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STEPWISE GRADIENT DEVELOPMENT IN THIN-LAYER CHROMATO-GRAPHY

II. TWO-DIMENSIONAL GRADIENTS FOR COMPLEX MIXTURES

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SUMMARY

A modified technique of two-dimensional thin-layer chromatography using an equilibrium sandwich chamber with a glass distributer is described. The sample is introduced from the edge of the layer as a rectangular zone 1.5 cm long and developed using a stepwise gradient of eluent composition. After preconcentration of the rectangular spots in the second direction, the chromatogram is divided into four zones, each being developed in the second direction using eluents of solvent strengths suitable for the individual fractions. In the first stage the sample capacity is significantly increased and the separation of components having large differences in capacity factors is possible, being enhanced by strong displacement effects occurring under gradient conditions; in the second stage the preconcentration of the zones and adaptation of the solvent strength to the individual groups of solutes ensures the distribution of the components over the whole plate area. Owing to the greatly increased sample size, trace components can also be isolated from complex samples such as plant extracts.

INTRODUCTION

Two-dimensional thin-layer chromatography (2D-TLC) is a very effective method of separation of multicomponent samples such as protein hydrolysates or plant extracts; its numerous applications have recently been reviewed by Zakaria *et al.*[1] who also discussed the general principles of optimization of adsorbent–eluent systems for the two perpendicular directions of development. In the ideal case, the number of spots separated is equal to the square of the number of spots separated in a single direction[2,3].

The limitations of 2D-TLC result from three main causes:

(1) The "general elution problem"[4]: solutes having great differences in polarity accumulate in the proximity of either the start line or the end line.

(2) Increased sample size leads to large initial spot diameters and deterioration

of the separation of spots in the resulting chromatogram. For instance, if the limiting capacity is assumed to be 1 mg of sample per g of adsorbent, then the initial spot containing 1 mg sample should have an area of 10 cm² corresponding to 1 g of a 0.2-mm layer.

(3) For analogous adsorbent–eluent systems used in the two directions, the spots tend to accumulate near the straight or curved diagonal line[5-7], leaving the opposite angle areas free of spots. One solution to this problem is to use layers composed of two different adsorbents, e.g., silica and silanized silica RP-2, RP-8 or RP-18[1,8].

The three limitations are illustrated in Fig. 1.

The use of the horizontal sandwich chamber with a glass distributer, constructed by one of the authors (E.S.)[9-13], permits complete or partial elimination of the limitations of conventional TLC.

"The general elution problem" is solved by multistep gradient elution according to a predetermined program, as described in the preceding paper[14]. The space under the distributer of the equilibrium sandwich chamber (ca. 0.4 ml for a 190 mm × 190 mm layer, distributer dimensions 185 mm × 5 mm and slit height 0.4 mm) is consecutively filled with 0.4-ml volumes of eluents of increasing strength; thus, the first eluent fraction is 10% ethyl acetate in chloroform, and the last is pure ethyl acetate[13-16]. Twelve eluent fractions are necessary for development along a distance of 180 mm, since a single distributer volume (0.4 ml) develops the 0.25-mm layer along a distance of 1.5 cm. Each eluent fraction is introduced under the distributer after the complete absorption of the preceding fraction (Fig. 2a). The concentration steps are relatively small and are partially smoothed in the chromatographic system so that the gradient profile is approximately linear (Fig. 2b, c; the graphical program corresponds to the compositions of the eluent fractions in the layer).

For gradient elution (single-direction development, continuous or stepwise program), approximately double the number of separated spots were obtained for complex plant extracts[15,16].

To increase the sample size without excessive zone spreading, the sample is introduced as a zone 15 mm in length; in parallel, the eluent is delivered as a zone 175 mm long which prevents radial spreading of the initial zone[17]. In the rectangular initial zone (Fig. 3a) the components are partially separated owing to the mutual displacement characteristic of frontal chromatography[17-19]. Further development, using a single distributer 185 mm long, separates the initial zone into a series of rectangular zones 1.5 cm in length (Fig. 3b), the resolution being significantly improved by gradient development[15-16]
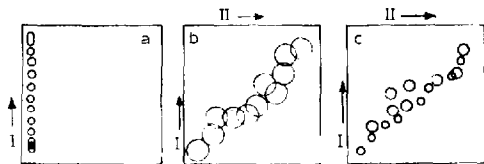

Fig. 1. Limitations of 2D-TLC due to the general elution problem (a), to a large starting zone for point application of a large sample (b) and to the tendency of the spots to accumulate along the diagonal line (c).
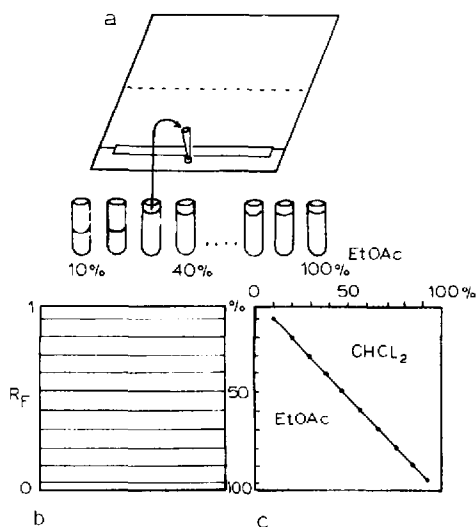
Fig. 2. Stepwise gradient elution in sandwich chambers with a glass distributer of the eluent: (a) 0.4-ml portions of eluents of increased solvent strengths are introduced under the distributer and from the edge of the layer; (b) developed chromatogram with zones of the mobile phase and a stepwise profile of the gradient; (c) corresponding graphical representation of the (approximated) continuous gradient.

