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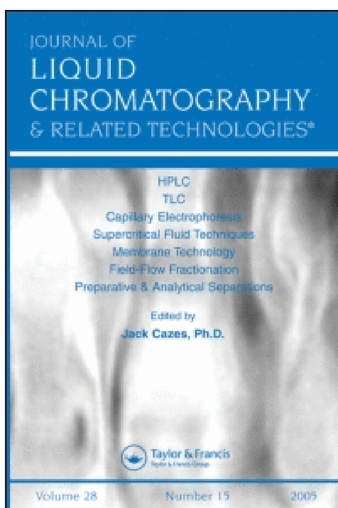

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A GUIDE TO THIN-LAYER CHROMATOGRAPHIC SYSTEMS FOR THE SEPARATION OF AFLATOXIN B_1, B_2, G_1 and G_2

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ABSTRACT

The separation of aflatoxin B_1, B_2, G_1 and G_2 was compared on six commercial silica gel plates in twelve solvent systems. Two of the solvent systems, chloroform:acetone:ammonium hydroxide (90:10:0.25) and chloroform: acetone:hexane (85:15:20) resolved the four aflatoxins on all the tested plates. The solvent modifier played an important role in the resolution of these compounds. The effect of the hardness of the plate is also discussed.

INTRODUCTION

Aflatoxins are well known toxic compounds which have been shown to be carcinogenic in a number of animal species (1). The principal technique for the separation of aflatoxin B_1, B_2, G_1 and G_2 is thin-layer chromatography (TLC), and many chromatographic system have been described for the separation of the different forms in which this carcinogen occurs. Manufacturers often recommend specific solvent system be used with their plates. For example Applied Science (2) recommend chloroform:acetone:water (88:12:1.5) with their Adsorbsil

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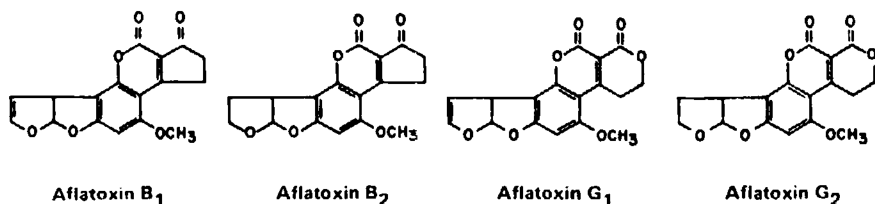
plate, and Whatman, Inc. (3) chloroform:tetrahydrofuran (90:10) with their K5F silica gel plate. The literature is also full of different systems which include, chloroform, acetone, water, acetic acid, hexane, benzene, tetrahydrofuran...etc. in varying proportions. We undertook this study to help the analyst find the best solvent system for the separation of the four aflatoxins (B_1, B_2, G_1 and G_2) using pre-coated, commercial silica gel plates.



EXPERIMENTAL

TLC plates were purchased from different manufacturers, Table I, and used directly from their containers without preconditioning, unless indicated otherwise. All solvents were distilled in glass (Burdick & Jackson, Muskegon, MI). Acetic acid and ammonium hydroxide solutions were obtained from J.T. Baker. Aflatoxin B_1, B_2, G_1 and G_2 were purchased from Applied Science Laboratories and used without further purification. The individual aflatoxins were dissolved in benzene:acetonitrile (98:2). The plates, after spotting development and drying, were observed under an excitation wavelength of 366 nm in a viewing box (Brinkman, Westburry, NY). Quantitative fluorescent measurements were made with a Perkin-Elmer model MPF-3 Fluorescence Spectrophotometer equipped with a special TLC scanning attachment. All TLC work was performed under soft fluorescent light (General Electric F40GO) which is low in voltage output below 500 nm. The solvent systems compared were selected from the literature (2-12) as the ones most often used for aflatoxin separations, and are listed in Table II.

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Method: The 5 X 10 cm plates were quantitatively spotted using a micropipet, and developed in a standard developing tank. Plates were developed for 7 cm, and then left to dry in a hood. Quantitative fluorescence measurements were made at an excitation wavelength of 366 nm and emmission wavelength of 425 nm.

