Journal of Chromatography, 108 (1975) 131–140
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 8117

IMPROVEMENTS IN THE THIN-LAYER CHROMATOGRAPHY OF NATURAL PRODUCTS

I. THIN-LAYER CHROMATOGRAPHY OF THE AFLATOXINS

J. G. HEATHCOTE and J. R. HIBBERT
University of Salford, Salford (Great Britain)
(First received September 6th, 1974; revised manuscript received November 19th, 1974)

SUMMARY

During the period which has elapsed since the aflatoxins were first isolated, one of the main problems has been the separation of the individual aflatoxins in pure form from aflatoxin-containing extracts. This separation has been best effected by thin-layer chromatography, and in this paper we describe how some of the difficulties may be overcome by using an appropriate combination of solvent system and silica gel preparation. For the examination of aflatoxin-containing extracts from the mycelia of *Aspergillus flavus* moulds, an initial freeze-drying step has been found to improve appreciably the quality of the chromatograms obtained.

LYOPHILIZATION IN THE PREPARATION OF AFLATOXIN-CONTAINING MYCELIAL EXTRACTS FOR TLC

During the investigation of metabolites from the mycelium of *Aspergillus flavus*, one of the difficulties encountered was the occurrence of a considerable amount of a dark pigment which remained immobile on thin-layer chromatography (TLC) plates. In most cases, this interfered seriously with the chromatography by upsetting the even flow of the developing solvent, causing streaking and tearing of the bands (Fig. 1). As trial experiments had indicated that this dark pigment was a decomposition product, formed during air-drying of the mycelium at 60–65°, it was decided to investigate freeze-drying as an alternative method for drying the mycelium, which might avoid degradation of the metabolites and thus improve the TLC separation.

Method

The mycelium of *A. flavus* 91019b obtained from culture experiments was washed well with warm water to remove traces of medium, cut up into small pieces and placed in a 150-ml beaker. Liquid nitrogen was slowly poured into and around the beaker which was placed in a wide Dewar flask until the mycelium was frozen. The beaker was then placed inside a special, wide-necked flask which was attached to the freeze-drying apparatus, and the contents were dried over a period of 30 h.

The dried mycelium was extracted in darkness with chloroform–methanol (3:1) for 24 h in a Soxhlet apparatus which was modified for running under reduced pressure in order to prevent the temperature rising above 35°. This extract, which contained both aflatoxins and pigments, was evaporated to dryness *in vacuo*, prior to application to a TLC plate for development in a suitable solvent. Such plates were compared with TLC plates of extracts prepared similarly from air-dried mycelium.

Results

From Fig. 1 it will be seen that there is a vast improvement in the quality of the pattern obtained on TLC when the freeze-dried mycelial extract is substituted for the air-dried extract. The large, blue-fluorescing bands of high R_F value which are normally found towards the top of plates developed in chloroform–methanol (49:1), and which arise from aerial oxidation of lipids, have disappeared. The absence of

