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STEPWISE GRADIENT DEVELOPMENT IN THIN-LAYER CHROMATOGRAPHY

I. OPTIMIZATION OF GRADIENT PROGRAM

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SUMMARY

The application of an equilibrium sandwich chamber with a glass distributer for stepwise gradient development in thin-layer chromatography is described. Six to ten fractions of the eluent having increasing concentrations of a polar modifier are programmed graphically and introduced directly under the distributer with a micropipette. A comparison of the gradient profile with the obtained chromatogram permits adjustment of the gradient program in order to improve the positions of the spots on the chromatogram. The technique is relatively simple and permits the use of multicomponent gradients; it is especially suitable for the separation of complex samples, *e.g.*, plant extracts, including zonal micropreparative separation. It is possible to improve the spot separation in a fragment of the plate without changing the positions of the remaining well separated spots.

INTRODUCTION

Complex mixtures containing components having a wide range of capacity factors cannot be separated by isocratic elution owing to the "general elution problem"[1]: eluents of low strength separate the less strongly retained solutes, while the strongly retained components are eluted much later as low intensity, diffuse peaks. On the other hand, strong eluents do not separate weakly retained components, which are eluted together as a common or partly resolved peak. In high-performance liquid chromatography (HPLC) it is usual to apply gradient elution, analogous to temperature programming in gas chromatography (GC) of solutes which have a wide range of volatilities.

Gradient elution has also been employed in thin-layer chromatography (TLC). The general elution problem in TLC is illustrated in Fig. 1. Gradient elution in TLC can be carried out by several methods[2–6].

(a) Eluent demixing, especially with multicomponent eluents which form a gradient of eluent strength leading to the so-called multizonal chromatography, see ref. 3, p. 177.

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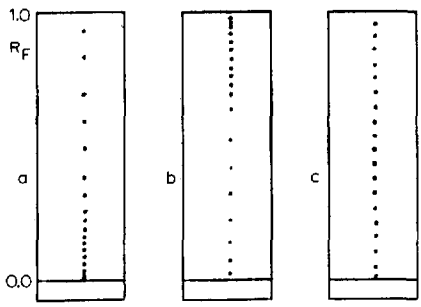

Fig. 1. The general elution problem in TLC: isocratic development (a,b) is not sufficient to separate all components. Eluent: ethyl acetate in chloroform; (a) 10%, (b) 50%, (c) gradient elution, 30 to 70%.

(b) Preadsorption of vapours in chambers of the Vario-KS type where the plate is placed over a tray containing a solvent. Thus, Geiss demonstrated that a plate with preadsorbed benzene vapours and developed with acetone has a very steep gradient of solvent strength (ref. 3, p. 247). These two methods of generation of gradients are relatively simple, however the actual composition of the gradient in the layer is virtually unknown and the control of its shape is difficult.

(c) Controlled changs of the eluent composition in a manner similar to gradient HPLC. Several earlier methods of this type have been discussed in monographs on liquid chromatography[3–6]; also a review by Berezkin *et al.*[7]. In a recent paper[8] a miniaturized device[9] for gradient elution is equilibrium sandwich chambers equipped with a glass distributer was described; the component solvents, A (weaker) and B (stronger), were chosen so as to ensure spontaneous mixing due to molecular interactions and differences in densities, *e.g.*, A = chloroform, B = ethyl acetate.

Another method is to introduce the eluent in small portions[10–12], *e.g.*, six to ten, having increasing concentration of the stronger solvent. The stepwise gradient thus obtained is analogous to a continuous gradient because the steps become diffuse in the development process. Any gradient programs, including multicomponent ones, can be generated in this way and although the gradient shape in the layer may be somewhat distorted relative to the initial program, it is possible to vary the program to fit the actual analytical problem.

EXPERIMENTAL AND METHODS

Apparatus

An equilibrium sandwich chamber (for 20 cm × 5 cm plates) with a glass distributor was used (Polish Reagents POCh, Lublin, Poland)[7–12]. The distributor was 5 or 10 mm wide; in the latter case the volume between the distributor and the carrier plate was about 0.2 cm^3, which for 0.3-mm layers corresponded to development along a distance of 3 cm; six to seven distributor volumes were thus required for full development. The narrower distributor corresponded to a volume of 0.1 cm^3 and about fifteen distributer volumes were necessary to complete the development. To simplify the procedure, a special microfunnel (Fig. 2) was used. It could be filled with up to 0.5 cm^3 of the eluent which was introduced gradually under the distributer by capillary forces.