Caution: Aflatoxins are carcinogens, and should be handled carefully in a chemical fume hood with appropriate precautions (13).

RESULTS & DISCUSSION

One objective of this study was to find a solvent systems that will separate aflatoxin B_1, B_2, G_1 and G_2 on any commercially available pre-coated silica gel plates. The plates most commonly used, and which were selected for this study, are listed in Table I. The solvent systems frequently mentioned in the literature for the separation of the aflatoxins are listed in Table II. Another objective was to see whether aflatoxins could be separated on pre-coated plates without preactivation before development in an unequilibrated tank. Stoloff et al (5) suggests that the plate be stored in a dessicator and be developed to a height of 16 cm in an equilibrated tank. Pons (14) spotted the aflatoxins on a warm plate which was then developed in an equilibrated tank for 1 hour. Beljaars (15) recommends the activation of the plate before

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TABLE 1
SILICA GEL PLATES USED

PLATE	NAME	LAYER THICKNESS	MANUFACTURER
1	Silica Gel 60	0.25 mm	E. M. Laboratories Inc.
2	K5F	0.25 mm	Whatman Inc.
3	HPTLC	0.20 mm	E. M. Laboratories Inc.
4	Sil G 25 HR	0.25 mm	Brinkmann Instruments, Inc.
5	Adsorbsil I	0.25 mm	Applied Science Laboratories, Inc.
6	Silica Gel IBF	0.20 mm	J. T. Baker Chemical Co.

TABLE II
SOLVENT SYSTEMS EXAMINED

	SOLVENT	RATIO
A	Chloroform:Acetone:Water	88:12:1.5
B	Chloroform:Tetrahydrafuran	90:10
C	Chloroform:Acetone	90:10
D	Benzene:Methanol:Acetic Acid	90:5:5
E	Chloroform:Acetic Acid:Ether	17:1:3
F	Chloroform:Acetone:Ammonium Hydroxide	90:10:0.25
G	Chloroform:Acetone:Hexane	85:15:20
H	Chloroform:Methanol	4:1
I	Tolune:Ethyl Acetate:Formic Acid	6:3:1
J	Tolune:Ethyl Acetate:Chloroform:Formic Acid	70:50:50:20
K	Benzene:Ethanol	95:5
L	Chloroform:Methyl Isobutyl Ketone	4:1

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and after spotting, removal of the edges to prevent edge effects, development in the dark, and preparation of the solvent mixture just before use.

To save on material and time, we used 5 X 10 cm plates. Although we found the use of 5 X 20 cm plates gave better resolution, the separations on 5 X 10 cm plates (7 cm development) were adequate for this study. This was especially true when HPTLC plates were used. Since commercial pre-coated plates are activated before shipment, we did not find it necessary to activate the plates again.

Our results indicate that preactivation does not improve separation. On the contrary, separations were better and plates required less time to develop without preactivation. Table III shows the separations achieved on six different plates developed in the each of 12 solvent systems listed in Table II.

Table III reveals much which merits discussion. Although on plate 6 (Silica Gel IBF, plastic backing) the four aflatoxins were not resolved in

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TABLE III

COMPARATIVE AFLATOXINS B_1, B_2, G_1 and G_2 SEPARATION ON SILICA GEL PLATES USING DIFFERENT SOLVENT SYSTEMS AND PLATE PREPARATIONS.

