6

Gradient Development in Thin-Layer Chromatography

Władysław Gołkiewicz

Medical University, Lublin, Poland

I. INTRODUCTION

The separation of multicomponent mixtures by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) under fixed experimental conditions is often complicated by large differences in the polarity of the various components. To deal with this problem, eluents of low strength are needed to separate the less strongly retained solutes, whereas the strongly retained components of the mixtures can be separated by eluents of high strength. This is referred to as the general elution problem (1), and in TLC it can be handled in various ways: gradient elution (stepwise or continuous), stationary-phase gradient, polyzonal TLC, or temperature programming. These various techniques are based on different band migration rates of the components of the mixture during the separation process.

Gradient development in liquid chromatography stands in contrast to isocratic elution, in which the conditions of separation are not changed throughout the time required for the sample separation. In gradient development the situation is different: The conditions of separation (mobile-phase concentration, composition of the adsorbent layer, temperature, etc.) are changed during the separation. These continuous or stepwise changes in the separation conditions lead to changes in the relative migration velocity of the components of a sample. For example, if the concentration of the stronger solvent in a binary mobile phase increases, the eluent strength and R_f values of all solutes are also increased. As a result, separate optimization of the R_f values of individual bands is possible.

Gradient development in TLC is a technique that allows one to improve the resolution of a given pair of adjacent bands, to accelerate a separation, to concentrate the sample band and lower the detection limit, and to speed up the search for an optimal chromatographic system.

Successful separations of many complex mixtures by HPLC gradient elution have demonstrated the utility of this technique (1–5). In contrast to HPLC, gradient development in TLC has been applied relatively rarely, owing to the rather complex devices required for the generation of reproducible gradients and the lack of a simple theory of gradient development. Niederwieser and Honegger (6,7) systematized many experimental results and outlined some theoretical problems.

Recently, gradient development in TLC has become more popular, as evidenced by papers on theory (6–15), devices for gradient development (16–23), and the preparative mode (24).

The purpose of this chapter is to acquaint the reader with the most popular gradient techniques in TLC, including their characteristics, advantages, and limitations.

A. History of Gradient Development in TLC

The idea of using gradient development in column chromatography is ascribed to work by Tiselius and coworkers in 1952 (25), but as early as 1949, Mitchell et al. (26) used salt and pH gradients for the separation of some enzymes.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Gradient elution was applied in TLC in 1962 by Wieland and Determan (27) and by Rybicka (28,29). Wieland and Determan (27) used gradient elution to separate LDH isozymes and nucleotides on DEAE-Sephadex. Rybicka (28,29) used gradient elution to separate glycerides and pentaerythritol esters. Later, Niederwieser and coworkers (6,7,30,31) worked intensively to improve this technique.

Gradients in the stationary phase made slower progress, probably owing to the difficulties with devices for spreading the adsorbent layer. Berger et al. (32) used a modified spreader usually used for normal TLC. Later, improved devices for spreading layers were described by Stahl (33,34) and Warren (35).

The use of a temperature gradient was introduced in 1961 by Liteanu and Gocan (36), whereas Turina et al. (37) described an adapter for evaporation of the solvent during development of a plate.

Geiss et al. (38,39) and De Zeeuw (40) described a special chromatographic chamber for impregnation of adsorbent layer with vapors of various solvents. These resulted in the formation of an activity gradient of the adsorbent layer.

B. Nomenclature in Gradient Development

In TLC, in contrast with column chromatography, it is possible to apply a gradient in a direction other than the direction of flow of the eluent.

Niederwieser (31) introduced a rational system for full description of gradients. According to the definition given by that system (31), the arrow of gradient direction ponts to the chromatogram region where the sample components show their greatest mobility. In the case of an adsorbent gradient, the arrow points to the region of lowest activity. In the case of a mobile-phase gradient, the arrow points in the direction of greatest solvent strength.

Each separation process using a gradient development is based on a combination of two vectors that define the gradient direction and solvent flow direction (Fig. 1). When the gradient direction is congruent with the solvent flow direction, the gradient arrangement is termed parallel (p); in the reverse case, when the solvent flow direction is opposite to the gradient direction, the gradient arrangement is said to be antiparallel (ap). The stationary-phase gradient can exist either parallel to the solvent direction flow or at right angles to the solvent flow. In the latter case, the term orthogonal (o) gradient is used.

Definitions of gradient directions (31) are illustrated in Fig. 1.

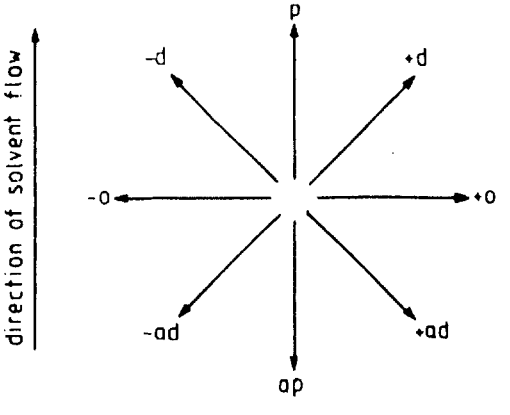

Figure 1 Nomenclature of gradient arrangement related to the direction of solvent flow. For definition of the gradient direction, see the text: p = parallel, d = diagonal, o = orthogonal, ad = antidiagonal, ap = antiparallel. (Reprinted from Ref. 31 with permission.)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

C. Classification of Gradients According to Their Shape

According to Niederwieser's (31) definition, gradient TLC is "a chromatographic technique using within the separation area locally different separation conditions." Separation conditions can vary in both the stationary and mobile phases. Taking into account these variations, chromatographic gradient techniques can be classified (3) as follows:

Mobile-phase gradients
- Composition
- pH
- Ionic strength

Stationary-phase gradients
- Composition
- Impregnation
- Activity

Gradients connected with change
- Temperature
- Flow rate
- Vapor pressure

The greatest possibilities of achieving gradients are offered by changing the mobile-phase concentration. Some examples of different shapes of gradients are presented in Fig. 2. The concentration of the more efficient solvent in the mobile phase can vary linearly (Figs. 2b and 2e) or curvilinearly (Figs. 2a, 2c, 2d, 2f). In practice, a continuous gradient is preferred (1,4,5), but stepwise gradients are much easier to obtain. It should be emphasized that if several steps are used in a stepwise gradient, then the gradient obtained is almost identical with a continuous gradient (41,42).

II. APPARATUS FOR GRADIENT DEVELOPMENT

Which device is used for generating the gradient depends on the type of gradient desired. The greatest number of devices have been described for generating mobile-phase gradients. Some of the most typical devices are presented here; however, so far there is no single best one.

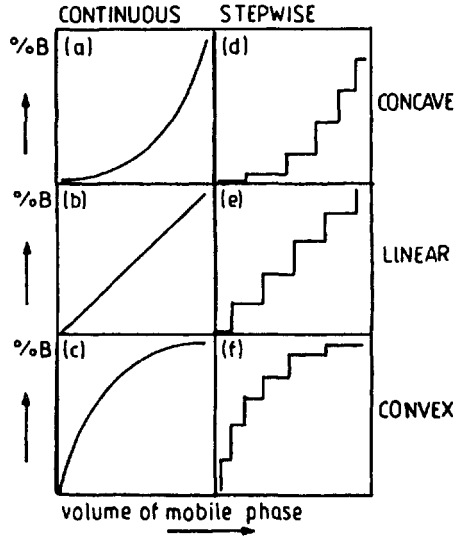

Figure 2 Classification of gradients according to their shape.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

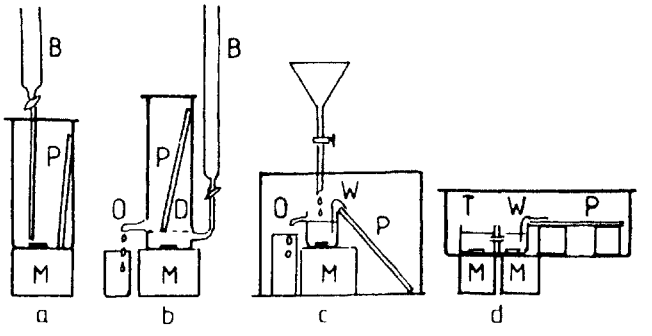

Figure 3 Devices for gradient elution in TLC. (Reprinted from Ref. 7 with permission.)

A. Devices for Achieving a Mobile-Phase Gradient

In gradient elution, devices for generating both continuous and stepwise gradients are used. Details related to the devices are described by Liteanu and Gocan (3) and by Niederwieser and Honegger (6). Some devices for generating continuous gradients are presented in Fig. 3.

Rybicka (28,29) employed a normal separation chamber (Fig. 3a) equipped with a magnetic stirrer (M) and a buret (B) containing the stronger solvent. Wieland and Determan (27) used a glass cylinder divided by a filter plate into a 1 cm deep mixing chamber equipped with a magnetic stirrer and an upper separating chamber (Fig. 3b).