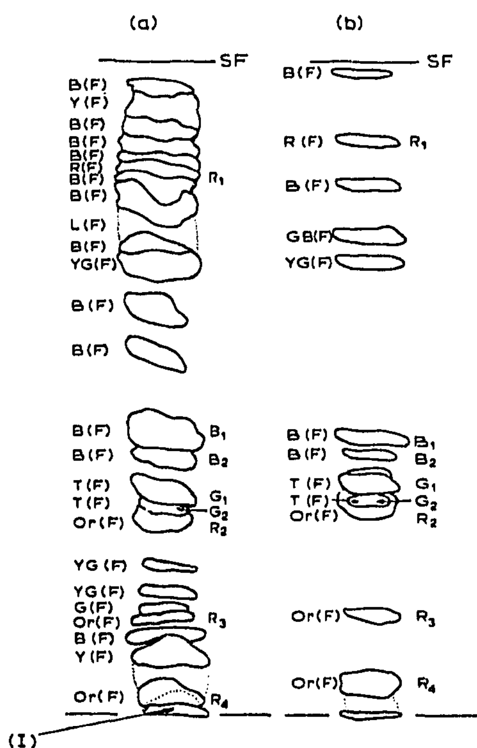

Fig. 1. Thin-layer chromatographs of extracts of (a) air-dried and (b) freeze-dried mycelia. Layer, SilicAR TLC-7G. Solvent system, chloroform–methanol (49:1). Spot (I) represents dark brown residue streaking up into fluorescent bands. Abbreviations: SF = solvent front; (F) = fluorescence in ultraviolet light; B = blue; G = green; L = lilac; Or = orange; Or.R = orange red; PB = pale blue; R = red; T = turquoise; Y = yellow; YB = yellowish blue; YG = yellowish green; R_1, R_2, etc. = pigment fractions; R_{1a} = O-methylsterigmatocystin; R_{1b} = sterigmatocystin; R_{2a} = dihydroaverufin; R_{2b} = versicolorin C; R_{2c} = versicolorin-type pigment; R_{2d} = versicolorin B; Asp = aspertoxin. Unidentified metabolites: BB_1, BB_7, XB_7, XB_8, XB_9 and XM_{2a}. Aflatoxins: B_1, B_2, G_1, G_2, B_3, M_1, GM_1, GM_{2a}, M_{2a}, B_{2a} and G_{2a}. M and GM series of aflatoxins: M and GM represent $M_1 + M_2$ and $GM_1 + GM_2$, respectively.

these oxidation products reduces the smearing which otherwise would occur on the top half of the plates. The technique demonstrates also that some of the additional blue, fluorescent bands of high R_F value, observed in the culture extracts were, in fact, decomposition products produced during the extraction. The heavy pigment band which was found normally on the baseline of extracts from heat-dried mycelium was also absent in extracts of freeze-dried mycelium and this resulted in less tailing and interference at the bottom of the plate.

The use of freeze-drying was thus found to improve the purity of the metabolites obtained from a given weight of mycelium.

EFFECT OF GRADE OF SILICA GEL ON TLC RESOLUTION OF AFLATOXIN-CONTAINING EXTRACTS

In the purification of aflatoxin-containing extracts, the extract is most conveniently resolved into its component fractions by TLC on silica gel layers. In order to achieve good TLC resolution many solvent systems and adsorbents have been experimented with. Most of the early solvent systems reported for the TLC separation of aflatoxins were based on chloroform with 2–7% methanol added according to specific requirement (De Iongh *et al.*[1], Chang *et al.*[2], Andrellos and Reid[3], and Stoloff *et al.*[4]). These systems, however, were found to be sensitive to environmental changes and many laboratories carried out tests to try to find a solvent system which was less susceptible to such changes (Stubblefield *et al.*[5], Velasco[6], and Nesheim[7]).

In our laboratory, reproducibility of resolution was markedly affected by variations in humidity and temperature, and was a serious problem when analysing mixtures of metabolites containing aflatoxins. Nesheim[7] had previously indicated that the grade of silica gel used affects the quality of the chromatogram obtained and the suitability of the solvent system, finer grades of silica giving a higher resolution. We decided, therefore, to carry out a series of tests in an attempt to determine the combination of solvent system and adsorbent needed to overcome these difficulties[8].

In order to check whether the rather indifferent results obtained when using a solvent system recommended by other workers was due to the grade of silica gel (Kieselgel G, Merck) currently being used in the laboratory, we tested four other grades of silica, which have been recommended for the separation of aflatoxins. Kieselgel G (Merck) was included in these tests as a control. Three solvent systems were used in all, two from a number recommended by other workers and one (toluene–ethyl acetate, 8:1) developed in this laboratory (see Table I).