$R_F \times 100$

PLATE	1				2				3				4				5				6			
SOLVENT	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
A	51	45	40	35	84	82	77	72[b]	70	63	59	52[a]	80	76	72	68[b]	87	80	74	65	63	63	52	52
B	47	44	38	33	76	72	61	57[b]	44	37	33	27[a]	93	84	80	74[a]	80	61	53	42	48	48	33	33
C	44	37	34	29[b]	74	71	67	63	52	46	42	38[a]	74	68	65	59[b]	78	68	65	52[b]	42	37	37	31
D	26	23	20	19	68	65	55	52	29	27	21	20[b]	57	52	48	43[a]	50	44	38	33[a]	27	27	20	20
E	52	47	40	35[b]	74	74	57	57	55	49	39	34	70	65	59	55	96	79	79	69[c]	99	99	88	88
F	33	29	23	20	87	84	76	71	48	44	37	33[a]	87	82	72	65	44	37	31	25	63	57	52	46[b]
G	48	43	36	30	59	54	46	43[b]	48	41	36	30	54	46	41	36	80	71	63	54	68	61	52	45
H	99	99	99	99	98	98	98	98	98	98	98	98	93	93	89	89	99	99	99	99	96	96	96	96
I	35	28	25	15[a]	52	44	39	31	35	28	25	15	50	40	40	29	55	52	52	46[c]	99	76	76	61
J	38	32	28	21[a]	52	46	40	33	38	33	28	22	50	43	38	31[b]	60	55	53	49[c]	99	89	89	76
K	25	23	23	20	50	46	46	44[b]	25	23	23	20[b]	46	42	42	38	36	33	31	27[c]	95	95	82	82
L	29	20	20	14	44	37	37	29[b]	44	34	32	24[b]	42	34	34	27	44	31	31	21	99	95	45	76

a - compact spots

b - diffuse spots

c - bands

10 of the 12 tested solvent systems, they were resolved by solvents F and G. The solvent systems recommended by Applied Science (Solvent A), and by Whatman, Inc. (Solvent B), for their plates, did not give the best separations. Table III also shows that when the Applied Science Laboratories Systems was used (plate 5 with solvent A) the R_fX100 values were 87, 80, 74, 65. However, when the same plate was developed in solvent B, better separations were obtained (80, 61, 53, 42). The same was true of the Whatman, Inc. System (plate 2 with solvent B). R_fX100 values were 57, 61, 72, 76, while when the same plate was developed in solvent I or J, the results were 52, 44, 39, 31, and 52, 46, 40, 33 respectively, which gives better resolution of B_1 from B_2, and G_1 from G_2. It was also observed that all six plates would resolve the four aflatoxins if the right solvent system was used (solvents F and G). When the wrong solvent system was used (solvent H), none of the aflatoxins was resolved. Solvents K & L performed a little better than Solvent H but not much, under our experimental conditions. This shows the importance of the role played by the mobile phase in thin layer chromatography. The Adsorbsil plate gave better separation than any other plate in 6 of the 12 solvent systems, namely (A, B, C, D, G and K), while the HPTLC plate gave better separations than others with solvents E and L. The Brinkman plate gave better separations with solvents F and J, and Whatman, Inc., K5F plate with solvents I and J.

Looking at the plates individually, the solvent system giving the best separation is listed in Table IV.

Solvent systems F and G were found to give good separations with all the plates we tested. As a matter of fact, solvents F and G were the only solvents to resolve the four aflatoxin on plate 6.

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TABLE IV

Plate#	1	2	3	4	5	6
Solvent	G	I or G	E	F	B	G

In this study, 4 of the 12 solvent systems were a combination of chloroform and acetone, with or without a modifier. When solvent C (chloroform: acetone 90:10) was used, the resolution of aflatoxin B_2 from G_1 was poor. However, the addition of a drop of ammonium hydroxide (solvent F) improved the overall resolution and the aflatoxins were separated on plate 6. When water was added to solvent A, a slight improvement over solvent C, was observed. With solvent G, the addition of hexane and an alteration in the ratio of chloroform and acetone noticeably improved the resolution on all the tested plates. When the acetone in solvent C was replaced by tetrahydrofuran, (solvent B) a better overall resolution was observed (see Table III).

These results indicate that the modifier in the solvent system can play a major role in achieving separations in thin layer chromatography.

