CHROM. 17 877

THIN-LAYER CHROMATOGRAPHIC SEPARATIONS USING A TEMPERATURE GRADIENT

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(First received February 4th, 1985; revised manuscript received May 7th, 1985)

SUMMARY

A normal chromatographic chamber was adapted for work with a temperature gradient. The influence of the magnitude of the temperature gradient for different chromatographic times was studied. Application of the procedure to the separation of aflatoxins and to dansyl-amino acids, including the chiral resolution of the latter, is described.

INTRODUCTION

In liquid chromatography, separation takes place by establishing a partition equilibrium of solutes between a mobile phase and a stationary phase. The conditions of the medium (*e.g.*, temperature and flow-rate of the mobile phase) have a great influence on the process. Obviously, when a sample is complex its separation is difficult under constant medium conditions. Indeed, it is hardly likely that a given set of conditions would be suitable for the separation of *all* the components of a mixture. Various modifications of the chromatographic procedure have been introduced to overcome this problem, *e.g.*, the use of gradients[1–3] and two-dimensional development[4,5]. Thus, Johnson and Nurok[6] reported the resolution of a number of hormones using two-dimensional thin-layer chromatography (TLC) coupled with continuous development, and Gulyas[7] used over-pressure TLC (OPTLC) for the separation of aflatoxins.

It has been shown with many examples that the use of gradients in TLC permits the resolution of mixtures that under normal conditions are difficult to separate. According to Stahl's[8] principle, the gradients are classified as follows[9]:

      Mobile phase gradients:
           concentration;
           polarity;
           pH;
           ionic strength.
      Stationary phase gradients:
           composition;
           impregnation;
           activity.

Medium gradients:

> temperature;
> vapour tension;
> flow-rate;
> particle size;
> cross-section;
> layer thickness.

Combined gradients:

> vapour composition/mobile phase concentration;
> mobile phase concentration/layer thickness;
> temperature/pH.

This paper deals with the application of a temperature gradient in TLC. This technique was introduced in 1961 by Liteanu and Gocan, who built the first chromatographic chamber specially equipped for this purpose. Since that time, they have improved the procedure, and shown it to be very suitable for mixtures of compounds which under the usual conditions are difficult or impossible to resolve. Thus, the method was applied to the separation of some inorganic ions such as Ni^{2+} and Cu^{2+}[10] and to mixtures of amino acids[11,12] using different stationary phases.

Later development led to a classification of the procedures in accordance with the direction of the movement of the solvent front (Fig. 1):

(a) parallel temperature gradient[10–13];
(b) antiparallel temperature gradient[14];
(c) orthogonal temperature gradient[15–17].

In this paper, we report on the effect of a parallel temperature gradient on separation. Our work was carried out on dansyl-amino acids (Dns-AAs) and aflatoxins B_1, B_2, G_1 and G_2, using plates coated with the reversed phase RP-18 and silica gel, respectively.
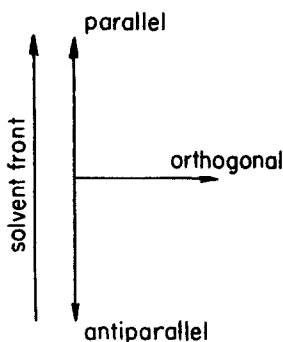

Fig. 1. Classification of temperature gradients in relation to the direction of movement of the mobile phase.

EXPERIMENTAL

FRP-18 (10 × 20 cm cut into pieces of 5 × 10 cm) and FRP-18 (10 × 20 cm) TLC plates were purchased from Merck (Darmstadt, F.R.G.). Multilayer K with a

3-cm strip of silica and a 17-cm strip of RP-18 (Whatman) were cut to obtain a 10 × 10 cm surface coated with RP-18. Pre-coated silica gel plates were obtained from the Analytical Chemistry Laboratory (Cluj-Napoca, Romania).

The Dns-AAs were purchased from Sigma (St. Louis, MO, U.S.A.) or were prepared according to ref. 18. The aflatoxins were supplied by Makor (Jerusalem, Israel).