Before development in the second (perpendicular) direction the long zones should be focused by elution with a volatile, strong eluent under the narrow cover plate (preconcentration step, Fig. 3c).[20] The preconcentrated zone is developed in the second direction using a suitable eluent or gradient program (Fig. 3d).

To avoid the accumulation of spots along a straight or curved diagonal line, after the preconcentration step (Fig. 3c) the chromatogram is cut (in the direction of the second development) into several, e.g., four, zones. This is easy for precoated
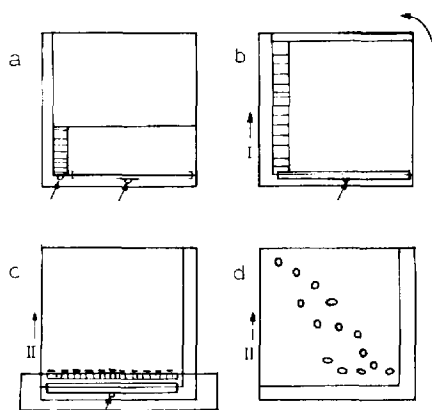

Fig. 3. (a) Increasing the capacity of the system by zonal application of the sample with the short distributer; (b) elution in the direction I; (c) preconcentration of the wide zone in the second direction by development under the narrow cover plate; (d) elution in the direction II. In (a)-(c) the cover plates are omitted; only the positions of the distributer are shown.

aluminium or plastic foils. The zone borders should be chosen between spots visible under an UV lamp so that a single component is not divided between two zones. Each point of the chromatogram is considered as a separate analysis with the initial spots applied on the preconcentration line: the top zone A (relative to the first direction), containing the least polar components, is developed with the weak eluent, *e.g.*, 10% ethyl acetate in chloroform; the middle zones B, C, with a moderately strong eluent, *e.g.*, 20 and 40% ethyl acetate, and the bottom zone, containing the most polar solutes, with a strong eluent, *e.g.*, 10% ethanol in ethyl acetate. Since the eluent strengths are individually chosen for the four fractions obtained by gradient elution in the first direction of development, each fraction will be separated into spots in the optimum range of moderate R_F values. In the integral square chromatogram the spots are thus spread over the whole area of the plate, which is advantageous for a high degree of separation.

EXPERIMENTAL AND RESULTS

Precoated 0.2-mm layers of silica on 20 cm × 20 cm aluminium foil or glass carrier plates with a preconcentration zone were used (E. Merck, Darmstadt, F.R.G.).

The gradient program was optimized for zonal application of the sample which comprised non-volatile compounds of Azulane (extract of Matricaria chamomila flowers; Herbapol, Warsaw, Poland) dissolved in ethyl acetate (3.45%, w/v). one distributer volume (*ca.* 100 μl) of the solution was introduced from the edge of the layer on a 200 mm × 50 mm plate. The first stepwise gradient program in the range 5–30% ethyl acetate in chloroform resulted in the accumulation of zones in the lower part of the chromatogram (Fig. 4a), which indicated that a steeper gradient program
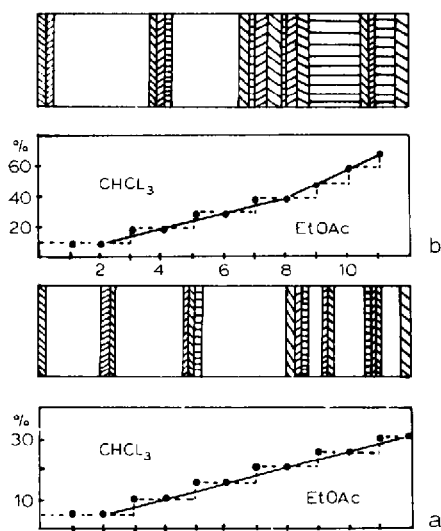

Fig. 4. Zonal chromatograms of an Azulane extract. Stepwise gradients: (a) 5–30%; (b) 5–70% ethyl acetate in chloroform.

was necessary. A steeper gradient, 5–70% ethyl acetate in chloroform, resulted in a better separation of the zones (Fig. 4b).

Although the TLC conditions were rather complex owing to variations in the vapour composition over the layer, the reproducibility was satisfactory as illustrated in Table I. However, the reproducibility of the R_F values depends on the type of eluent used. For non-polar and weakly polar solvents and their mixtures, *e.g.*, heptane, benzene, chloroform, the R_F values depend strongly on the water content of the adsorbent (its activity) and the solvent. In the case of eluents containing polar solvents, *e.g.*, ethyl acetate (such eluents were used in this study), the water content is less critical owing to the reactivation of the adsorbent by the polar solvent and the low activity of water in the mobile phase. The diffusion of vapours over the layer and their adsorption and desorption may be reduced by decreasing the space between the layer and the cover plate to a fraction of a millimetre. For gradient programs at lower eluent strengths, greater care should be taken regarding the standardization of the experimental conditions, *e.g.*, rapid transfer of the plate to the chamber, control of the water content in the solvent, etc.

