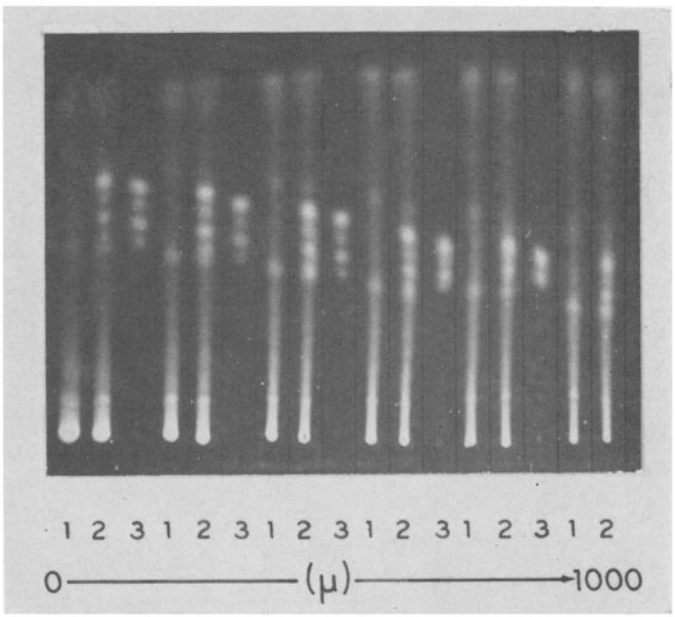

FIG. 6. The effect of variable thickness of silica gel MN G-HR layer. Thickness continuously decreases from 1000 μ on the left side to near 0 μ on the right side of the chromatogram. Developing solvent, methanol-chloroform (7:93). Test mixtures as in Figure 2. Alternate spot patterns consist of peanut butter extract plus standard aflatoxins followed by the standard aflatoxins reading from left to right.

quality of the $CaSO_4$ proved to be important to the chromatography when methanol-chloroform (7:93) was the developer. A TLC plate was coated with a gradient concentration from 0% to 100% $CaSO_4$ (a precipitated $CaSO_4$ for TLC, Mallinckrodt Chem. Works) in SilicAR TLC-7 across the plate from left to right. The gradient coating was spread with the Desaga-Brinkmann Model GM TLC applicator with gradient mixing mechanism. Excess silica gel resulted in fuzzy spots; excess $CaSO_4$ resulted in higher R_f values and impaired resolution. Results were improved, as shown in Figure 3, by adding 10% (by weight) precipitated $CaSO_4$ to SilicAR TLC-7. Precipitated $CaSO_4$ was superior to Plaster of Paris (native $CaSO_4$) when the two were compared at 10% concentrations in SilicAR TLC-7.

Commercial silica gels differ in range and distribution of particle sizes. Figure 4 shows the results obtained from the commercially available Bio Sil A silica gels with particle size ranges of 2–10, 10–30 and 30–60 μ. The silica gel of the 2–10 μ range gave excellent resolution, whereas the others did not. Truter (5) reported that resolution will deteriorate if the average particle size exceeds about 5 μ, although particle sizes up to 70 μ can be used. The incomplete resolution with the two largest size ranges (Fig. 4) could also be attributed to lot variation since the three materials were not from the same batch of silica gel. Similarly, the lot-to-lot variation observed for silica gels could be due to undisclosed differences in particle size.

To overcome the difficulties of variable chromatographic properties of the adsorbent preparations described above, practical silica gel specifications related to chromatographic properties are greatly needed. Stahl has suggested a number of parameters and tests as a basis for standards (6).

The temperature at which the chromatoplates were dried was not critical from 24 to 150 C but moisture proved to be an important variable. Dry silica gel plates adsorb moisture at a rapid rate. Dallas (7) pointed out that half the total amount of moisture adsorbed by silica gel at equilibrium in an atmosphere of 50% relative humidity is taken up within 3 min and that even breathing on a plate during spotting can markedly affect the R_f values. To determine the effect of moisture, TLC plates were equilibrated at various levels of relative humidity (RH) and were tested with two solvent systems. With Bio Sil A silica gel (5% $CaSO_4$ binder) and methanol-chloroform (7:93) as the developing solvent, the spots were most widely separated and most compact on the plates exposed to 35% RH or less (Fig. 5). The equilibrium moisture content of the silica gel at 35% RH was 16.7% of the dry silica gel. An interfering fluorescent spot from the peanut butter extract was most completely separated from aflatoxin G_2 on plates exposed to 52% RH or higher. Plates exposed to 66% RH or higher did not resolve the aflatoxins. Results were similar with MN G-HR silica gel and with acetone-chloroform (10:90) as the developing solvent.