Luzatto and Okoye (45) used a descending chromatographic technique (Fig. 3c) and a paper wick (W) as a capillary bridge between the mixing chamber and the chromatographic plate.

In Strickland's (46) device (Fig. 3d), a polyethylene trough (T) is divided along its entire length into two equal compartments filled with different solvents and stirred by magnetic stirrers (M). The partition wall between the compartments has two holes through which solvents are able to mix. The eluent from the trough is delivered to the plate (P) by means of a filter paper strip (W).

The devices described (Fig. 3) have some disadvantages: They produce only one type of gradient profile (mostly a convex gradient, Fig. 2c), and they require magnetic mixing and a considerable excess of solvent.

The delivery of the solvent to the adsorbent layer should be determined by the migration rate of the eluent front; otherwise deformation of the gradient shape will occur (6).

Niederwieser and coworkers (7,43,44) described a system that allows free choice of gradient shapes, involves reproducible partial mixing of two neighboring solvents in a capillary tube, and requires only as much solvent as the adsorbent layer can absorb. Their device (Fig. 4) differs

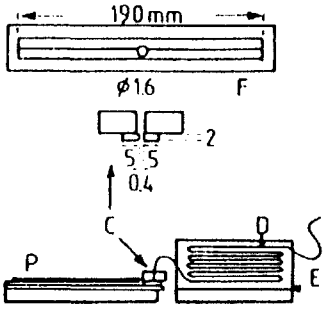

Figure 4 Device for solvent gradient TLC according to Niederwieser et al. (7). (Reprinted from Ref. 7 with permission.)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

from the other devices in that a long PTFE capillary tube serves as an eluent reservoir. A PTFE capillary (D), with an inner diameter of approximately 1.5 mm and several meters long, is mounted wavelike on a table (E). The eluent fractions are sucked into the capillary tubing in reverse order. The device (3,6,7) basically consists of a chromatographic plate (P) (Fig. 4), covered with glass plate, the all-glass distributor (C), Teflon tubing (D), and the table (E).

Consecutive portions of the eluents, with increasing amounts of the more efficient solvent, are introduced and stored in a length of PTFE tubing. The outlet of the PTFE tubing is put into the distributor hole, and the eluent coming out of the tube is distributed along the lower edge of the adsorbent layer. The stepwise gradient thus obtained is analogous to a continuous gradient because the profile becomes diffuse in the development process.

Sander and Feld (16) used a liquid chromatograph (solvent programmer in conjunction with two pumps) to generate a mobile-phase gradient. The eluent was introduced into the developer trough and distributed across the layer.

Soczewiński and Matysik (21) proposed a simple device, without a magnetic mixer, coupled with a horizontal sandwich chamber. The device consists of two vessels with two solvents, which mix spontaneously owing to density differences and the formation of molecular complexes (e.g., chloroform–ethyl acetate). They also showed (22) that stepwise gradient elution can be easily performed in a sandwich chamber with a glass distributor (41,47) (Fig. 5). Matysik and Soczewiński (23) also described a device that is a modification of the system introduced by Niederwieser and coworkers (7,43,44).

Burger (17) and Jaenchen (18,19) described a fully automatic machine for multiple development of a plate. An elution gradient is employed in accordance with the gradient program (see also Chap. 5 in this Handbook).

Vajda et al. (20) applied a device originally used for overpressured layer chromatography (OPLC) to multiple step-gradient development. The modified OPLC equipment, with loops filled with the different solvents, can generate a stepwise gradient by switching solvents with a two-position, 10-port valve (for details, see Chap. 7 in this Handbook).

B. Devices for Achieving Stationary-Phase Gradients

Discontinuous gradients in the stationary phase can be conveniently produced using a normal TLC spreader. The spreader cylinder is divided into two (32) or more (31) compartments by the intro-

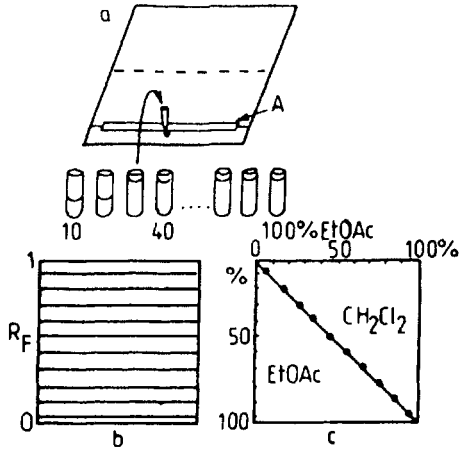

Figure 5 Stepwise gradient elution in a sandwich chamber with a glass distributor (A) of the eluent. (a) 0.4 mL portions of eluents of increasing solvent strength are introduced under the distributor and from the edge of the layer; (b) developed chromatogram with zones of the mobile phase and a stepwise profile of the gradient; (c) corresponding graphical representation of the (approximated) continuous gradient. (Reprinted from Ref. 22 with permission.)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

duction of close-fitting pieces of PTFE. The compartments are filled simultaneously to equal height with suspensions of different adsorbents. The plates are coated in the usual way.

Impregnation gradients are usually obtained by immersing a chromatographic plate for a moment in a solution of the impregnation agent or by suspending the adsorbent in a solution of the impregnation agent and simultaneously spreading the different suspensions on the plate (3,31).

Stahl (33,34) described an apparatus for obtaining continuous stationary-phase gradients that maintained the basic construction principle of the normal spreader. A rectangular case divided diagonally into two compartments by a partition wall is filled with two different adsorbent suspensions. When the sliding bottom of the case is opened, the suspensions fall into the spreader cylinder, which is divided into several small compartments, and mix in various proportions. After mixing of the compartments' contents, the plates are coated in the usual way (for details see Refs. 3, 6, 31, 33, and 34).

Activity gradients on adsorbent layers are very convenient (48,49). The Vario-KS chamber permits preadsorption of vapors on the adsorbent layer, which is placed face down over a tray that contains various solvents. The removable tray consists of many rectangular troughs that can be filled with different solvents or humidity-controlling liquids (details are in Ref. 49). The eluent is in a separate trough and can be delivered to the adsorbent layer by a wick.

III. GRADIENT ELUTION

A. Polyzonal Thin-Layer Chromatography

Polyzonal TLC (6,7) can be carried out only in a cooled sandwich chamber. Experience has shown that the phenomenon of solvent demixing can take place mainly in sandwich chambers. If a binary mobile phase migrates through an adsorbent layer, e.g., silica gel, the molecules of stronger solvent are preferentially adsorbed, resulting in demixing of the mobile phase. This effect is the basis of frontal analysis and polyzonal chromatography (6,7). The demixing effect is more pronounced when a cooled sandwich chamber is used (for example, a Brenner-Niederwieser chamber). The demixing effect is also more pronounced if the components of the mobile phase differ strongly in eluent strength.

When the solvent molecules are selectively adsorbed during the separation process and solvent demixing occurs, the α zone, containing only the weak solvent, is formed. Behind the α zone, the β zone, containing in the stationary phase the molecules of the stronger solvent, is formed. The β zone is separated from its predecessor by the β front. Zone and front formation with a ternary mobile phase are illustrated in Fig. 6.

The migration rates of the fronts are different and can be expressed by the retardation factor

$$k_\beta = \frac{\text{Distance from immersion line to } \beta \text{ front}}{\text{Distance from immersion line to } \alpha \text{ front}}$$

The k_β factor for a given adsorbent and mixed eluent is a function of the concentrations of

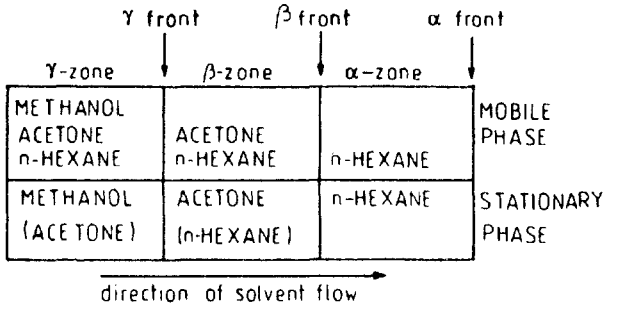

Figure 6 Phase formation with multicomponent solvents (polyzonal TLC) in an unsaturated sandwich chamber.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

the individual components in the developing solvent; it increases with an increase in concentration of the stronger solvent. The height and steepness of the gradient and length of the zones depend on the nature and the concentration of the eluent components. [The gradient steepness for linear gradients can be expressed as the percent per minute change in the concentration of solvent B (Fig. 2) or, for nonlinear gradients, as the average percent per minute change in the concentration of solvent B.]