Method

A set of individual TLC plates, 20 × 20 cm, was coated with 0.3 mm of one of the five commercial silica gel preparations selected. The plates were dried in air for 4 h and then activated at 130° for 4 h. Five microlitres of a standard solution of each of the known aflatoxins and of a mixture of the aflatoxins (a culture fluid extract) were applied as bands, 1 cm in width, along a line 3 cm from the bottom of each plate. The chromatograms were then developed in one of the solvent systems described in Table I. After development, the plates were viewed under ultraviolet light and the fluorescent bands were marked out on the plate with a scribe. AnalaR solvents were used throughout and the plates were prepared under standard conditions to minimize

TABLE I

EFFECTIVENESS OF SILICA GEL PREPARATIONS IN TLC OF AFLATOXINS AND PIGMENTS

The quality of the chromatogram was scored for excellence, ranging from 1 (poor) to 5 (excellent), depending upon the resolution of the fluorescent bands.

Silica gel	*Score*		
	Methanol–chloroform (3:97)	*Toluene–ethyl acetate (8:1)*	*Benzene–ethanol–water (46:35:19)*
Kieselgel G*	2	3	1
SilicAR TLC-7G**	5	5	5
SilicAR TLC-4G**	5	5	5
SilicAR TLC-4GF**	4	5	4
Bio-Sil A***	4	5	5

* Merck, Darmstadt, G.F.R.
** Mallinckrodt, St. Louis, Mo., U.S.A.
*** Bio-Rad Labs., Richmond, Calif., U.S.A.

possible sources of error due to variation in the materials. When possible, all the silica gel used was from the same batch as it was noticed during the course of routine work that the quality of the plates varied from batch to batch, an observation frequently reported by other workers. Similar procedures were carried out with each grade of silica gel in combination with each type of solvent system. During the course of our tests, particular attention was given to recording any variation in temperature or humidity, and its effect on the resolution and quality of the chromatogram. The quality of the plates was estimated on a scale ranging from 1 (poor) to 5 (excellent).

Results

The results show that the grade of silica, Kieselgel G (Merck), used until the start of the investigation (1969), was inferior to any of the other grades tested. The results were also in general agreement with the observations of Truter[9] and Nesheim[7], who reported that when the particle size exceeds 7 μm the resolution of the TLC plates deteriorates. This would account for the excellent resolution obtained using Bio-Sil A, which has a particle range of 2–10 μm. The rather indifferent separation obtained under identical circumstances, using Kieselgel G of particle range 5–40 μm, is noteworthy by comparison. However, during this experiment it was found that the Bio-Sil A layer was unstable, tending to flake off the bottom of the plate when dipped in certain solvents. This flaking appeared to be related to the amount of binder (calcium sulphate) in the silica, because the SilicAR TLC preparations, with 15% binder, against only 5% in the Bio-Sil A, did not flake from the plate.

The difference between the three SilicAR TLC preparations was minimal but, as the acid pH of SilicAR TLC-4G and SilicAR TLC-4GF could cause the formation of catalysed hydration derivatives, it was thought unwise to use these grades. SilicAR TLC-7G proved to be the most reliable and, consequently, this preparation was used in future work.

During the course of this investigation, we also tested the effect on the quality of resolution of aflatoxins when a mixture of different grades of Mallinckrodt SilicAR

gels was used as recommended by Teng and Hanzas[10], but found that the change in TLC resolution was not significant. We also found that the fluorescence under ultraviolet light masked many of the minor fluorescing bands, particularly when examining crude extracts containing the G series of aflatoxins. It was also observed that the use of SilicAR TLC-4G or TLC-4GF in conjunction with solvent systems containing alcohol resulted in the appearance of traces of O-alkyl derivatives[11] in the aflatoxins recovered from the silica gel plates.

Comparisons with ready-made, commercial plates were not easy to carry out, partly because of the difficulty of obtaining consistency in these plates at the time and, more seriously, because of attack by some of the organic solvents on the supporting base.

With all the silica gel preparations tested it was found that optimum resolution of the aflatoxins was achieved when the plates were exposed to 40% relative humidity, or less. Plates exposed to 65% RH, or more, did not resolve clearly any of the aflatoxins. When TLC had to be carried out under conditions of high humidity, the plates were stored *in vacuo* and equilibrated in an atmosphere of the solvent before being used, in order to reduce the deleterious effect.