Resolution of the aflatoxins was also dependent on plate thickness with methanol-chloroform as the developer. The chromatoplate shown in Figure 6 was spread to give a wedge layer increasing in thickness from left to right. In the same direction resolution and R_f values decreased. Other experiments showed that resolution becomes unsatisfactory on layers which are spread thicker than 0.5 mm with methanol-chloroform (7:93) as the developing solvent. With acetone-chloroform (10:90) as the developer, resolution was not affected significantly by thickness over the range 0.25–1.0 mm although the R_f decreased with increasing thickness. The adsorbent layer thickness usually reported in the literature is that of the wet slurry, not that of the dry layer. To determine the dry layer thickness as a function of variation in the technique with the Desaga-Brinkmann applicator, plates were prepared in several ways. The thickness of the layer at 16 uniformly distributed points on each plate was measured by determining the amount of micrometer screw adjustment needed to change the

TABLE II
Properties of 8 × 8 in. TLC Plates in Relation to Spreading Procedure Variables[a]

Description of variable	Thickness (μ)[b]	High-low difference[c] (μ)	Density[d] (g/cm^3)
Plate spread in 3.3 sec	177	12	0.403
Plate spread in 0.2 sec	151	59	0.385
30 g Silica gel with 80 ml water	148	46	0.354
Control	173	49	.....
Control	176	41	0.414

[a] Unless otherwise shown, slurry was prepared from 30 g MN G-HR silica gel and 60 ml water. Spreader gate setting was constant at 0.25 mm. Control plates were spread at intermediate speed between 0.2 and 3.3 sec.
[b] Average thickness measured with a microscope at 16 uniformly distributed points.
[c] The difference between the highest and lowest value of the 16 measurements.
[d] Based on weight of silica gel after drying at 105 C.

focus of a microscope from the top to the bottom of the layer.

The slurry was made thinner than the normal consistency by increasing its water concentration from the standard amount of 60 ml to 80 ml/30 g of silica gel, and the plates were spread quickly (0.2 sec/plate) or slowly (3.3 sec/plate). All plates were prepared with a gate setting of 0.25 mm. Two plates spread with a slurry of normal consistency at an intermediate speed between 0.2 and 3.3 sec/plate had dry layers 173 and 176 μ thick; a thin slurry produced a dry layer 148 μ thick. The normal slurry, when spread slowly, produced a dry layer 177 μ thick and when spread quickly, a layer 151 μ thick. The effect of these variables on layer thickness, uniformity and density is presented in Table II.

During early work with the aflatoxins, methanol-chloroform was the most frequently used developing solvent, and various ratios were recommended for development and for equilibration of the development chamber (3,8–10). The presence of methanol in the development chamber atmosphere was considered essential for resolution. Therefore, the following variables which might affect the concentration of methanol in the chamber atmosphere and on the silica gel layer were investigated: methanol concentration in the chloroform used to wet the chamber liner; the area of solvent trough surface; the position of the plate in relation to the liner; the loss of chamber vapor when the equilibrated chamber is opened to insert the spotted plate; and equilibration time. Methanol was also sprayed directly on the silica gel layer. A methanol concentration greater than 7% in the chloroform used to equilibrate the development chamber gave poor resolution. No correlation could be found between resolution and any of these other variables examined.

Water absorbed by the developing solvent from exposure to the atmosphere can affect the chromatography. Addition of as little as 0.035% water to the methanol-chloroform (7:93) developing solvent almost doubled the R_f of aflatoxin B_1 and the four aflatoxins were separated into only two spots. However, water added to the acetone-chloroform (10:90) developing solvent at concentrations up to saturation (0.3%) had no deleterious effect on resolution.

During this work we experienced intermittent problems such as streaking of plates, fading of the aflatoxins or poor resolution, for no apparent reason. These troubles disappeared as mysteriously as they appeared. The only plausible explanation we can offer is that the problems were caused by accidental plate contamination, for example, from alkaline residues on the glass as a result of inadequate rinsing after washing or from adsorption of contaminants from the laboratory atmosphere by the silica gel layer.

Our investigations indicate that the following variables have relatively little or no effect on chromatogram quality with methanol-chloroform (7:93) as the developer.

Variables in Plate Preparation

Preheating the silica gel as high as 170 C before plate preparation; the amount of water used to make the slurry; the speed of spreading the slurry; the type of spreader; aging of plates before drying or agitation of wet plates. Although these factors did not significantly affect resolution, some did affect the layer thickness and uniformity. The plates that were spread slowly were the most uniform, even more so than commercially available machine-coated plates.

Variables in Spotting Technique

The presence of 0.7% or 2% ethanol in the chloroform spotting solution; evaporation aids used during spotting; spotting under nitrogen or spotting the sample quickly versus slowly.

Variation in Developing Chambers

Chambers varying in shape and in volume from 0.4 to 20 liters.

Development Variables

Ethanol preservative concentration from 0% to 2% in the chloroform used for the developing solvent, development distance from 10 to 15 cm, reusing the 50 ml portion of developing solution to develop as many as eight consecutive plates, resulting in satisfactory resolution but lower R_f values.

The variables described in this report have been studied in conjunction with one or more of the following systems of developing solvents: acetone-chloroform (10:90), benzene-ethanol-water (46:35:19) and methanol-chloroform (7:93), all described in the AOAC methods for aflatoxin in peanut products (3,4). An attempt was also made to find better solvent systems. Of all those examined, the three that were most satisfactory were: acetone-chloroform (10:90) (4), the upper phase of benzene-ethanol-water (46:35:19) (3) and benzene-methanol-acetic acid (90:10:10). Other solvents which were not as effective in resolving the aflatoxins but which may be useful to separate the aflatoxins from particular interferences are: benzene-acetone-acetic acid (80:10:10), acetic acid-benzene (10:90), acetic acid-chloroform (3:97) and chloroform-acetone-ethyl ether (5:1:4).

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