A developing solvent that contains n components will give n zones separated by $n - 1$ fronts. In polyzonal TLC, it is of particular interest to vary the distance between the immersion line and the starting point of the mixture. This can be done by applying the mixture solution several times at different distances from the immersion line. Any changes in the mobile and stationary phases during chromatography influence the behavior of the solute, depending on the distance between the starting point and the immersion line. As can be seen in Fig. 7, the complete chromatographic separation of a complex mixture can often be conveniently carried out by the use of two or more different starting points. Spots 4 and 7 from the first starting point (first mixture from the left side) are not separated, although spots 8 and 9 are well separated. The situation is different for the second starting point: Spots 8 and 9 are not separated, in contrast to 4 and 7.

During the chromatographic process, molecules of each new solvent displace the solvent molecules of lower eluent strength and push the demixing front nearer to the α front. Generally, it is advisable to create conditions that allow these fronts to spread in equal proportions over the entire development distance (6).

The greater the differences between the components of the mixture to be separated, the greater must be the range of solvent strengths of the components of the eluent.

In general, mixtures in equimolar amounts of the lower representatives of any homologous series are frequently used. The following mixtures are useful (6):

Chlorinated hydrocarbons: carbon tetrachloride–chloroform–methylene chloride (96:80:64)

Ethers: diisopropyl ether–diethyl ether–dioxane (141:104:85)

Esters: *n*-butyl acetate–*n*-propyl acetate–ethyl acetate–methyl acetate (132:115:98:80)

Ketones: cyclohexanone–diethyl ketone–methyl ketone–acetone (103:106:90:73)

Alcohols: *n*-butanol–*n*-propanol–ethanol–methanol (92:75:58:40)

Polyzonal TLC with a multicomponent mobile phase represents the simplest technique for stepwise gradient elution. A continuous gradient can be realized only if the eluent contains a great number of different components with very small increments in solvent strength. However, because

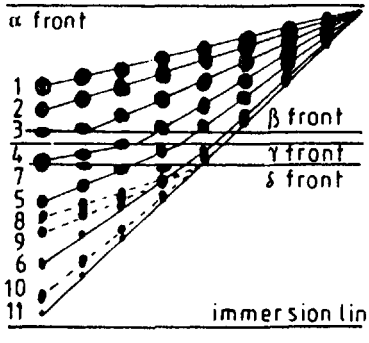

Figure 7 Polyzonal chromatogram of a mixture containing, in 0.5 μL, 1 μg each of the 2,4-dinitrophenyl derivatives of the following amines and amino acids: (1) *n*-amylamine, (2) *n*-butylamine, (3) *n*-propylamine, (4) ethylamine, (5) methylamine, (6) tyramine, (7) leucine, (8) methionine, (9) proline, (10) hydroxyproline, and (11) glutamine, with separation starting from eight increasingly higher origins. Silica gel G (Merck), air-dried layer (relative atmospheric humidity, 50%), BN chamber, solvent isopropylether–propionic acid–acetic acid–formic acid (100:0.66:0.66:0.66). (Reprinted from Ref. 30 with permission.)

each solvent has a different elution effect, such a mixture can seldom be realized. For a general discussion of polyzonal TLC, see the review by Niederwieser and Honegger (6).

B. Mobile-Phase Gradient

Complex, multicomponent mixtures containing components with a wide range of R_f values ($0.01 \leq R_f \leq 0.9$) cannot be separated by isocratic elution owing to the general elution problem (1,50). Eluents of low eluent strength separate the less strongly retained solutes, whereas the strongly retained components are eluted with very low R_f values. On the other hand, strong eluents do not separate weakly retained components, which migrate together and exist on a chromatogram as common or partly resolved spots.

The general elution problem (1) in HPLC is usually solved by application of gradient elution (1–5,41,42). The technique can also be applied in TLC (3,6–16,21–24,28–31).

Usually we are concerned with two-component gradients composed of a weak solvent A and a strong solvent B. The concentration of the stronger solvent B can be varied linearly or curvilinearly (convex or concave, Fig. 2), from pure A to pure B (for details, see Ref. 50, pp. 668–686), so the concentration of B in the mobile phase entering the chromatographic plate increases throughout the separation. The eluent is initially weak and becomes progressively stronger as separation proceeds. In this case, the gradient applied is antiparallel.

It is well known that the sample R_f values depend on the concentration of the stronger solvent in a binary mobile phase, so in gradient elution variations in sample retention are achieved almost exclusively by changes in the mobile-phase concentration.

A stepwise gradient, which is more easily achieved in practice and easier to understand, is considered first. In most cases, the stepwise gradients are produced in sandwich chambers equipped with special solvent distributors (6,7,9–16,21–24).

The space under the distributor, a strip of glass ($1.3 \times 5 \times 95$ mm for a 100×200 mm plate) placed over a margin of the carrier plate cleaned of adsorbent (see illustrations in Refs. 11, 22, and 51), is consecutively filled with up to 0.5 mL of the eluent. The first eluent fraction is, e.g., 10% ethyl acetate in chloroform, the second 20%, and the last is pure ethyl acetate. Each eluent fraction is introduced under the distributor with a micropipet after complete adsorption of the preceding fraction by the layer. If the difference between concentrations in two consecutive steps is relatively small and the gradient is partially smoothed during the separation process, the profile becomes approximately a continuous gradient (51). Five eluent fractions of increasing eluent strength are usually sufficient to avoid marked accumulation of spots on the front between two consecutive zones, as can occur in polyzonal TLC.

C. Optimization Strategy in Gradient Elution

Analysis of the distribution of the spots along a chromatogram enables the formulation of simple quantitative rules of gradient optimization for a particular gradient program. Some of the most important rules were given by Soczewinski (51).

1. Choice of Eluent Strength Range

Soczewinski (51) proposed the following series of solvents for use on silica gel [eluent strength, ε° values (52), in parentheses]: heptane (0.0), trichloroethylene, dichloromethane (0.32), diisopropyl ether (0.34), ethyl acetate (0.38), isopropanol.

The gradient program should start from weak 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 if the R_f values of the sample components in several mixtures of A and B are determined. A plot of R_f versus %B will guide the choice of the optimum range of the gradient. For instance, Fig. 8a shows that a gradient of 10–80% B should be suitable; the mixture in Fig. 8b (51) cannot be separated by a gradient of 10–80% B, because some of the components have R_f values that are too low, even with pure

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

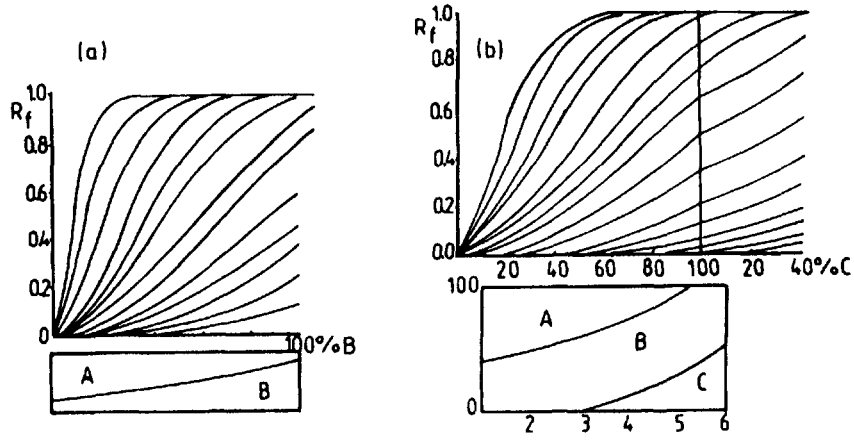

Figure 8 Examples of the relationships between R_f and the modified concentrations for multicomponent samples and the corresponding profiles required for their separation ($\varepsilon^\circ_A < \varepsilon^\circ_B < \varepsilon^\circ_C$). (Reprinted from Ref. 51 with permission.)

solvent B. In this case, it is necessary to use a wider eluent strength range by using a three-component mixture, A + B + C ($\varepsilon^\circ_A < \varepsilon^\circ_B < \varepsilon^\circ_C$).

2. Gradient Elution and Correction of Gradient Program

With a good gradient elution program, no sample component moves with the solvent front or remains at the starting point.

The gradient program chosen from the preliminary experiments may require correction of eluent strength range and profile. Comparison of the gradient program and the resulting chromatogram (Figs. 9 and 10) shows that changes in gradient shape are required. Two examples of the correction of gradient profiles are given in Figs. 9 and 10.

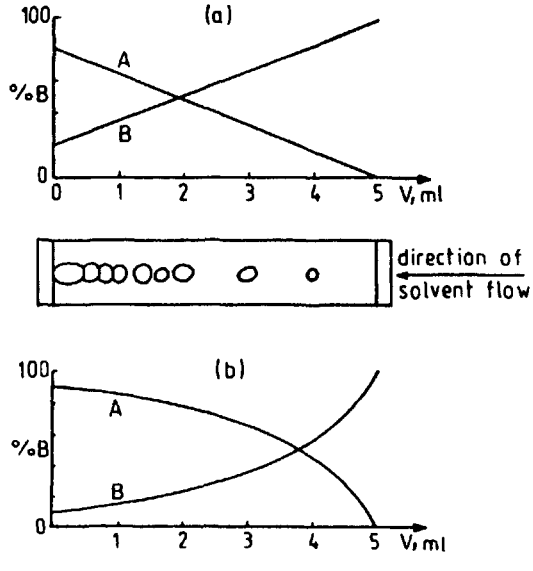

Figure 9 Adjustment of the gradient profile to improve the distribution of spots along a chromatogram (see text).