SOLVENT SYSTEMS FOR THE TLC OF AFLATOXINS AND RELATED PIGMENTS

The above series of tests showed that the most suitable adsorbent for TLC was Mallinckrodt SilicAR TLC-7G in combination with the solvent systems used. We attempted next to find out whether other solvent systems, in combination with this particular grade of silica gel, would result in a more efficient TLC system for the resolution of the aflatoxins. Twenty-four solvent systems in all were tested, including a number already published by other workers. The quality of the chromatograms, as judged by resolution of the metabolites, was scored for excellence from 1 (poor) to 5 (excellent), covering certain different categories of metabolite. During the experi-

TABLE II

SOLVENTS USED IN THE TLC SEPARATION OF MOULD METABOLITES

Range of score from 1 (poor) to 5 (excellent).

Solvent system	*Quality of chromatogram*			
	Pigment R_1 group	*Pigment R_2 group*	*Aflatoxins B_{2a} group*	*All metabolites*
Benzene–ethanol–water (46:35:19)	1	1	1	2
Chloroform–methanol (49:1)	3	1	2	5
Chloroform–methanol (97:3)	1	1	3	3
Chloroform–methanol (94:6)	—	—	4	1
Cyclohexane–chloroform (1:1)	5	2	—	1
Toluene–ethyl acetate–acetone–acetic acid (50:35:15:2)	1	1	4	4
Toluene–ethyl acetate (18:1)	2	3	—	1
Toluene–ethyl acetate (8:1)	2	5	—	1
(1) Chloroform (2) Chloroform–cyclohexane (1:1) Develop in (1), then redevelop in (2)	4	1	—	1

ments particular attention was given to recording the effect of any variation in temperature and/or humidity on the quality of the chromatogram. Solvent systems less susceptible to such variations received a higher score.

Method

A set of individual TLC plates (20 × 20 cm), coated with 0.3 mm of SilicAR TLC-7G, was prepared, using a Shandon "Unoplan" spreader. Special care was taken to see that all the SilicAR TLC-7G was from the same batch, so that consistency in quality and particle size of the adsorbent would be common to all tests. The plates were air-dried for 4 h and then activated at 130° for 4 h before storing in a desiccator until required.