Temperature-gradient TLC was performed in a modified chromatographic chamber, as shown in Fig. 2.
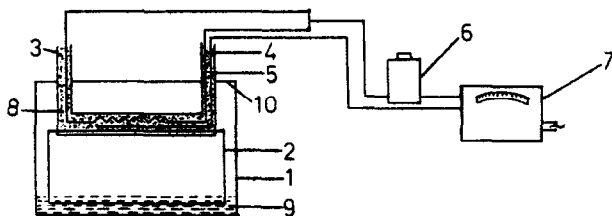

Fig. 2. Chromatographic system for temperature gradient. 1 = Chamber; 2 = TLC plate; 3 = U-shaped tube; 4 = heating element; 5 = thermocouple; 6 = adjustable autotransformer; 7 = temperature controler; 8 = silicone oil; 9 = mobile phase; 10 = lid.

The mobile phases for the separation of Dns-AAs in the achiral mode were as follows: for the RP-18 plates, 0.3 M sodium acetate in water–acetonitrile (65:35, v/v) (apparent pH = 6.6) (A) and for the multi-layer plates dichloromethane–diethyl ether–acetic acid (20:30:10, v/v) followed in the second dimension by A. In the chiral mode an 8 mM solution of N,N-di-n-propyl-L-alanine (DPA) and 4 mM copper(II) acetate in water–acetonitrile (95:5, v/v) was used as a spray reagent for the impregnation of the FRP-18 TLC plates. The plates were then developed with a mobile phase containing 8 mM DPA and 4 mM copper(II) acetate dissolved in 0.3 M sodium acetate in water–acetonitrile (70:30, v/v) (apparent pH = 7). The mobile phase for the separation of aflatoxins was toluene–ethyl acetate–diethyl ether–formic acid (5:4:1:1, v/v).

The detection of the separated compounds was effected under UV light at 366 nm.

RESULTS AND DISCUSSION

In order to establish the kinetics of migration of the mobile phase through the layer under temperature-gradient conditions, we measured the path lengths of the mobile phase as a function of time. For this purpose, we attached to the RP-TLC plates a strip of millimetric paper and determined the height reached by the solvent at different time intervals. The results are shown in Fig. 3. Examining the shape of the curve, it can be suggested that the movement of the mobile phase through the layer obeys the following equation[10]:
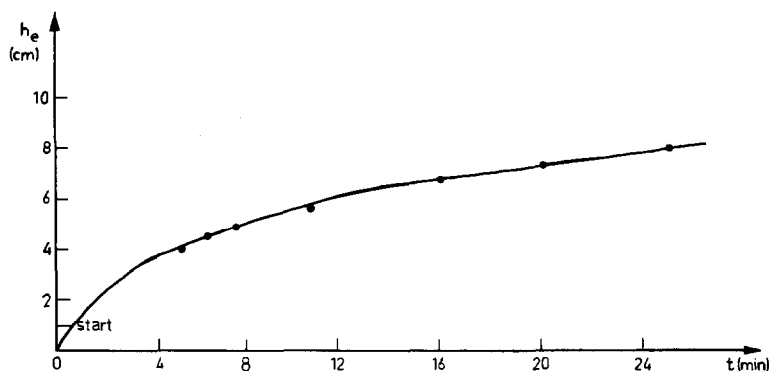
$$h_e = t/(A + Bt) \tag{1}$$

Fig. 3. Migration of the mobile phase as a function of time.

where t is time and A and B are constants that characterize the mobile phase–stationary phase system. The magnitudes of A and B also depend on the temperature interval in which the gradient is produced. To determine constants A and B, it is necessary to write eqn. (1) in a linear form:

$$1/h_e = B + A/t \qquad\qquad (2)$$

Fig. 4 shows the regression curve obtained from eqn. 2, which gave values of 0.852 and 0.091 for A and B, respectively. From eqn. 1, it follows that the average speed of the eluent is given by

$$v_s = h_e/t = [t/(A + Bt)]/t = 1/(A + Bt) \qquad\qquad (3)$$

As is shown in Fig. 5 and Table I, the average speed decreases continuously with time and finally falls to zero. This behaviour is caused by the evaporation of the solvent in the heated part of the plate, but does not mean that chromatography has stopped. Obviously, an equilibrium between the eluent that ascends the plate and that which leaves the plate by evaporation is established. The amount of the eluent that is evaporated increases with time and, if the chromatography is not stopped at an optimum time, all the components of the mixture move into the solvent front, reaching $R_F = 1$. Therefore, the separation should be stopped as soon as resolution is achieved. The data given in Table I show good agreement between the predicted and found values of the average speed of the mobile phase.