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

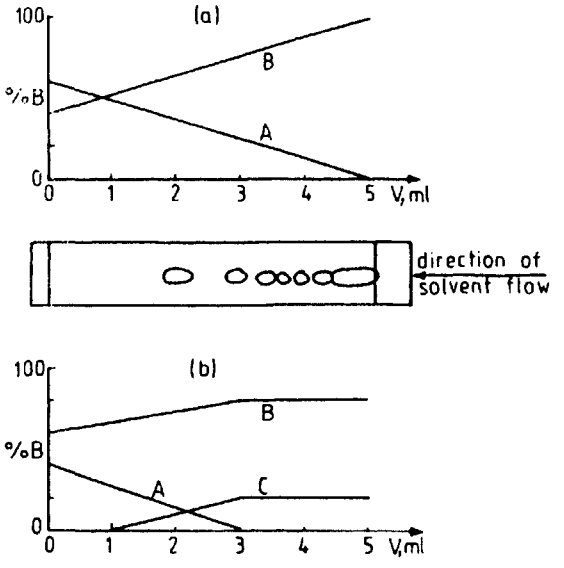

Figure 10 Adjustment of the gradient profile to improve the distribution of spots along a chromatogram (see text).

1. For the linear gradient from, for example, 20% ethyl acetate in methylene chloride to 100% ethyl acetate (Fig. 9a), most of the spots accumulate in the upper part of the chromatogram. This means that the initial concentration of B and the range of eluent strength were too high. Suggested changes in the gradient profile and initial concentration of B are illustrated in Fig. 9b. Changing the shape of the gradient from linear to concave and lowering the initial concentration of ethyl acetate to 10% should improve the distribution of spots along the plate.
2. Most spots on the chromatogram presented in Fig. 10 accumulate in the lower part. Suggested changes include the use of a stronger solvent C in a mixture of A and B, or a ternary gradient, as shown in Fig. 10b.

For other examples, see Ref. 51.

It should be emphasized that the high efficiency of gradient elution is caused by flattening of the spots due to varying eluent strength and mutual displacement of the sample components. In many cases, it is possible to detect about double the number of spots relative to isocratic elution. It is also possible to vary the R_f values in a poorly separated region of the chromatogram without changing those in the remaining part (51).

The same rules can be applied to continuous gradients, but in this case the situation is more complex. Continuous gradients provide better separation of complex samples, but their applications are relatively scarce because rather complex devices are required to generate reproducible gradients. In many devices, a mixer is used and excess overflowing eluent is discarded, so the user cannot know which section of the elution gradient is responsible for the separation of which fractions.

D. Stationary-Phase Gradient

A stationary-phase gradient in TLC involves a continuous or discontinuous change in the composition or activity of the adsorbent layer along the plate (8). A gradient of the stationary phase can be applied either parallel to the direction of solvent flow or at right angles to it (orthogonal gradient). The latter gradient is equivalent to using several different plates of varying adsorbent composition in searching for the best TLC system.

As was shown in Section II.A, adsorbent gradients can be achieved in several different ways. For example, a strong adsorbent (e.g., silica gel) is mixed with varying proportions of a weak adsorbent (e.g., kieselguhr). As a result, an adsorbent gradient is formed along the plate. In fact, gradients composed of silica gel and kieselguhr have not fulfilled expectations. Greater dilution of the silica gel with kieselguhr (or other adsorbent of low surface area) results in reduced capacity and overloading of the initial part of the plate (31).

Layers containing a discontinuous adsorbent gradient usually consist of a narrow zone of adsorbent A along the lower edge of the plate and an adsorbent B on the remaining part of the plate [layers with five zones of different adsorbents were also proposed (53)]. Discontinuous adsorbent gradients are used for three purposes:

1. To adsorb some interfering components of the sample at the starting point (31,32). The adsorbent in zone A strongly retains the unwelcome substances, e.g., an ion-exchange of complexing mechanism, but it does not retain the rest of the components of a mixture.
2. To carry out two-dimensional TLC. In the first direction, isocratic TLC occurs along the zone of adsorbent A. In the second direction, prefractionated sample components enter the layer of adsorbent B, which differs as much as possible from adsorbent A, for example, in pH or the presence of a complexing agent (31).
3. To concentrate the spot applied on a narrow preconcentration zone of a very weak adsorbent (e.g., kieselguhr). During development by an eluent, the spot is concentrated into a narrow band because the solvent strength is too high for such a weak adsorbent.

Many examples of continuous and discontinuous adsorbent gradients applied in practice are given by Niederwieser (31) and by Liteanu and Gocan (3).

The adsorbent layer can also be exposed to solvent vapors in special sandwich-type chambers that permit various solvent vapors to contact different parts of the plate, resulting in an adsorbent activity gradient along the plate. This technique is called preloading (43) or vapor-programmed gradient TLC (40).

If the chromatographic plate is exposed to the vapors of a strong solvent such as acetone, the adsorbent layer is highly deactivated and high R_f values are obtained. The opposite effect would occur for a weak solvent such as hexane. A vapor-programmed gradient can also be applied either parallel to the direction of solvent flow or at right angles to it (for details, see Ref. 49). This method of gradient generation is relatively simple. However, the actual composition of the adsorbent layer and the gradient shape are virtually unknown.

E. Automated Multiple Development

Perry et al. (54,55) introduced in 1973 a new technique called programmed multiple development (PMD), in which the TLC plate was repeatedly developed in the same direction with the same solvent. Burger (17) improved this technique but maintained the general principles of PMD. The Burger (17) method is called automated multiple development (AMD). The characteristics of the AMD system are as follows (17–19):

1. A TLC plate is repeatedly developed in the same direction with solvents that differ from one step to the next.
2. Each developing step is longer than the previous one (approximately 3 mm per step).
3. From step to step, the solvent strength is decreased.
4. Gradient elution is used, but, in contrast to HPLC, the gradient starts with the most polar solvent (usually a mixture of methanol and dichloromethane, 50:50) and ends with the weakest solvent (e.g., a mixture of dichloromethane and *n*-hexane).
5. Solvent is completely removed from the plate after each developing step so that the composition of the solvent introduced in the next step is not changed.
6. From 10 to 25 steps are necessary to develop a plate, which corresponds to a total developing time of 0.5–3 h and a total migration distance of 3–10 cm.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

A typical gradient in AMD usually consists of three or four solvents: methanol, acetonitrile, dichloromethane, and hexane.

In AMD, the chromatogram is developed under reproducible conditions so the user can compare it or its densitometric scanning curve with the profile of a elution gradient. This is demonstrated in Fig. 11 (19). Such a diagram allows the user to conclude which part of the gradient is ineffective and can be omitted (e.g., steps 1–18 for sample d) and which part should be modified. The samples of PTH amino acids (a), analgesics (b), and barbiturates (e) are resolved sufficiently, but some corrections of the gradient profile and eluent strength are necessary. Using the methanol–dichloromethane gradient over the full length of all 25 steps would probably improve the separation (19).

The dye mixture (Fig. 11d) migrates through 18 steps as a narrow band and begins to resolve when the hexane–dichloromethane gradient starts, so the first 18 steps should be omitted and a new experiment started with the dichloromethane–hexane gradient.

For mixtures of wide polarity differences, such as the pesticides (56), amino acid derivatives (57), alkaloids (58), or drugs (59), multiple development becomes the obvious choice. It enables the convenient stepwise application of solvent gradients for optimization of the separation of each group of compounds that migrate in a given solvent. Universal (60) solvent gradients are generated in a stepwise fashion, with as many solvents as required being employed to achieve the desired separation.

Universal AMD gradients (60) have found wide application, particularly for the analysis of crop protection agents in surface water (61–63), plant extracts (64), psychopharmaceutical drugs (65), and steroids (66). It was shown in many papers (61,63,67,68) that automation of the multiple development procedure increased the reliability and reproducibility of the method while minimizing operator time and errors.

AMD gradient elution was used for quantitative determination of eight pesticide residues (69) in soil that was considerably contaminated with petroleum derivatives. The excess of the petroleum derivatives was removed by solid-phase extraction. Another application of AMD gradients (70) was for the analysis of pesticide residues in drinking water. This method, elaborated for identification and quantification of active ingredients of plant-protecting agents in drinking and mineral water, has been accepted as standard in Germany.