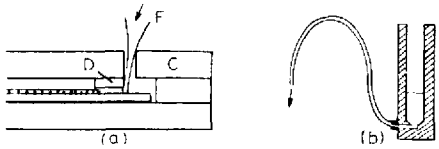

Fig. 2. Cross-section of the equilibrium sandwich chamber: (a) Introduction of eluent between the margin of the carrier plate (cleaned of adsorbent) and the distributor (D) through a microfunnel (F), *e.g.*, the tip of an automatic micropipette, placed in the orifice of the cover plate (C). (b) Another type of microfunnel (0.5–1.0 cm^3) made of PTFE with a capillary elastic PTFE siphon. The siphon connection with the distributer is produced by temporarily lifting the container above the plate level or producing an over-pressure in the container with a syringe.

Gradient programming

A graphical method of gradient programming can be used. A rectangle 20 cm × 10 cm is drawn on millimetre paper (Fig. 3): the vertical side represents solvent composition (and partial volumes of solvents; in Fig. 3 the total volume is 5 cm^3); the horizontal side represents consecutive fractional volumes of the eluent. A line representing the gradient shape is drawn from the starting to the final concentration of the stronger solvent. Vertical lines are divided by this line into parts corresponding to the contents of solvents A and B in the consecutive portions of the eluent; the volumes of A and B necessary to prepare 5-ml portions of the eluent can be read directly from the right-hand ordinate. This is very convenient especially for complex gradient programs as illustrated below.

After development the first eluent fractions are near the end line while the last fractions are at low R_F values; thus, the gradient of the mobile phase composition in the layer is obtained by juxtaposing the chromatogram and the gradient program as shown in Fig. 4.

The calculation of the consecutive spot positions *a priori* is rather difficult and requires knowledge of the quantitative relationships between k' and the composition (see the studies of Golkiewicz and co-workers[13–17] relating to stepwise elution). Moreover, the actual gradient profile in the layer gradually becomes distorted in compar-

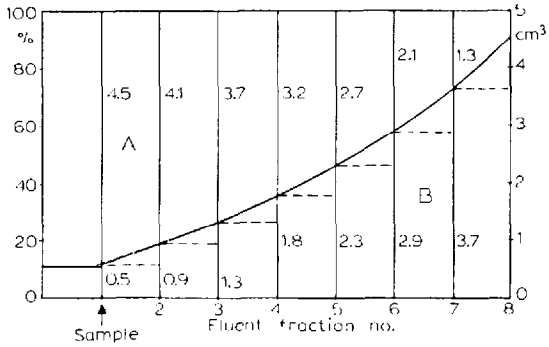

Fig. 3. Graphical programming of an eight-step gradient. After choosing the suitable gradient shape, the volumes of the components A and B are determined from the lengths of the vertical lines dissected by the gradient profile. The sample is spotted after introduction of one fractional volume of the eluent to eliminate eluent demixing.

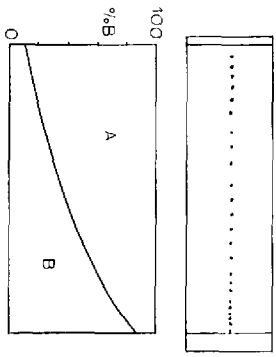

Fig. 4. Comparison of the gradient profile and the resulting chromatogram: the first eluent fractions correspond to the upper part of the plate.

ison to the initial one due to (a) eluent demixing (frontal chromatography of the mobile phase)[2–6,18] and (b) exchange of the stagnant mobile phase in the pores for the mobile phase of increased concentration. The first effect may cause strong deformation of the gradient profile and formation of steep gradients[12,18] especially for low-percentage B[18,19]; it is thus advantageous to start the program not from zero concentration of solvent B but from, *e.g.*, 10% and to spot the sample behind the solvent front. Prewetting with one distributor volume is usually sufficient to displace the deformed part of the gradient profile ahead of the leading zones[12,18].

In spite of the complexity of the gradient development, simplified, general, quantitative rules of gradient optimization can be formulated, based on the analysis of the distribution of the spots along the chromatogram corresponding to the gradient program used. The procedure can be useful in the separation of multicomponent samples, *e.g.*, plant extracts.

Choice of eluent strength range

The sample is chromatographed using solvents of different eluent strengths. On silica, the following series can be recommended: heptane (0.0), trichloroethylene; toluene (0.22); dichloromethane (0.30) or chloroform (0.26); diisopropyl ether (0.42); ethyl acetate (0.94); isopropanol (1.8) (eluent strength, ε^0 values[1], in parentheses).