   We also followed the migration of the spots in the temperature-gradient regime. The results for the four amino acid derivatives (Dns-Ser, Dns-Asp, Dns-Gly and Dns-Phe) are plotted in Fig. 6. The shape of the curve obtained can be expressed mathematically in the following form:

$$h_s = bt^m \qquad\qquad (4)$$

where b and m are constants that depend on the nature of the solutes and the experimental conditions. This equation becomes linear, when written in logarithmic form:
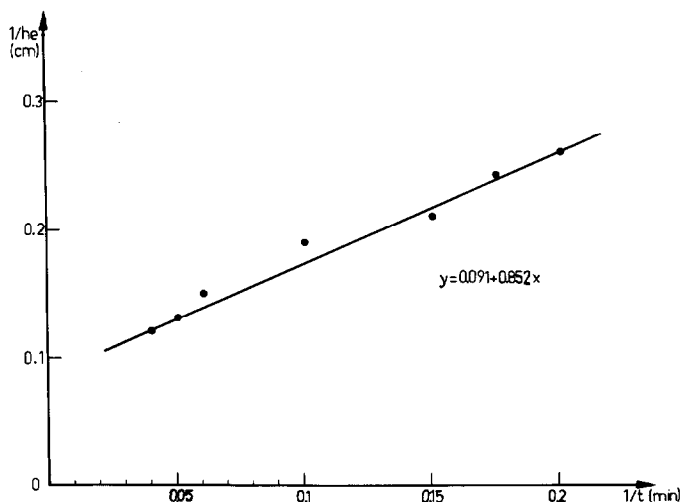
Fig. 4. Plot of $1/h_e$ versus $1/t$.

$$\log h_s = \log b + m \log t \tag{5}$$

When plotted as in Fig. 7, a straight line was obtained from which the parameters b and m were determined. The h_s values calculated with these constants are compared with the experimental values in Table II. Again, good agreement was observed between the two sets of data.

Chromatography with a temperature gradient leads, as mentioned above, to evaporation of the solvent and a consequent increase in the amount of eluent fed to the plate. This is equivalent to the use of a longer migration path and thus the better separation is explained.

Our experiments were effected on several classes of compounds and showed improved resolution. We carried out the separation on TLC plates having a strip of 3 × 10 cm of silica and 10 × 10 cm of RP-18. Two-dimensional development was used first on the silica gel strip and then perpendicularly to it. The chromatography was initially carried out isothermally, and it can be seen that under these conditions
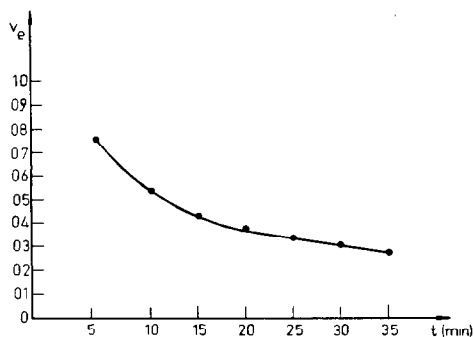

Fig. 5. Average speed of the mobile phase through the layer as a function of time.

TABLE I

AVERAGE SPEED OF THE MOBILE PHASE *VERSUS* TIME OF CHROMATOGRAPHY

Gradient = 5°C/cm; A = 0.852 min/cm; B = 0.091 cm^{-1}; stationary phase, RP-18; mobile phase, 0.3 M sodium acetate in water–acetonitrile (65:35, v/v); apparent pH = 6.6.

t (min)	V_s (cm/min)	
	Calculated	Found
5	0.765	0.760
10	0.567	0.530
15	0.451	0.440
20	0.374	0.375
25	0.320	0.328
30	0.250	0.293
35	0.280	0.263
40*	0.222	
45	0.202	
50	0.185	
90	0.111	
130	0.079	
170	0.061	
210	0.050	
245	0.042	

* Starting at 40 min, only the calculated values are listed.

almost the whole group of the Dns-AAs were badly separated (see Fig. 8). However, when a temperature gradient was applied in the second step, resolution of all the Dns-AAs was achieved. The optimum time for chromatography was found to be 3 h. The spots were compact and well defined, as shown in Fig. 9.