TABLE I

REPRODUCIBILITY OF $100 \cdot R_F$ VALUES OF COMPONENTS OF A CHAMOMILE EXTRACT IN STEPWISE GRADIENT TLC

Program: 10, 10, 20, 30, 40, 50, 100% ethyl acetate in chloroform. Precoated HPTLC aluminium foil (silica) with a preconcentration zone. Two experiments were carried out in six consecutive days.

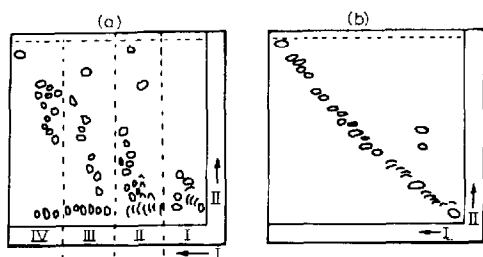| Solute No.	Colour in daylight	Colour in UV_{254}	$100 \cdot R_F$					
			1	2	3	4	5	6
1	—	Pale	79	83	84	79	80	81
		green	79	81	82	80	81	79
2	Green	Pink	76	80	81	76	76	72
			75	80	79	76	76	73
3	Pale	Pink-	72	76	77	73	72	70
	green	violet	72	75	75	73	72	72
4	Pale	Pink	54	57	58	53	55	54
	green		54	56	56	53	54	54
5	Pale	Blue-	43	40	45	41	40	43
	yellow	violet	42	40	44	41	40	42
6	Yellow-	Brown	30	29	32	27	29	30
	green		30	29	30	28	30	30
7	Pale	Pink	18	21	24	19	18	21
	yellow		18	20	22	19	19	20
8	Pale	Red	17	14	16	13	15	14
	green		15	14	15	14	15	15

Fig. 5. 2D-TLC chromatograms of an Azulane extract on glass plates (200 mm × 200 mm) with a preconcentration zone (silica): (a) stepwise gradient elution in the first direction (3 × 10, 20, 30, 40, 50, 60, 70, 80, 90, 100% ethyl acetate in chloroform) and isocratic elution after preconcentration of four zones in the second direction (I, 80%; II, 40%; III, 20%; IV, 10% ethyl acetate in chloroform); (b) stepwise gradient development in both directions (2 × 10%, 2 × 20%, 2 × 30%, 2 × 40%, 2 × 50% and 2 × 70% ethyl acetate in chloroform).

The second, steeper gradient was used in 2D-TLC experiments. Margins 1 cm wide were cleaned of adsorbent on two adjacent sides of the foil. To introduce the sample and solvent in the first (19 cm) direction a two-part distributer (15 + 170 mm) was used (Fig. 3a). The sample was applied as a 1.5-cm wide band parallel to the eluent to secure an even linear flow of the mobile phase. After introduction of a *ca.* 2-cm long zone, the small cover plate was replaced with another one with a single 185-mm distributer. The elution was carried out (Fig. 3b) using portions of the eluent having increasing contents[14] of the polar modifier (ethyl acetate), to the farther end of the layer thereby completing the development in the first direction. The volume of the sample corresponding to the 2 cm × 1.5 cm starting zones was *ca.* 20 μl which corresponds to 0.7 mg of dry sample. After preconcentration to narrow bands by development with acetone for a short distance in the second direction (Fig. 3c), the chromatogram was cut into four zones corresponding to hR_F values in the ranges 0–25, 25–50, 50–75 and 75–100.

The four chromatograms were developed in four separate 10 cm × 5 cm sandwich chambers using eluents expected to be suitable for the four fractions, *i.e.*, 80% (I), 40% (II), 20% (III) and 10% ethyl acetate in chloroform (IV). The criterion of success was the number of spots visible under UV light (360 nm) or after exposure to iodine vapours (Fig. 5a).

To compare the results obtained with those from the traditional 2D-TLC technique, in the second experiment the whole plate was developed in the second direction using the same gradient program. The spots were mostly accumulated along a diagonal line (Fig. 5b) and their number was somewhat greater than that obtained using development in a single direction, but much lower in comparison to those in Fig. 5a. The good separation of spots indicates that a greater sample load could be applied.

CONCLUSIONS

The chromatographic technique proposed is somewhat more complex and time-consuming in comparison to the traditional technique. However, the analysis time for low-viscosity solvents is short, about 40 min, and it is possible to carry out several parallel experiments using several sandwich chambers and an automatic mi-

cropipette to introduce the eluent fractions. The greatly increased separation efficiency and purity of the separated components permits direct structure determination of the extracts by mass spectrometry[21]. The zonal application of greatly increased sample sizes (frontal + elution chromatography) together with gradient elution permits the isolation of trace components.

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