The gradient program is started from the solvent (A) with which low R_F values are obtained for most components of the sample; with the second solvent (B), most components should have high R_F values and even the strongly retained components should have $R_F > 0$.

The eluent strength range can be chosen more accurately by determination of the R_F values of the sample components in several mixtures of A and B. A plot of R_F *vs.* % B yields the optimum range of the gradient. For instance, Fig. 5 shows that a gradient of 10 to 80% B should be suitable; the mixture in Fig. 5b cannot be separated by a gradient of 10 to 100% B because some of the components have too low R_F values even with pure solvent B and it is necessary to use a wider eluent strength range by the addition of a third solvent C ($\varepsilon^0_A < \varepsilon^0_B < \varepsilon^0_C$).

Gradient elution and correction of gradient program

The gradient program chosen from the preliminary experiments may require

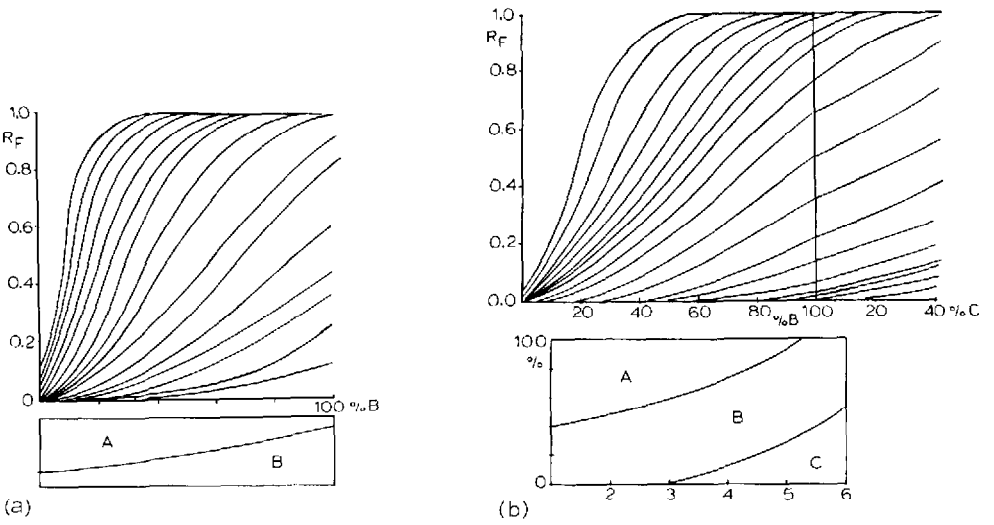

Fig. 5. Examples of the relationships between R_F and the modifier concentration for multicomponent samples and the corresponding gradient profiles required for their separation ($\varepsilon_A^0 < \varepsilon_B^0 < \varepsilon_C^0$).

correction of its eluent strength range and profile. Comparison of the graphical program and the resulting chromatogram according to the principles illustrated in Fig. 6a shows the changes in the gradient shape required to improve the distribution of zones along the plate. Several examples of the correction of gradient profiles are illustrated in Fig. 6a–c.

(a) For the program 10% ethyl acetate (in chloroform) to 100% ethyl acetate most of the spots are accumulated in the upper part of the chromatogram: the range of eluent strength was too high or the profile too steep. Suggested change: replace chloroform and ethyl acetate by weaker solvents, *e.g.*, trichloroethylene and diisopropyl ether, respectively or use a less steep gradient, *e.g.*, 5% ethyl acetate (in heptane) to 30% ethyl acetate.

(b) Most zones are accumulated in the lower part of the chromatogram. Suggested change: use stronger component solvents (B and C instead of A and B) or a ternary gradient A + B + C.

(c) Upper spots are well separated, accumulation of spots having low R_F values with a gap between the two groups of components. Suggested change: steeper gradient of B in the middle fractions and addition of a stronger modifier C to the last fractions of the eluent.

(d) Most spots are accumulated in the central part of the plate. Suggested change: increase slope at the beginning and the end of the gradient and use isocratic elution in the middle. This will decrease the distances between vicinal zones near $R_F = 0$ and $R_F = 1$ and increase the distances near $R_F = 0.5$.