The plates were spotted with known aflatoxins and pigments in a line 3 cm

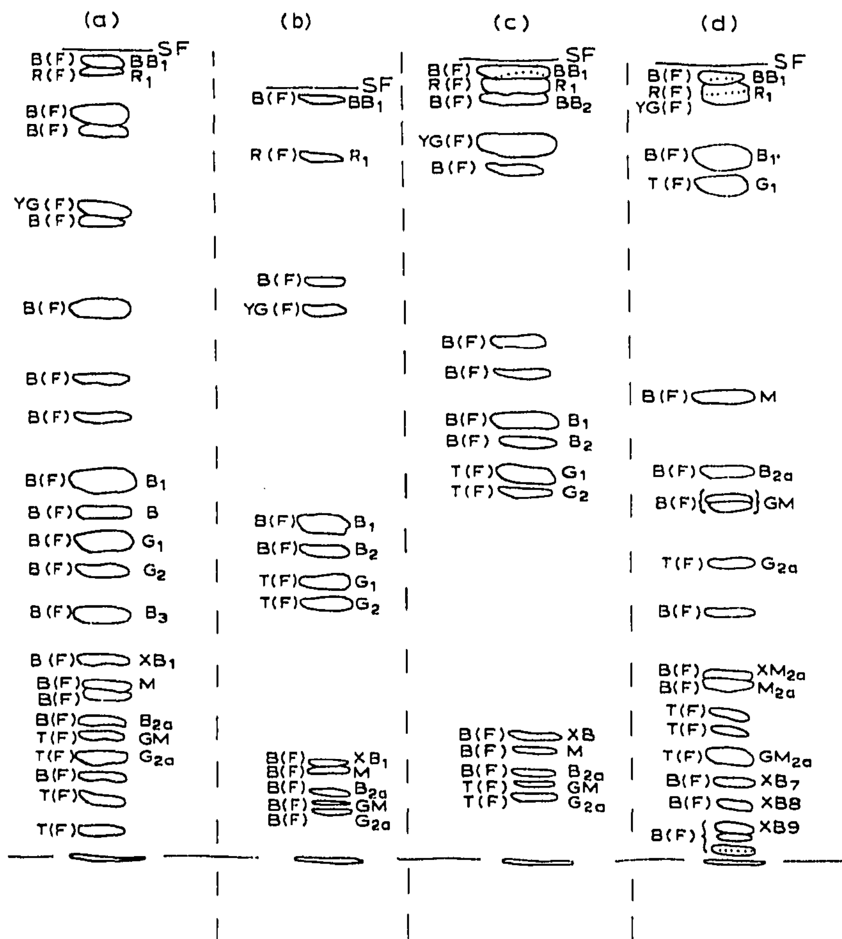

Fig. 2. Thin-layer chromatographs of culture fluid extracts of *A. flavus* 91019bii developed in various solvent systems, *viz.* (a) benzene–ethanol–water (46:35:19), (b) chloroform–methanol (49:1), (c) chloroform–methanol (97:3) and (d) chloroform–methanol (94:6). Layer, SilicAR TLC-7G. For abbreviations, see the legend to Fig. 1.

from the bottom of the plate, using a micropipette. Each of the solvent systems was used to develop two such chromatograms. The purity of the solvents was carefully controlled by using AnalaR solvents to eliminate, as far as practicable, possible sources of error due to any variation in materials.

Results

A few selected results are shown in Table II and Figs. 2-4.

The benzene–ethanol–water system of Eppley[12] gave good separation of aflatoxins B_1, B_2, G_1 and G_2 (Fig. 2), only when used under optimum conditions of temperature and humidity, which were very critical; otherwise separation was poor. It was also noticed that the appearance of the saturated benzene layer, which was used to develop the chromatogram, changed from that of a clear liquid to a milky emulsion when the temperature and humidity in the laboratory became elevated. This change, though not always apparent, invariably coincided with poor resolution of the aflatoxins on TLC. The addition of methanol to chloroform resulted in a

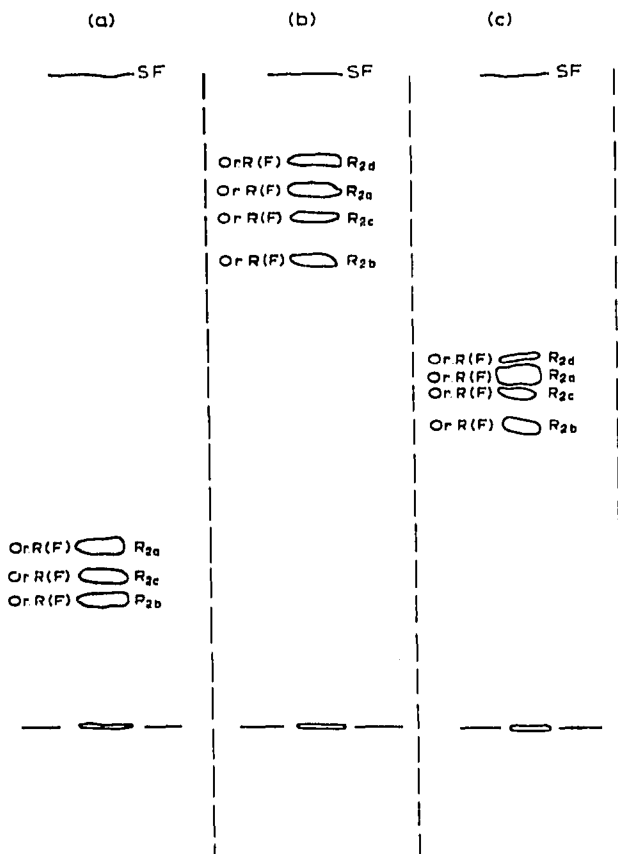

Fig. 3. Thin-layer chromatographs of metabolites extracted from cultures of *A. flavus* 91019bii developed in various solvents, *viz.* (a) toluene–ethyl acetate (18:1), (b) benzene–ethyl acetate (9:1) (first solvent system) toluene–ethyl acetate (16:1) (second solvent system) and (c) toluene–ethyl acetate (8:1). Layer, SilicAR TLC-7G. For abbreviations, see the legend to Fig. 1.