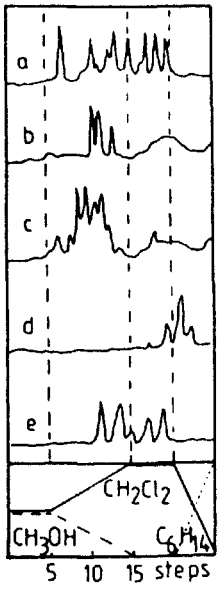

Figure 11 An application of the AMD technique. The densitometric scanning curve is superimposed on the diagram of the gradient profile. (Reprinted from Ref. 19 with permission.)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

A 25-step gradient based on methanol, diethyl ether, and hexane was used to separate the six major human plantar stratum corneum lipids (71). Peak heights as well as peak areas were used for densitometric quantification of separated lipids.

AMD-HPTLC gradient development enabled the separation and quantification of forskolin and its 10 derivatives (72). These diterpenoids have interesting pharmacological properties.

Multistep gradient elution can also be carried out with modified overpressured TLC equipment (20,74), described in Chapter 7 of this Handbook. Vajda et al. (20) applied the method to the analysis of the components of total lipid extracts from various human blood samples. Pick (74) used it for the chromatographic separation of membrane gangliosides. The advantage of the procedure consists in the removal of less polar solutes in the first stages of the gradient and separation of the polar gangliosides in the last stages.

IV. OPTIMIZATION OF STEPWISE GRADIENT ELUTION

A. Graphical Method

Consider the elution of a given solute by a two-component mobile phase on a chromatographic plate during stepwise gradient elution (10,12). The length of the plate is assumed to be unity. The composition of the binary mobile phase is defined in terms of the concentration of the stronger solvent. It is assumed that the composition of the mobile phase changes gradually during elution but is constant in each step. The elution model is presented in Fig. 12 (12).

Assuming a constant mobile-phase flow rate, the straight line $0Y$ shows the migration of the mobile-phase front. The migration rate of compound A is lower than that of the mobile phase, and after one dead volume of eluent has passed through the bed, the R_f value of compound A is 0.2 (point A in Fig. 12). When the front of the mobile phase of 5% concentration reaches the end of the plate ($R_f = 1.0$), the concentration of the eluent is changed stepwise. The solvent front is observed by means of a marker (azulene or azobenzene) whose R_f value in the solvent system is close to unity. The line $0'Y'$ in Fig. 12 indicates the migration of the mobile phase of 10% concentration. Obviously, the front of 10% mobile phase will, after some time, overtake spot A, which traveled until then in the mobile phase of 5% concentration (section AA'). From point A' onward, the spot travels in the mobile phase of 10% concentration. It is assumed that the R_f value for compound A in the mobile phase of 10% concentration is 0.3. To find point B, a length $A'C$ corresponding to one dead volume V_m is marked, and a section equal to 0.3 R_f unit from point C is measured. Upon connecting points A', B, B', the migration of the spot A in the 10% mobile phase and the final R_f value are obtained.

If the R_f values obtained in several isocratic elution steps are known, the program for gradient elution can be constructed (11,12). Results of stepwise gradient elution of DABS-amino acids are

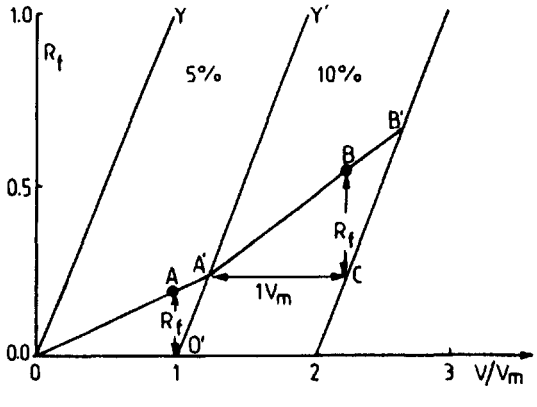

Figure 12 Graphical representation of the movement of sample A during stepwise gradient elution. (Reprinted from Ref. 12 with permission.)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

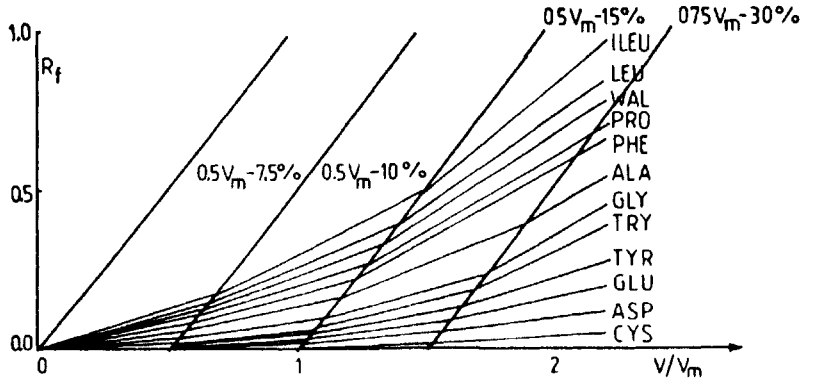

Figure 13 Results of thin-layer chromatography of DABS–amino acid derivatives. (Reprinted from Ref. 12 with permission.)

presented in Fig. 13 (12). The solvent concentration for the first step was chosen from the plot of R_f versus percent of the more efficient solvent (it is still better in normal-phase TLC to use R_m versus log % of the more polar solvent) by assuming that for the first eluted compound the R_f value should be equal to 0.25. In fact, half of the dead volume V_m of the eluent was used, so the R_f value in the first step is equal to 0.25/2 = 0.125 (see Fig. 13). Knowing the concentration in which the R_f for the first compound is equal to 0.25, the R_f values for the rest of the compounds were obtained in the same way from a plot of R_f versus %B. In the next steps, $0.5V_m$ of 10% and $0.5V_m$ of 15% concentrations were used (12).

If experimental conditions in isocratic and gradient elution are comparable (constant flow rate, temperature, thickness of layer, etc.), the R_f values for gradient elution determined graphically from $R_f = f(\%$ concentration), or better, $R_m = f(\log \%)$, and also experimentally differ by not more than 0.01–0.03 R_f unit (12). Both the shape of the gradient and the number of dead volumes of the eluents required to ensure that the final R_f values of compounds do not exceed $R_f = 1.0$ can be determined. This is particularly important in the separation of colorless compounds.

B. Numerical Method

1. Stepwise Gradient Elution

All recent gradient theories are based on the linear relationship, obtained under isocratic conditions, between log k (or $R_m = \log 1 - R_f/R_f$ in TLC) values and the logarithm of the molar fraction of the more efficient solvent in the binary eluent (in normal-phase chromatography) and between log k (or R_m in TLC) and the volume fraction of the organic solvent (e.g., methanol or acetonitrile) in an aqueous–organic eluent in reversed-phase chromatography (1,4,5,9,41,42).

Soczewinski and coworkers (13,75) derived an equation for the R_f values of solute chromatographed under stepwise gradient elution. Assuming a definite relationship between the k value and the modifier concentration, the final R_f values of solute j (considering that the last, hth, development step is incomplete) is

$$R'_f = \sum_{i=1}^{h-1} \frac{V_{(i)} R_{f(j,i)}}{1 - R_{f(j,i)}} + R_{f(j,h)} \left[1 - \sum_{i=1}^{h-1} X_{(j,i)} \right] \qquad \text{for } h = 1, 2, 3, \ldots \tag{1}$$

where

j = the number of the solute (the code)
i = the sequential number of the elution step (eluent fraction)
h = the number of the last step (in which the solute migrates through part of the concentration zone)
$R_{f(j,i)}$ = the R_f value of the solute (isocratic value) in the ith concentration zone

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

$V_{(j,i)}$ = the volume of eluent introduced in the ith step expressed as a fraction of total eluent volume used in the gradient elution

$X_{(j,i)}$ = the volume of mobile phase corresponding to the migration of solute j through the ith concentration zone

$r_{(j,i)}$ = the fractional distance traveled by solute in the ith step

The volume $X_{(j,i)}$ of mobile phase for sample j in the ith step can be calculated from the equation

$$X_{(j,i)} = \frac{V_{(i)}}{1 - R_{f(j,i)}} \tag{2}$$

As an example of the application of the present method, consider the stepwise gradient elution of a hypothetical sample j. R_f values of solute j in the mobile phase of different concentrations (fraction volume) are as follows:

Volume fraction of solvent B in eluent	0.05	0.1	0.2	0.3	0.4
R_f value	0.09	0.12	0.27	0.48	0.62

Assume that a five-step gradient with equal volumes of mobile phase in each step will be applied, so that $v = 0.2$ (one-fifth of the total volume of solvent used for gradient elution). The concentrations expressed as volume fractions in subsequent steps are 0.05, 0.1, 0.2, 0.3, 0.4.