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Effect of ammonia in solvent F on aflatoxin separation and decomposition. Solvent F, chloroform:acetone:ammonium hydroxide (90:10:0.25) gave good separation of the four aflatoxins on all the plates tested in this study (Table III). We were apprehensive about using ammonium hydroxide because this compound has been recommended as a detoxification agent for aflatoxins (7). However, we found that the level we used did not appear to lead to any loss of the aflatoxins. Using fluorescence scanning of two plates spotted with the four aflatoxins and developed in solvents G and F, we found that the areas under the peaks (Figure 1) were the same. However, when the level of ammonium hydroxide was increase to 1% we did find evidence of destruction of the starting material. We recommend that, when ammonium hydroxide is incorporated into the solvent system, the level be no more than 0.5%.

The Effect of the Softness or Hardness of the Coating on Separation. Of the six plates, 4 and 5, have soft layers, while the other four have hard coats. Softness or hardness is determined by the binder used and the way the plate is made. In plates 1, 2, 3 and 6 the binder is a polymer, sodium polyacrylamide. Gypsum, which is used as a binder in plate 4, does not give as hard a coating,

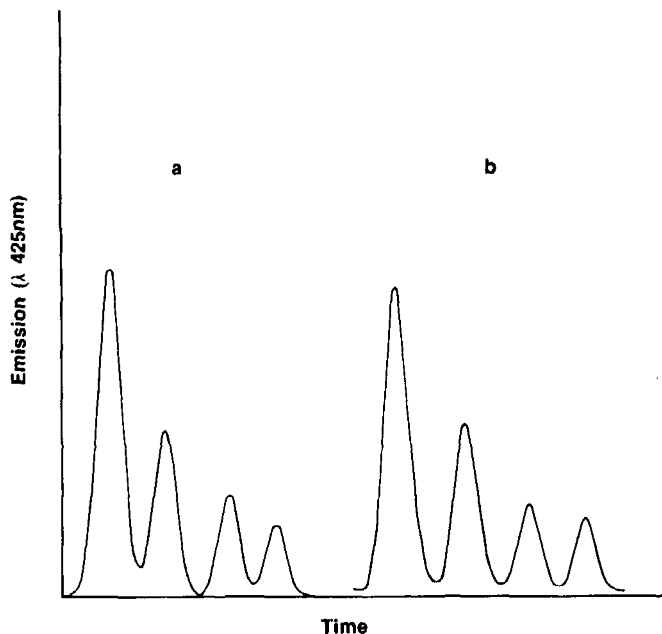

Figure 1. A trace of the fluorescent scan of the four aflatoxins after development in (a) solvent G, and (b) solvent F, at an excitation and emission wavelengths of 366 nm and 425 nm, respectively.

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as the polymer. Plate 5 does have a gypsum binder that gives a very loose coat, which is difficult to handle, without damage. The softest coating, the Adsorbisil plate (plate 1), gave the best separation in 6 of the 12 solvent systems.

Plates 3 and 6 have the same binder and the same coating thickness but a different type of plate-backing glass - plastic poly(ethylene terphthalate), and the separations are very different (see Table III). This may be due to the particle size or the backing. We believe that the smaller particle size gives a more effective separation. When plates 1 and 2, were compared the results were comparable except in solvent E, with plate 1 giving slightly better resolution.

Another aspect that should be taken into consideration is the size of the spot after development. The HPTLC plate gave a more compact spot than the other plates. This means that resolution, detection limits and sensitivity are better than with the other plates. They also develop faster. Although the separation of the aflatoxins on the Adsorbsil - 1 plate were better than with the other plates (Table III) the resulting spots were diffused, which means less molecules/unit area. These diffused spots may arise from the size and uniformity of the silica gel particles and softness of the coating.

CONCLUSION

The objective of this study was to find out if it is possible to separate aflatoxins B_1, B_2, G_1 and G_2 on six precoated, commercial silica gel TLC plates, without preactivation. Two solvent systems, chloroform:acetone: ammonium hydroxide (90:10:0.25), and chloroform:acetone:hexane (85:15:20) resolved the four aflatoxins on all the silica gel plates tested.

It was also shown that (a) the solvent modifier plays an important role in achieving a good resolution of these compounds on silica gel TLC plates, and that (b) when the right solvent system is selected separation can be achieved. This shows the importance of the role played by the mobile phase in liquid chromatography.

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