In other experiments we applied this technique to the separation of enantiomers, a recent extension of the applications of TLC. Only a few papers have appeared on this topic so far. Wainer et al.[19] described the RP-TLC separation of 2,2,2-tri-
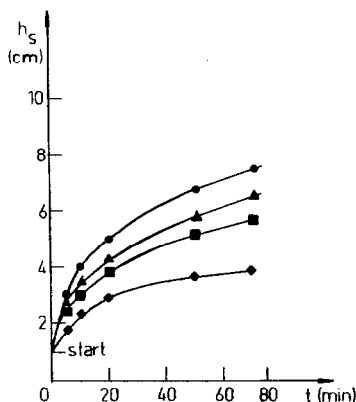

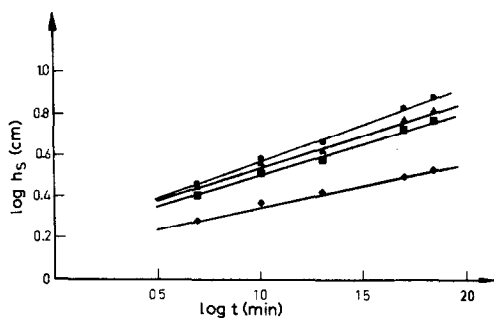Fig. 6. Migration of Dns-AAs as a function of time. ●, Dns-Ser; ▲, Dns-Asp; ■, Dns-Gly; ◆, Dns-Phe.

Fig. 7. Plot of log h_s versus log t. ●, Dns-Ser, $y = 0.22 + 0.35\ x$; ▲, Dns-Asp, $y = 0.23 + 0.31\ x$; ■, Dns-Gly, $y = 0.21 + 0.30\ x$; ◆, Dns-Phe, $y = 0.15 + 0.20\ x$.

TABLE II

CALCULATED AND EXPERIMENTAL VALUES OF h_s

The temperature gradient used was 5°C/cm, calculated as described in ref. 9. The calculated h_s values were obtained using eqn. 5.

Dns-AA	t (min)	b	m	h_s^* (cm) Calculated	Found
Ser	30			5.4	5.6
	40	1.65	0.35	6.0	6.0
	50			6.5	7.0
Asp	30			4.9	4.9
	40	1.72	0.31	5.4	5.2
	50			5.8	6.2
Gly	30			5.4	5.6
	40	1.61	0.30	4.8	4.6
	50			5.2	5.6
Phe	30			2.8	2.8
	40	1.41	0.20	2.9	2.8
	50			3.1	3.7

* h_s is defined as the distance between the start and the middle of the spot. For chromatographic conditions, see Table I.