The volume X of mobile phase for sample j in the first step of gradient elution can be calculated by using Eq. 2:

$$X_{(j,i)} = \frac{0.2}{1 - 0.09} = 0.22$$

The volume X in the second step is

$$X_{(j,i)} = \frac{0.2}{1 - 0.12} = 0.23$$

The volume X for the next two steps is

$$X_{(j,3)} = 0.27 \quad \text{and} \quad X_{(j,4)} = 0.38$$

The sum of the fractional volumes X is

$$X_{(j,1)} + X_{(j,2)} + X_{(j,3)} + X_{(j,4)} = 1.1$$

This is greater than 1.0, which means that solute j migrates through three concentration zones and partly into the fourth zone.

Knowing the R_f value of solute j under isocratic conditions, the value of the fractional distance $r_{(j,i)}$ can be calculated with the help of Eq. 1 (neglecting the second term) as

$$r_{(j,i)} = \frac{V_{(i)}R_{f(j,i)}}{1 - R_{f(j,i)}}$$

Then the fractional distance $r_{(j,1)}$ and $r_{(j,2)}$ values in the first and second steps are

$$r_{(j,1)} = \frac{0.2 \times 0.09}{1 - 0.09} = 0.02 \quad \text{and} \quad r_{(j,2)} = \frac{0.2 \times 0.12}{1 - 0.12} = 0.03$$

and for the third step, $r_{(j,3)} = 0.07$.

Now the final R_f value can be calculated for solute j during a four-step gradient:

$$R_f = (0.02 + 0.03 + 0.07) + 0.48(1 - 0.22 - 0.23 - 0.27) = 0.25$$

Markowski et al. (75) elaborated a microcomputer program for the calculation of final R_f

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

values obtained under stepwise gradient conditions. After introduction of R_f values of the sample components obtained for several isocratic runs, the microcomputer calculates R_f values for any gradient program and displays the paths of migration of the spots through the concentration zones. It is thus possible to study by computer simulation the final arrangement of spots for chosen programs of stepwise gradients.

2. Automated Multiple Development

Optimization of gradients in automated multiple development (AMD) can be achieved in three steps:

1. Selection of the "base" solvent (i.e., medium polarity) and at least two modifiers (very polar and nonpolar solvents)
2. Improvement of the separation by development of a final gradient with the appropriate range of eluotropic strengths of the solvent mixtures
3. Development of a suitable slope of a gradient (i.e., rate of change of the eluotropic strength with time)

Solvents with the selectivity necessary for the separation of the mixture are usually selected (57,58) with the help of the PRISMA model, based on Snyder and Kirkland's (76) solvent selectivity scheme. Selection of the correct base solvent from the different Snyder classes turned out to be critical to the optimization of selectivity.

The eluotropic strength of the binary solvent mixtures can be calculated using Snyder's equation (77).

When the individual components of the mixture to be analyzed are available, preliminary experiments based on isocratic development may be useful for selection of suitable solvents. The preliminary investigation may be performed as follows (56). The retention behavior of high and medium polarity standards in binary mixtures of strong and "base" solvent is carried out to determine the solvent strength range of the AMD gradient. Successive investigations using binary mixtures composed of the base solvent and (usually) hexane are carried out to optimize the separation of low polarity standards. The isocratic data obtained for different concentrations of binary mixtures are conveniently plotted as the relationship between R_m and solvent composition (9–12). Inspection of these plots gives useful information about the adequate solvent strength and the change in selectivity resulting from the change of base solvent and modifiers.

If the polarity range of an AMD gradient is such that insufficient resolution is obtained, the separation might be optimized by changing the gradient slope. Queckenberg and Frahm (58) stated that, in general, steeper gradients improve peak shape but reduce the resolution, whereas flatter gradients generate broader but better separated peaks.

Two gradient profiles are recommended: universal (56,61) and linear (58,59). Some authors (58,59) prefer a linear gradient because abrupt changes in eluotropic strength occur within the universal gradient (59), and some components of a complex mixture might coelute. The concentration of mobile phase at which the coelution occurred corresponded to an abrupt change in the eluotropic strength, thus explaining the results observed (59).

The optimization procedure is frequently carried out by the trial-and-error method (56–60,67) owing to the lack of a theoretical model of the multiple development process. Markowski and Soczewinski (78,79) formulated the physical model for AMD, which is useful for describing the migration of the solute zones and computer analysis of various parameters determining the final optimization of gradient.

Let us consider two-step gradient development (80). After a first development to the distance z_1, the R_f of the solute is equal to

$$y_1 = z_1 R_{f1}$$

where R_{f1} is the R_f value for the first eluent. The chromatogram is now dried and developed to distance z_2 with the second eluent, for which the solute R_f is equal to R_{f2}. However, the spot does not move until the solvent front overtakes it; thus, the real solute migration distance in the second step is $z_2 - z_1 R_{f1}$. The final R_{fg} value for the two steps of gradient is

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

$$R_{fg} = \underset{\text{first development}}{z_1 R_{f1}} + \underset{\text{second development}}{(z_2 - z_1 R_{f1}) R_{f2}}$$

Generalizing the situation for an n-step gradient, we can write

$$R_{fg} = \sum_{i=1}^{h=(n-1)} y_i + y_n = \sum_{i=1}^{h=(n-1)} y_i + \left(z_n - \sum_{i=1}^{h=(n-1)} y_i \right) R_{fn}$$

where R_{fg} is the final R_f value after the n-step gradient, $\sum_{i=1}^{h=(n-1)} y_i$ is the sum of the preceding fractional migration distances, y_n is the real R_f value in the last step, z_n is the development distance in the last step, and R_{fn} is the isocratic R_f value of the solute for the solvent used in the last step.

A computer program for the calculation of the final R_{fg} value, taking into account the development distances z_i, compositions of consecutive eluents, and the retention–modifier concentration relationship, was elaborated by Markowski (79).

V. GRADIENT ELUTION IN ANALYTICAL AND PREPARATIVE TLC

As demonstrated in many papers (23,81–83), much better separation efficiency is obtained for stepwise gradient elution than for continuous elution, especially in the case of plant extracts, owing to enhanced displacement effects.

Matysik and Jusiak (82) used stepwise gradient development for the separation of chelidonium alkaloids in waste industrial fractions. Binary (toulene–methanol) and ternary (toulene–ethyl acetate–methanol) mobile phases were used, and a six-step program was performed. Eight-step stepwise gradient elution was also used for separation of glycosides from *Digitalis* species (83).

Ergot alkaloids (84) and coumarin derivatives (85) were separated on TLC silica plates by using stepwise gradients with different solvents. Stepwise gradients have also been used to separate anthocyanins (86) in the petals of red poppy, furocoumarins (87), and anthraquinones (88).

Marked improvement of the separation of two plant extracts by the use of a modified program of stepwise multiple gradient development was reported (89). Modification lies in the fact that the chromatographic plate was developed over decreasing distances with eluents of increasing eluent strength.

Gradient development combined with densitometry is an efficient method for the analysis of plant extracts, because it eliminates preliminary purification of extract. Examples of such a procedure are presented in some papers, e.g., perstilbene (3,5-dimethoxy-2-hydroxy-*E*-stilbene) was satisfactorily separated by use of two-step gradient elution and quantified by densitometric techniques (90). In another work (91), plant extracts containing flavonoids were separated on HPTLC silica plates by two- and three-step gradient elution.

An HPTLC method with densitometric detection was used to determine the convallatoxine content of extracts from flowers, leaves, and underground parts of *Herba convallariae* (92). Plant extracts were separated on HPTLC silica plates by multiple gradient development.

Mycotoxins such as alternariol and alternariol methyl ether, produced by fungi of the genus *Alternaria*, were analyzed by stepwise gradient TLC (93). The obtained chromatograms were well suited for quantitative densitometric determination.

Two-step gradient elution was applied to separate the colored pigments of *Trichoderma harzianum* fermentation broth (94). The main fractions were identified by instrumental methods (IR, DAD detector, and MS) after gradient reversed-phase TLC. Additionally, multistep gradient elution developed for RP-TLC was successfully used as a pilot method for the rational design of a gradient elution program in RP-HPLC.

Fluorescein, the active component in the French preparation "fluoresceine," was quantitatively determined after gradient HPTLC development (95). Gradient mobile-phase TLC was also applied to the quantitative determination of prednisolone acetate in a Polish preparation "prednisolon" and in the aqueous humor of rabbit eyes (96).

Gradient development has occasionally been employed in preparative TLC chromatography. Soczewinski and coworkers (24,97) applied an equilibrium sandwich chamber (47) for systematic investigations of the formation of zones and separation selectivity in overloaded preparative liquid chromatography.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

The sample solution band (test dye mixture), applied from the edge of the layer, formed a partly separated starting zone (frontal chromatography stage). After adsorption of the sample by the adsorbent layer, the eluent was introduced under the solvent distributor, and the marker (azobenzene) was spotted. The movements of the marker and the dye zones were recorded on a transparent foil (97). By connecting the points representing the upper and lower boundaries of the zones, a dynamic picture of the movement and separation of the zones could be obtained.