general movement of the aflatoxins further up the plate (Fig. 2), and this technique gave reasonable results up to a concentration of 6–7% methanol. However, chloroform–methanol systems were highly susceptible to changes in humidity, a low humidity allowing a higher concentration of methanol. At a concentration of 6% methanol, aflatoxin B_1 had an R_F value of 0.9, and aflatoxin B_{2a} an R_F value of 0.5, thus giving good separation of the bands below aflatoxin G_{2a}, including those of aflatoxin M_{2a} and aflatoxin GM_{2a}[13]. However, as the humidity rose, these more polar bands rapidly spread out and merged with each other.

The suitability of various toluene-based solvent systems for the resolution of pigment fraction R_2 is shown in Fig. 3. It would seem that a system based on toluene–ethyl acetate (8:1) or (7:1) is the most suitable method for resolving these pigment bands.

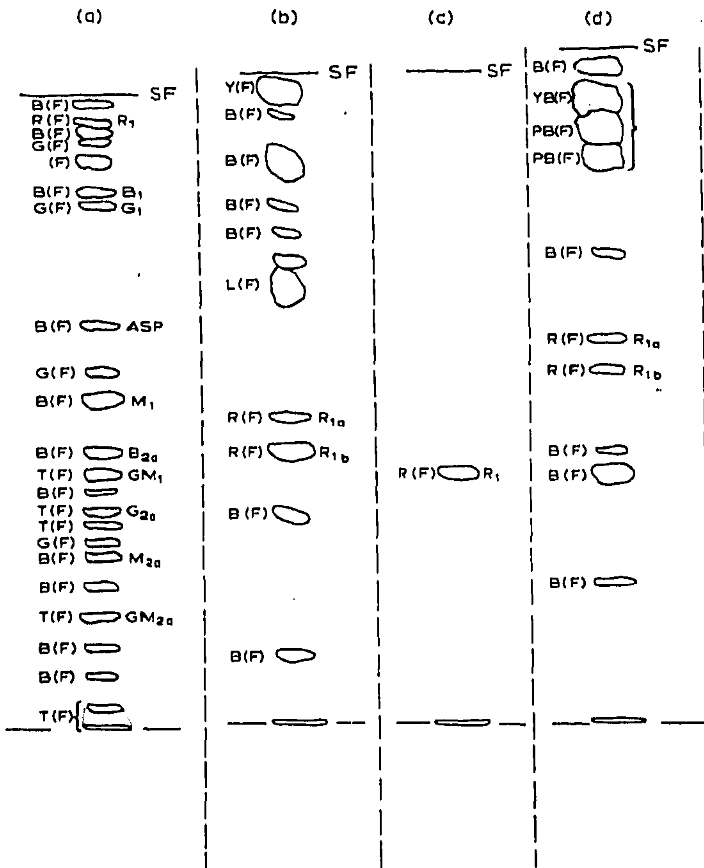

Fig. 4. Thin-layer chromatographs of metabolites extracted from cultures of *A. flavus* 91019bii developed in various solvents, *viz.* (a) toluene–ethyl acetate–acetone–glacial acetic acid (50:35:15:2), (b) chloroform–carbon tetrachloride (2:3) (first solvent system), chloroform–cyclohexane (1:1) (second solvent system), (c) cyclohexane-chloroform (1:1) and (d) chloroform (first solvent system), chloroform-cyclohexane (1:1) (second solvent system). Layer, SilicAR TLC-7G. For abbreviations, see the legend to Fig. 1.

A combination of toluene–ethyl acetate–acetone–glacial acetic acid (50:35:15:2) was found to give good separation of aflatoxin G_{2a} and the more polar derivatives of aflatoxins M_{2a} and GM_{2a} (Fig. 4). The resolution was not as susceptible to environmental changes as the chloroform–methanol (94:6) system, and consequently its use made possible the isolation and characterization of the more polar components. However, the system had one disadvantage in that it caused the formation of small quantities of the acetyl derivatives of the compounds. These could be removed by TLC, using chloroform–methanol (97:3).