fluoro-1-(9-anthracyl)ethanol into its enantiomers. More recently, Gunter et al.[20] reported the resolution of some underivatized amino acids by TLC plates coated with (2S, 4R, 2'RS)-4-hydroxy-1-(2-hydroxydodecyl)proline. Weinstein[21] achieved the enantiomeric analysis of Dns-AAs. In the last instance, the separation of Dns-AAs into enantiomers was performed on commercial RP plates treated with the copper complex of N,N-di-n-propyl-L-alanine and all the protein amino acids except proline could be resolved. The method cannot, however, be applied to a mixture of all the dansyl protein amino acids, because of overlap of some of the component spots. As it is desirable to analyse complex amino acid mixtures for many practical applications, especially in biological work, we have introduced a two-dimensional RP-TLC
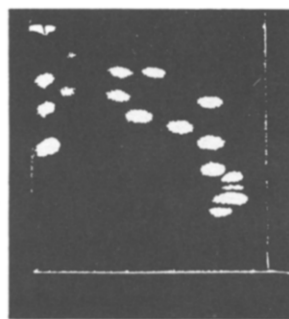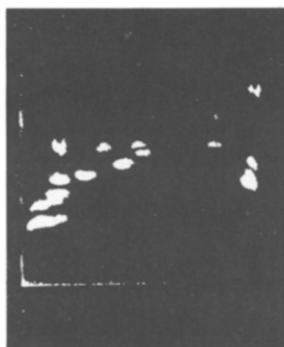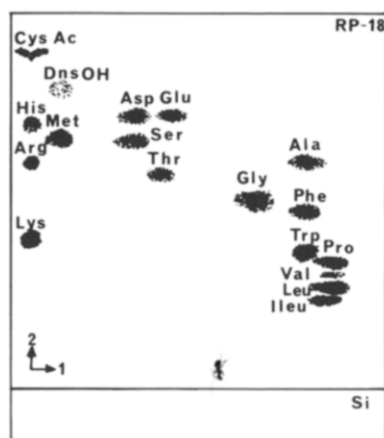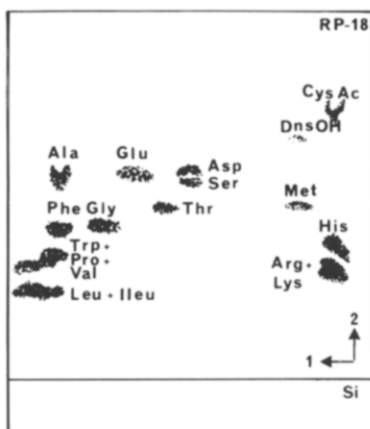
Fig. 8. Two-dimensional separation of Dns-AAs at room temperature (for other conditions, see text).

Fig. 9. Two-dimensional separation of Dns-AAs with a temperature gradient (for other conditions, see text).

technique[22]. To solve the problem of overlap, the Dns-AAs are first partially separated in a non-chiral mode, in the longer dimension of a 10×20 cm RP-TLC plate. To achieve the best results we used in this step convex gradient elution with an aqueous sodium acetate buffer and varying concentrations of acetonitrile. After this separation of Dns-AAs, the plates were dried with a fan, the strip of the chromatographic plates containing the amino acids was covered with a glass plate and the remaining exposed plate was sprayed with a solution of the copper complex of DPA (Cu:DPA = 4:8, mM/mM) dissolved in water–acetonitrile (5:95, v/v) and was then ready for the perpendicular run. In our experiments only 8 cm of plate were left for the second dimension, which is not sufficient for a good separation. In order to overcome this problem, we used a temperature gradient of 6.25°C/cm, which was found to be efficient. With this gradient, the amount of mobile phase evaporated in the heated part of the plate simulates a theoretical supplementary column sufficient for the resolution of Dns-AAs. Development in the second dimension required 150 min; throughout the D-enantiomers moved faster than the L-enantiomers.
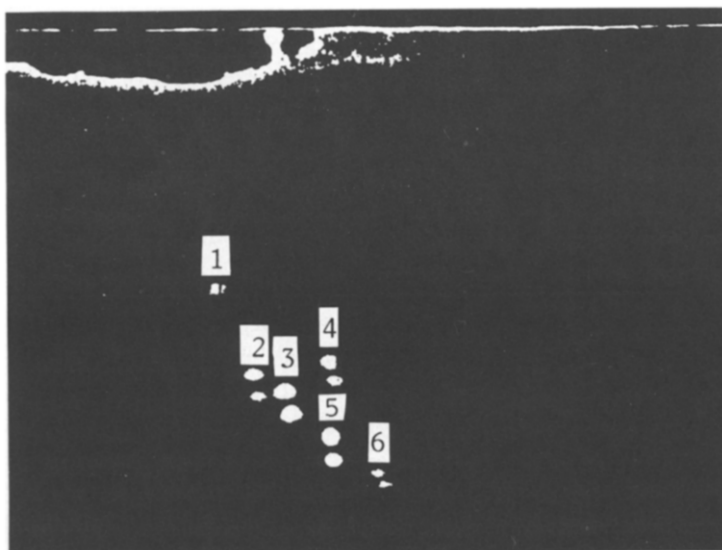
Fig. 10. Enantiomeric separation of Dns-AAs by RP-TLC using a temperature gradient. 1 = Dansylic acid; 2 = Asp; 3 = Ser; 4 = Met; 5 = Ala; 6 = Phe.