Stepwise gradient elution has been applied to the overloaded zonal preparative TLC of complex, multicomponent plant extracts of the herbal medicines azulan and hemorigen (98) used in therapy. Stepwise gradient elution combined with application of extract from the edge of the layer markedly improved the separation efficiency and purity of fractions, which was revealed by densitometry.

Theoretical and practical problems related to computer-aided optimization of stepwise gradient development in TLC of plant extracts containing biologically active compounds were reviewed by Matysik and Soczewiński (99).

Figure 14 (24) illustrates the separation of a dye sample during (a) isocratic and (b) stepwise gradient elution. It can be seen that full separation is obtained only for gradient elution; in isocratic elution, zones of dyes 3 and 4 overlap.

In the case of a stepwise gradient, the zones, instead of spreading, become narrower and more compact. In consequence, the sample capacity is markedly higher. The improvement of separation in preparative stepwise gradient elution is caused by two mechanisms: mutual displacement of the components of the mixture to be separated and compression of the zones, described earlier for continuous gradients in HPLC (1,4,5). The compression of the zones results from the fact that the lower edge of a zone is overtaken by the mobile-phase fronts of increasing eluent strength earlier than the upper edge, so that the upper edge of the zone moves in the mobile phase of a lower eluent strength than the lower edge.

VI. CONCLUSIONS

Gradient development can be applied for the following purposes:

Separation of samples that contain many compounds with widely different retention values
Lowering of the detection limit by sharpening of the chromatographic zones
Speeding up the search for a better chromatographic system

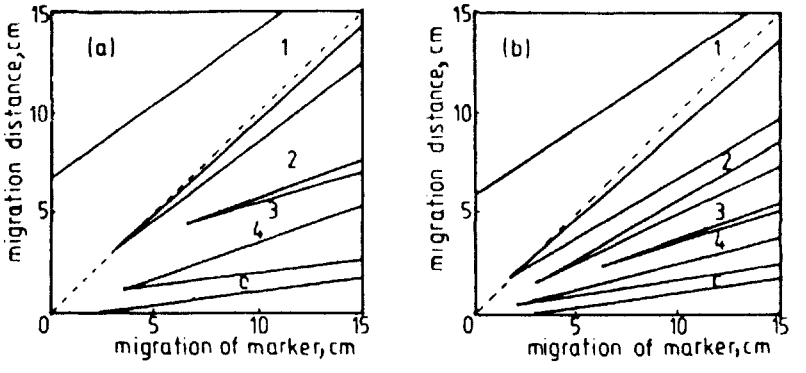

Figure 14 Dynamic representation of the migration of the bands of four test dyes. Sample: 1.5 mL of a 0.4% solution of 4-chlorobenzene-1-azo-1,4(*N*,*N*)-dimethylaminobenzene (1); disperse blue-Polanildunkelblau 3RT (2); disperse red-Polanilrubid FL (3); and disperse red-Polanilscharlach RP (4); c, contamination of No. 4. The dashed line represents the migration of the marker, azobenzene. (a) Isocratic elution with 30% ethyl acetate in trichloroethylene. (b) Five-step gradient elution, 10–20–30–40–50% of ethyl acetate in trichloroethylene. (Reprinted from Ref. 24 with permission.)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

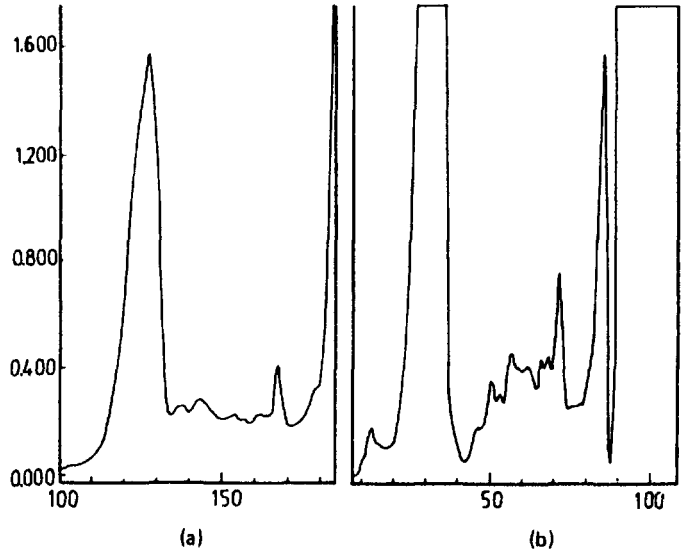

Figure 15 Densitograms (Shimadzu CS-930, 254 nm) of Seboren (plant drug). (a) Isocratic elution, ethyl acetate–chloroform (1:1); (b) stepwise gradient (10–20–30–40–50–70% ethyl acetate in chloroform). (Reprinted from Ref. 100 with permission.)

Increasing the loading capacity of the sample in preparative TLC

Separation of less strongly retained ballast components of the sample in the first gradient steps and chromatographic analysis of the remaining polar compounds in the last steps (69)

It should be noted that not every gradient arrangement is useful in practice. It has been shown (31) that the resolution of neighboring zones is better for antiparallel gradients than for parallel gradients. On the other hand, results of theoretical treatment (8) suggest that the four examined gradient TLC techniques can be arranged in the following order of decreasing resolution: adsorbent gradient layer (best), gradient elution TLC, polyzonal TLC, and vapor-programmed TLC (worst).

In most cases the optimum gradient profile is determined experimentally, but it is always possible to determine the optimum gradient profile, either graphically or numerically, with the help of a microcomputer.

Recently, a device for overpressured TLC and a fully automatic AMD machine for the complete plate-developing process were introduced. Both instruments can be used for gradient development. Gradient development can also be used in preparative TLC. In this case, the sample capacity for full separation of all components of the sample is several times larger for stepwise gradients than for isocratic elution.

In many cases, twice as many spots can be detected in gradient development as in isocratic elution. This is illustrated in Fig. 15, which presents copies of densitometer printouts obtained for Seboren extract (a plant drug) in two elution modes: isocratic and stepwise gradient (100).

REFERENCES

1. L. R. Snyder. In: C. Horvath, ed. High-Performance Liquid Chromatography, Vol. 1. New York: Academic Press, 1979, pp. 208–316.
2. L. R. Snyder. In: M. Lederer, ed. Chromatographic Reviews. Amsterdam: Elsevier, 1965.
3. C. Liteanu and S. Gocan. In: R. A. Chambers, ed. Gradient Liquid Chromatography. New York: Wiley, 1974.
4. P. Jandera and J. Churaček. In: J. C. Giddings, E. Grushka, J. Cazes, and P. R. Brown, eds. Advances in Chromatography. New York: Marcel Dekker, 1980, p. 126.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