Analogous rules can be formulated for reversed-phase systems. The type of relationship between the k' values and the modifier concentration should be taken into account[20–23]. For plain silica (and generally for polar adsorbents, *e.g.*, diol or aminopropyl silica)

$$\log k' = \text{constant} - n \log (\% \text{ B})$$

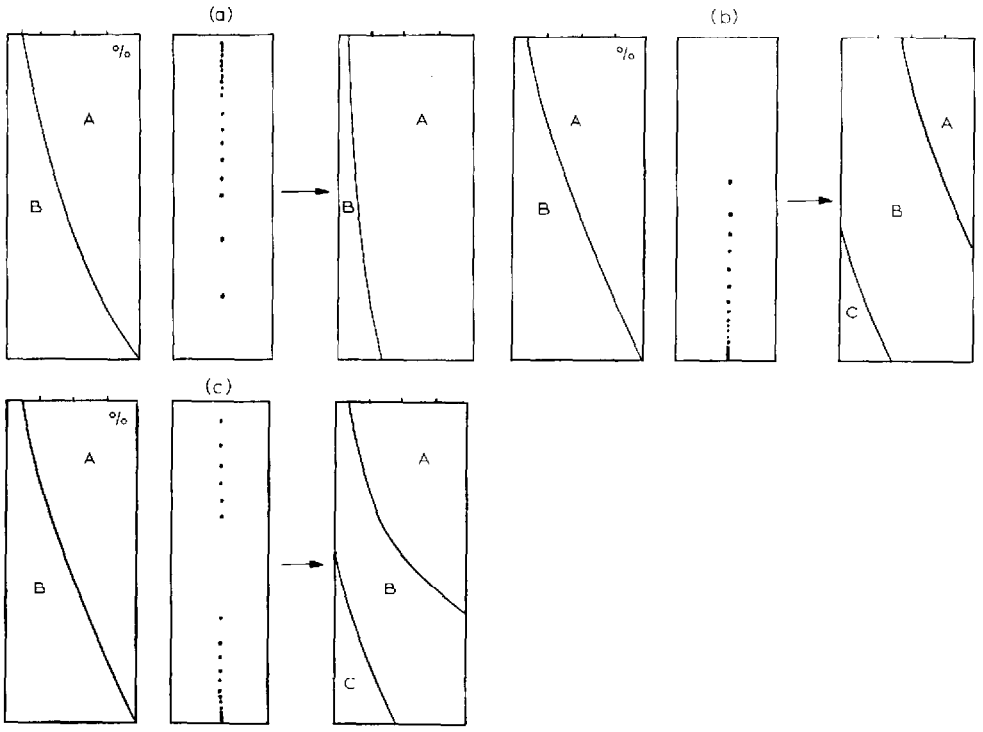

Fig. 6. Improvement of the distribution of spots along the chromatogram by adjustment of the gradient profile. (see text).

and a linear increase in eluent strength is obtained for exponential gradient profiles. For reversed-phase adsorbents and aqueous–organic eluents

$$\log k' = \text{constant} - n\ (\%\ \text{B})$$

and the eluent strength, ε^0, of the mixed solvent increases linearly with the concentration of the modifier B, so that a linear gradient profile is recommended. Owing to the limited wettability of most reversed-phase adsorbents at higher water concentrations, the program should be started from a suitable content of the modifier, *e.g.*, 60% B. It is recommended to prewet the layer with pure modifier; the sample is spotted 1 cm behind the eluent front.

It is advantageous to spot various volumes of the sample solution, *e.g.*, in the proportion 1:2:4, which enables an estimate of the optimum sample size for the detection of the maximum number of spots.

Gradient development of TLC plates is especially favourable in zonal separations carried out to isolate single components from complex mixtures. Even for mixtures of unknown components it is frequently possible to compare the chromatograms obtained from different elution programs owing to the presence of compounds that have a characteristic fluorescence under UV light. The high efficiency of gradient elution is caused by the flattening of the spots by the gradient and good

conditions for mutual displacement. Displacement effects are presumably strongest in the case of numerous adsorption–desorption processes of individual solute molecules, *i.e.*, in the range of moderate k' values. With increasing eluent strength of the mobile phase, consecutive components gradually attain this condition and separation takes place close to the start line[8]. The application of the sample from the edge of the layer (frontal + elution chromatography)[24,26] greatly increases the capacity of the system: even wide starting zones are partly separated and during gradient elution form narrow, well separated bands.

An important advantage of gradient TLC should be emphasized: for isocratic elution, a change in the eluent composition improves the separation in a fragment of the plate at the cost of a deterioration in the resolution in another fragment; all R_F values are changed (Fig. 1a,b). In gradient elution it is possible to vary the R_F values in a poorly separated fragment of the plate without changing those in the remaining part (Fig. 6c).

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