An example of the chiral separation of five amino acids in the second dimension is illustrated in Fig. 10. Table III indicates the resolution of the enantiomers in question. The resolution was so good that no overlap of the various enantiomeric spots occurred.

The aflatoxins constitute a very important class of compounds, derived from furocoumarins, which are highly toxic towards animals. They are secreted by the fungus *Aspergillus flavus*, which grows on various foods. Until now there has been no useful method for destroying their toxigenic power and the only safe method is to check the amount of alflatoxins by analysis of suspected foodstuffs. The most useful method for aflatoxin analysis is TLC, using different stationary and mobile phases[24]. However, there are two aflatoxins, B_2 and G_1, which under our experi-

TABLE III

RESOLUTION COEFFICIENT (α) OF SOME DL-DANSYL-AMINO ACIDS SEPARATED BY RP-TLC WITH A CHIRAL STATIONARY PHASE AND A TEMPERATURE GRADIENT

α defined according to Perry[23]. Chiral stationary phase: copper(II) acetate–DPA (4:8, mM/mM). Temperature gradient: 6.2°C/cm.

DL-*Dns-AA*	α
Asp	1.3
Ser	1.4
Ala	2.1
Met	1.3
Phe	2.0

mental conditions at room temperature overlap on the TLC plates. Further, on extraction of mycotoxins from the food sample, impurities are extracted that form tails on the TLC plates and interfere with the quantitative interpretation of the chromatogram. For this reason we applied the temperature gradient technique in the separation of aflatoxins B_1, B_2, G_1 and G_2, in order to improve the separation between aflatoxins B_2 and G_1. To find the optimum experimental conditions we operated in two ways. First the chromatographic time was kept constant and the temperature gradient was varied till its optimum value was reached; then we maintained the gradient constant and determined the time of chromatography for optimal separation.

The results of the separation are presented in Table IV and Fig. 11. It can be seen that with increasing temperature gradient, the R_F values increase. However, the resolution between aflatoxins B_2 and G_1 is achieved only with a temperature gradient of 2°C/cm. Fig. 12 shows the separation of aflatoxins when the temperature gradient was varied and the time was kept constant.

On varying the time of chromatography and maintaining constant the temperature at 2°C/cm, we obtained the results shown in Table V and Fig. 13. With such a gradient the resolution is achieved in 150–180 min. If the chromatography is pro-

TABLE IV

R_F VALUES OF AFLATOXINS FOR VARIOUS TEMPERATURE GRADIENTS AT A CONSTANT DEVELOPMENT TIME (80 min)

Stationary phase, silica gel R; mobile phase, toluene–ethyl acetate–formic acid–diethyl ether (5:4:1:1, v/v)

Aflatoxin	Temperature gradient (°C/cm)	R_F	Average reproducibility of R_F*
B_1	0.0	0.51	±0..03
	0.5	0.54	
	1.0	0.56	
	1.5	0.56	
	2.0	0.78	
B_2	0.0	0.45	±0.05
	0.5	0.47	
	1.0	0.48	
	1.5	0.50	
	2.0	0.65	
G_1	0.0	0.40	±0.05
	0.5	0.42	
	1.0	0.43	
	1.5	0.46	
	2.0	0.65	
G_2	0.0	0.33	±0.08
	0.5	0.35	
	1.0	0.36	
	1.5	0.38	
	2.0	0.55	

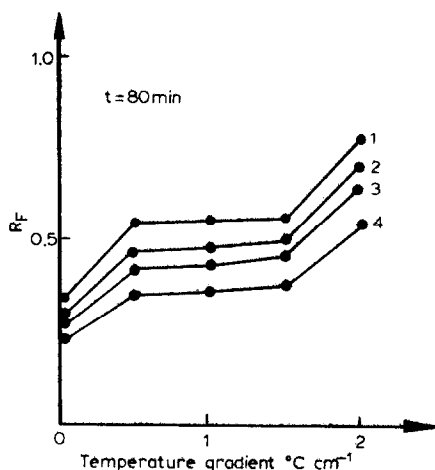* For each temperature gradient ten R_F measurements were made.