It was found that equilibrating an activated plate in the vapour of the solvent before applying the spots to the plate greatly reduced the effect of atmospheric moisture on the activity of the silica. This equilibration procedure was most effective when the toluene- or cyclohexane-based systems were being used, and least effective with chloroform.

The effect of developing a plate twice, using different solvents, was investigated as an alternative method for the separation of coalescing bands. This was a particular problem when separating the pigment fraction R_1 from the lipid fractions, and when separating the individual components of pigment fraction R_2 (Figs. 3 and 4). It was found that fraction R_1 could be separated from the interfering lipid fraction by developing the TLC plate in chloroform until the solvent front had travelled 7 cm from the origin. Then, after allowing the chloroform to evaporate, the plate was redeveloped in the same dimension, using chloroform–cyclohexane (1:1), and allowing the solvent front to travel approximately 17 cm (see Fig. 4). An alternative and rather more successful system utilized a chloroform–carbon tetrachloride mixture (2:3) as the first developer (Fig. 4). An important feature of this solvent system is that it gives good, consistent resolution of the components with minimum interference from changes in humidity, especially when the plates are equilibrated in the vapours of the second solvent system.

The addition of water to the TLC solvents, as suggested by Stubblefield *et al.*[5], was not a success. It was found to make the solvents too susceptible to variations in humidity and temperature. Although excellent resolutions of the aflatoxins were obtained under conditions of low humidity, the plates showed progressive streaking and loss of resolving power with small increases in humidity.

CONCLUSION

The *Aspergillus* moulds are ubiquitous, and the toxins produced by some species are hazardous to the health of both animals and man. Many of the aflatoxins elaborated by *A. flavus* are also highly carcinogenic, while others are not. Hence, satisfactory analysis of the components of crude aflatoxin extracts is essential to the control of contaminated foodstuffs. Furthermore, many workers now consider that carcinogenic aflatoxins hold the key to liver cancer studies (see, for example, Heathcote and Hibbert[14]). For these reasons it is important that satisfactory methods are available for separating the inert aflatoxins, such as B_{2a} and G_{2a}, or their O-alkyl derivatives, from highly carcinogenic analogues. It is hoped that this paper will assist other workers to achieve such separations, as the results of many pathological studies in the past have been confused by the use of impure mixtures of aflatoxins.

REFERENCES

1 H. de Iongh, R. K. Beerthius, R. O. Vles, C. B. Barrett and W. D.Ord, *Biochim. Biophys. Acta*, 65 (1962) 548.
2 S. B. Chang, M. M. Abdel-Kader, E. L. Wick and G. N. Wogan, *Science*, 142 (1963) 1191.
3 P. J. Andrellos and G. R. Reid, *J. Ass. Agr. Chem.*, 47 (1964) 801.
4 L. L. Stoloff, A. Graff and H. Rich, *J. Ass. Offic. Anal. Chem.*, 49 (1966) 740.
5 R. D. Stubblefield, G. M. Shannon and O. L. Shotwell, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 669.
6 J. Velasco, *J. Amer. Oil Chem. Soc.*, 46 (1969) 105.
7 S. Nesheim, *J. Amer. Oil Chem. Soc.*, 46 (1969) 6.
8 J. R. Hibbert, *Ph.D. Thesis*, University of Salford, 1972.
9 V. E. Truter, *Thin Film Chromatography*, Interscience, New York, 1963, pp. 14–17.
10 J. I. Teng and P. C. Hanzas, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 83.
11 M. F. Dutton and J. G. Heathcote, *Chem. Ind. (London)*, (1969) 983.
12 R. M. Eppley, *J. Ass. Offic. Anal. Chem.*, 49 (1966) 473.
13 J. G. Heathcote and J. R. Hibbert, *Trans. Biochem. Soc. London*, 2 (1974) 301.
14 J. G. Heathcote and J. R. Hibbert, *Brit. J. Cancer*, 29 (1974) 470.