5. P. Jandera and J. Churaček. Gradient Elution in Column Liquid Chromatography. Amsterdam: Elsevier, 1985.
6. A. Niederwieser and C. C. Honegger. In: J. C. Giddings and R. A. Keller, eds. Advances in Chromatography. New York: Marcel Dekker, 1966, p. 123.
7. A. Niederwieser. Chromatographia 2:362, 1969.
8. L. R. Snyder and D. L. Saunders. J. Chromatogr. 44:1, 1969.
9. W. Golkiewicz and E. Soczewinski. Chromatographia 11:454, 1978.
10. W. Golkiewicz and M. Jaroniec. J. High Resolut. Chromatogr. Chromatogr. Commun. 1:245, 1978.
11. E. Soczewinski and K. Czapinska. J. Chromatogr. 168:230, 1979.
12. W. Golkiewicz and T. Wolski. J. High Resolut. Chromatogr. Chromatogr. Commun. 4:115, 1981.
13. E. Soczewinski and W. Markowski. J. Chromatogr. 370:63, 1986.
14. E. Soczewinski, G. Matysik, and W. Markowski. J. Liq. Chromatogr. 10:1261, 1987.
15. J. K. Różyłło, I. Malinowska, and H. Kołodziejczyk. J. Planar Chromatogr.-Mod. TLC 1:24, 1988.
16. L. C. Sander and L. R. Feld. J. Chromatogr. Sci. 18:133, 1980.
17. K. Burger. Fresenius' Z. Anal. Chem. 318:228, 1984.
18. D. E. Jaenchen. Int. Lab., March 1987, p. 66.
19. D. E. Jaenchen. Instrumental High Performance Thin Layer Chromatography. Proc. Third Int. Symp., Würzburg, 1985, pp. 71–82.
20. J. Vajda, J. Pick, L. Leisztner, N. Anh-Tuan, and S. R. Hollan. Instrumental High Performance Thin Layer Chromatography. Proc. Third Int. Symp., Würzburg, 1985, pp. 339–349.
21. E. Soczewiński and G. Matysik. J. Liq. Chromatogr. 8:1225, 1985.
22. G. Matysik and E. Soczewinski. J. Chromatogr. 361:19, 1986.
23. G. Matysik and E. Soczewinski. J. Chromatogr. 446:275, 1988.
24. E. Soczewinski, K. Czapinska, and T. Wawrzynowicz. Sep. Sci. Technol. 22:2101, 1987.
25. L. Hagdahl, R. J. P. Williams, and A. T. Tiselius. Arkiv. Kemi. 4:193, 1952.
26. H. K. Mitchell, M. Gordon, and R. A. Haskins. J. Biol. Chem. 180:1071, 1949.
27. T. Wieland and H. Determan. Experientia 21:105, 1965.
28. S. M. Rybicka. Chem. Ind. (Lond.) 1962:308.
29. S. M. Rybicka. Chem. Ind. (Lond.) 1962:1947.
30. A. Niederwieser and M. Brenner. Experientia 21:105, 1965.
31. A. Niederwieser. Chromatographia 2:23, 1969.
32. J. A. Berger, G. Meyniel, G. Petit, and P. Blanquet. Bull. Soc. Chim. France 1963:2662.
33. E. Stahl. Z. Anal. Chem. 222:3, 1966.
34. E. Stahl. German Patent 1175912 (1964).
35. B. Warren. J. Chromatogr. 20:603, 1965.
36. C. Liteanu and S. Gocan. Studia Univ. Babes-Bolyai Chem. 6:99, 1961.
37. S. Turina, V. Marjanovic-Krajovan, and Z. Soljic. Anal. Chem. 40:471, 1968.
38. F. Geiss, H. Schlitt, and A. Klose. Z. Anal. Chem. 213:321, 1965.
39. F. Geiss and H. Schlitt. Chromatographia 1:392, 1968.
40. R. A. De Zeeuw. Anal. Chem. 40:2134, 1968.
41. W. Golkiewicz. Chromatographia 14:411, 1981.
42. W. Golkiewicz. Chromatographia 14:629, 1981.
43. A. Niederwieser and G. G. Honegger. Helv. Chim. Acta 48:893, 1965.
44. G. Pataki and A. Niederwieser. J. Chromatogr. 29:133, 1967.
45. L. Luzzatto and C. N. Okoye. Biochem. Biophys. Res. Commun. 29:705, 1967.
46. R. G. Strickland. Anal. Biochem. 10:109, 1965.
47. E. Soczewinski and G. Matysik. J. Planar Chromatogr.-Mod. TLC 1:354, 1988.
48. F. Geiss and H. Schlitt. Chromatographia 1:387, 1967.
49. F. Geiss. Fundamentals of Thin Layer Chromatography. Heidelberg: Huethig, 1987, Chaps. VI and IX.
50. L. R. Snyder and J. J. Kirkland. An Introduction to Modern Liquid Chromatography. 2nd ed. New York: Wiley-Interscience, 1979, pp. 54 and 663.
51. E. Soczewinski. J. Chromatogr. 369:11, 1986.
52. L. R. Snyder and J. J. Kirkland. An Introduction to Modern Liquid Chromatography. 2nd ed. New York: Wiley-Interscience, 1979, p. 366.
53. R. Lieberman and H. Schuhmann. Chem. Technol. 19:693, 1967.
54. J. A. Perry, K. W. Haag, and L. J. Glunz. J. Chromatogr. Sci. 11:447, 1973.
55. J. A. Perry, T. H. Jupille, and A. Curtice. Separ. Sci. 10:571, 1975.
56. G. Lodi, A. Betti, E. Menziani, V. Brandolini, and B. Tosi. J. Planar Chromatogr.-Mod. TLC 4:106, 1991.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

57. M. T. Belay and C. F. Poole. J. Planar Chromatogr.-Mod. TLC 6:43, 1993.
58. O. R. Queckenberg and A. W. Frahm. J. Planar Chromatogr.-Mod. TLC 6:55, 1993.
59. P. V. Colthup, J. A. Bell, and D. L. Gadsdon. J. Planar Chromatogr.-Mod. TLC 6:386, 1993.
60. D. E. Jänchen and H. J. Isaaq. J. Liq. Chromatogr. 11:1941, 1988.
61. K. Burger, K. Köhler, and H. Jork. J. Planar Chromatogr.-Mod. TLC 3:504, 1990.
62. U. De La Vigne and D. E. Jänchen. J. Planar Chromatogr.-Mod TLC 3:6, 1990.
63. U. De La Vigne, D. E. Jänchen, and W. H. Weber. J. Chromatogr. 553:489, 1991.
64. M. F. M. Trypsteen, R. G. E. Van Severen, and B. M. J. De Spiegeleer. Analyst (Lond.) 114:1021, 1989.
65. H. J. Bigalke, S. Ebel, W. Ullrich, and S. Voelki. In: R. Kaiser, ed. Proc. 4th Int. Symp. Instrum. High Performance Thin Layer Chromatogr. Bad Dürkheim, FRG: Inst. for Chromatography, 1987.
66. M. Matyska, A. M. Siouffi, and E. Soczewinski. J. Planar Chromatogr.-Mod. TLC 4:255, 1991.
67. C. F. Poole and A. T. Belay. J. Planar Chromatogr.-Mod. TLC 4:345, 1991.
68. S. K. Poole and C. F. Poole. J. Planar Chromatogr.-Mod. TLC 5:221, 1992.
69. J. Błądek, A. Kostkowski, and M. Miszczak. J. Chromatogr. A 754:273, 1996.
70. G. E. Morlock. J. Chromatogr. A 754:423, 1996.
71. S. Zellmer and J. Lasch. J. Chromatogr. B 691:321, 1997.
72. F. Bonté, P. Pinguet, A. Saunois, J. M. Chevalier, and A. Meybeck. J. Chromatogr. A 791:231, 1997.
73. C. F. Poole and S. K. Poole. J. Chromatogr. A 703:573, 1995.
74. J. Pick. J. Liq. Chromatogr. 10:1821, 1987.
75. W. Markowski, E. Soczewinski, and G. Matysik. J. Liq. Chromatogr. 10:1261, 1987.
76. L. R. Snyder and J. J. Kirkland. Introduction to Modern Liquid Chromatography. New York: Wiley, 1979, pp. 257–265.
77. L. R. Snyder. Principles of Adsorption Chromatography. New York: Marcel Dekker, pp. 257–333.
78. W. Markowski and E. Soczewiński. J. Chromatogr. 623:139, 1992.
79. W. Markowski. J. Chromatogr. 635:283, 1993.
80. W. Markowski and E. Soczewinski. Chromatographia 36:330, 1993.
81. G. Matysik and E. Soczewinski. Chromatographia 26:178, 1988.
82. G. Matysik and L. Jusiak. J. Chromatogr. 518:273, 1990.
83. G. Matysik, W. Markowski, E. Soczewinski, and B. Polak. Chromatographia 34:303, 1992.
84. W. Cisowski and E. Lamer-Zarawska. J. Planar Chromatogr.-Mod. TLC 3:47, 1990.
85. W. Cisowski, E. Palka-Gudyka, M. Krauze-Baranowska, and Z. Królicki. J. Planar Chromatogr.-Mod. TLC 4:471, 1991.
86. G. Matysik and M. Benesz. Chromatographia 32:19, 1991.
87. M. Waksmundzka-Hajnos and T. Wawrzynowicz. J. Planar Chromatogr.-Mod. TLC 7:58, 1994.
88. G. Matysik and E. Wojtasik. J. Planar Chromatogr.-Mod. TLC 7:34, 1994.
89. G. Matysik. Chromatographia 43:39, 1996.
90. H. D. Smolarz and G. Matysik. J. Planar Chromatogr.-Mod. TLC 14:199, 2001.
91. H. D. Smolarz, G. Matysik, and M. Wójciak-Kosior. J. Planar Chromatogr.-Mod. TLC 13:101, 2000.
92. G. Matysik, M. Wójciak-Kosior, and J. Kowalski. J. Planar Chromatogr.-Mod. TLC 14:191, 2001.
93. G. Matysik and H. Giryn. Chromatographia 42:555, 1996.
94. G. C. Kiss, E. Forgacs, T. Cserhati, and J. A. Vizcaino. J. Chromatogr. A 896:61, 2000.
95. G. Matysik. Chem. Anal. (Warsaw) 43:719, 1998.
96. G. Matysik, J. Toczołowski, and A. Matysik. Chromatographia 40:737, 1995.
97. T. Wawrzynowicz, E. Soczewinski, and K. Czapinska. Chromatographia 2:223, 1985.
98. G. Matysik, E. Soczewiński, and B. Polak. Chromatographia 39:497, 1994.
99. G. Matysik and E. Soczewiński. J. Planar Chromatogr.-Mod. TLC 9:404, 1996.
100. G. Matysik and E. Soczewinski. Chromatographia 26:178, 1988.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016