Fig. 11. Variation of R_F values of aflatoxins with the temperature gradient for a constant development time. 1 = Aflatoxin B_1; 2 = B_2; 3 = G_1; 4 = G_2.
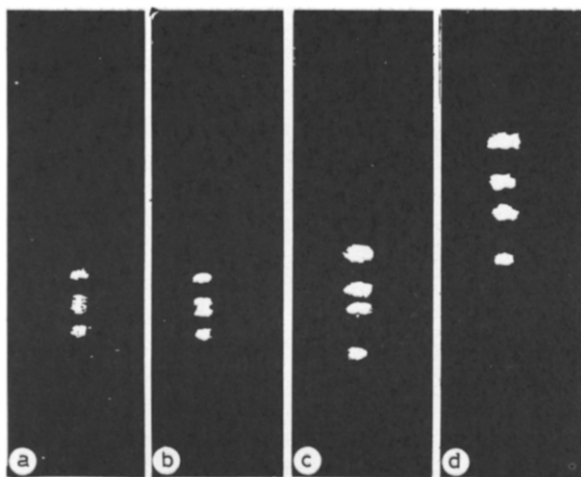

Fig. 12. Separation of the aflatoxins with different temperature gradients for a constant development time (80 min): (a) 0.5; (b) 1.0; (c) 1.5; (d) 2°C/cm.

longed to 240 min the resolution is lost and aflatoxin B_1 moves with the solvent front.

Recently, Gulyás[7] reported the separation of aflatoxins using the OPTLC technique. However, for certain samples his chromatograms show partial overlap of the peaks of aflatoxins B_2 and G_1, whereas under the conditions indicated above the temperature gradient gives complete separation.

This example clearly illustrates that TLC with temperature gradient is a useful technique for the analysis of compounds which under the usual conditions are difficult to separate. A temperature gradient also permits in some instances separation to be achieved with an eluent ineffective in development at room temperature. Com-

TABLE V

R_F VALUES OF AFLATOXINS FOR VARIOUS DEVELOPMENT TIMES AT A CONSTANT TEM-
PERATURE GRADIENT (2°C/min)

Stationary phase, silica gel R; mobile phase, toluene–ethyl acetate–formic acid–diethyl ether (5:4:1:1, v/v).

Aflatoxin	t (min)	R_F	Average reproducibility of R_F
B₁	80	0.78	±0.06
	120	0.81	
	150	0.92	
	180	0.93	
	210	0.98	
	240	1.00	
B₂	80	0.71	±0.04
	120	0.73	
	150	0.85	
	180	0.85	
	210	0.95	
	240	0.96	
G₁	80	0.65	±0.08
	120	0.73	
	150	0.78	
	180	0.78	
	210	0.91	
	240	0.93	
G₂	80	0.55	±0.06
	120	0.57	
	150	0.67	
	180	0.67	
	210	0.82	
	240	0.83	

* For each time interval ten R_F measurements were made.

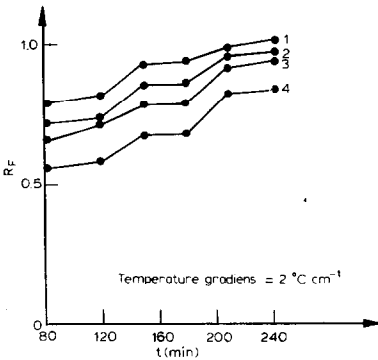
Fig. 13. Variation of R_F values of aflatoxins with time of development for a constant temperature gradient.
1 = Aflatoxin B₁; 2 = B₂; 3 = G₁; 4 = G₂.

pared with the other TLC techniques it has the advantage of not requiring a search for a suitable mobile phase system; the equipment necessary for carrying out the procedure can be easily prepared in the laboratory.

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