ELSEVIER

Journal of Chromatography A, 703 (1995) 573–612

JOURNAL OF CHROMATOGRAPHY A

Review

Multidimensionality in planar chromatography

Colin F. Poole*, Salwa K. Poole

Department of Chemistry, Wayne State University, Detroit, MI 48202, USA

Abstract

Multidimensionality in planar chromatography takes on a broader context than is common for column separations. Multidimensional methods used to improve the separation capacity in planar chromatography include unidimensional multiple development and two-dimensional development practiced under conditions of capillary controlled or forced flow of the mobile phase, mobile phase gradients and system automation. Theoretical considerations, the correct approach to methods development, instrumental requirements and contemporary applications of these approaches are outlined in this review. Interfacing column chromatographic methods, such as gas, liquid or supercritical fluid as one dimension and planar chromatography as the second, are not commonly mentioned in the literature but offer some unique possibilities for enhancing both separations and detection. These approaches are outlined here with a view to increasing the awareness of their potential for complex mixture analysis, component identification and trace analysis.

Contents

* Corresponding author. Present address: Department of Chemistry, Imperial College of Science, Technology and Medicine, South Kensington, London SW7 2AY, UK.

0021-9673/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(94)01286-5

1. Introduction

Thin-layer chromatography (TLC) is the dominant planar chromatographic method having largely superseded paper chromatography in contemporary laboratory practice. Modern TLC is a highly instrumental technique associated with the use of kinetically optimized layers prepared from particles of a narrow size distribution and a mean particle diameter between 5 and 15 μm; precise control over the operations of sample application, chromatogram development, and in situ chromatogram evaluation; and a high level of automation [1,2]. Modern TLC requires an investment in equipment and training that not all laboratories have chosen to make. Consequently, co-existing with modern TLC is the more widely practiced "conventional TLC", which requires minimal equipment support and provides qualitative results at low cost in a short time [2–4]. When modern high-performance column liquid chromatography (HPLC) was developed it virtually displaced conventional column liquid chromatography from chemical analysis because it provided fast, quantitative results with little manual effort, and although the instrumentation was expensive, the superiority in the quality of the results obtained and the time savings offset these costs. It can be seen that the comparison between conventional TLC and modern TLC and that of conventional column and HPLC are entirely different, and while it would be difficult to advocate continued use of conventional column liquid chromatography for general analysis, this is not the case for conventional TLC. If a quick, low-cost, qualitative answer employing a portable approach that can be applied almost anywhere, is sufficient to solve a problem then conventional TLC becomes legitimized. For improved quality of the results the approaches of modern TLC are obligatory. This review will try and respect the legitimacy of the dichotomy in TLC while emphasizing the recent advances in its practice; these largely fall under the heading of modern instrumental TLC.

Modern TLC and HPLC are complementary techniques. Separations occur according to the same retention mechanisms with the two techniques distinguished by their kinetic performance, stationary phase format, and use of the development mode in TLC and elution mode in HPLC to perform the separation. An elaboration on these features and what they mean in terms of the prospects of using either TLC or HPLC for a particular analytical problem is given by Siouffi et al. [5]. Those attributes of TLC that distinguish it from HPLC are summarized in Table 1. By using a planar format samples can be separated in parallel providing an increase in sample throughput. By using a disposable stationary phase sample cleanup need not be as rigorous and often sample cleanup and

Table 1
Advantages of TLC over column chromatographic methods

Attribute	Application
Separation of samples in parallel	Low-cost analysis of samples requiring minimal sample preparation
Disposable stationary phase	Analysis of crude samples (minimizing sample preparation requirements). When a single or small number of samples are to be analyzed and their composition and/or matrix properties are unknown. Analysis of samples containing components that remain sorbed to the separation medium or contain suspended microparticles
Static detection	Samples requiring a post-chromatographic treatment for detection. Samples requiring sequential detection techniques (free of time constraints) for identification or confirmation
Storage device	Separation can be archived. Separation can be evaluated in different locations or at different times
Sample integrity	Total sample occupies the chromatogram not just that portion of the sample that elutes from the column

separation can be performed simultaneously. Separations are achieved in space rather than time so that the detection step and separation step are effectively decoupled. Sequential detection by complementary techniques, the application of post-chromatographic derivatization techniques for identification and quantitation, and the possibility of archiving a separation for evaluation at a later time, combined with accessibility of the sample due to the planar format, allow greater flexibility and simplicity in sample evaluation. Consequently, TLC should be considered for those applications where many samples requiring minimal sample preparation are to be analyzed, where the use of TLC permits a reduction in the number of sample preparation steps, and where post-chromatographic reactions are required to detect separated analytes. Some of the reasons for prefering HPLC for an analysis are its greater separation capacity for mixtures containing more components than can be resolved by TLC, a greater variety of stationary phases are available for method development, a wider range of detection options exist, and automation for unattended operation is usually more straightforward. Other reasons such as familiarity, availability of equipment, and past experience tends to dictate that in many cases HPLC is used where TLC might logically be the first choice. However, it is more important to appreciate the complementary character of the two approaches to liquid chromatography, rather than to champion one approach to the exclusion of the other for all sample types.

Those applications for which TLC is often selected as the primary method of analysis are summarized in Table 2 [1]. The connection with the favorable attributes of TLC are now clear. In a significant number of highlighted applications the analytes lack a convenient chromophore, for example, organic acids, lipids, carbohydrates, surfactants, etc., and the ease of using post-chromatographic reactions for their determination is a primary reason for choosing TLC. The ability to analyze samples with minimal matrix simplification is important for the analysis of drugs in biological fluids, pesticide residues in crops, veterinary drugs in meat products and for authenticating plant products in pharmacy and commerce. The simultaneous separation of samples in parallel is important in screening studies and other applications were a large number of samples of a similar type have to be analyzed, for example, in industrial hygiene, detection of drugs of abuse in biological fluids, and regulatory analysis. Abjean [6] has commented on the economic advantage of using TLC for preliminary screening of meat samples for sulfonamide

Table 2
Major applications of modern TLC

Sample type	Application
Biomedical (indigenous compounds)	Organic acids and lipids Carbohydrates Porphyrins and bile pigments Amino acids and peptides Steroids
Pharmaceutical	Stability and impurities of synthetic drugs Pharmacokinetic studies Drug monitoring in biological fluids Antibiotics Plant taxonomy Enantiomeric purity Alkaloids Vitamins
Food science (agricultural products	Mycotoxins (including aflatoxins) Drug residues in meat and dairy products Antioxidants and preservatives Natural pigments Spices and flavors Flavonoids
Forensic	Drugs of abuse Poisons Alkaloids Inks
Clinical	Therapeutic drug monitoring Identification of metabolic disorders
Environmental	Pesticide residues in crops Crop-protecting agents in water Industrial hygiene
Industrial	Product uniformity Impurity determination Surfactants Synthetic dyes

residues with confirmation by HPLC compared to using a multiresidue HPLC method alone. In an analysis of 300 samples, 8 contained unacceptable drug residues. A single analyst required 50 days to complete the analysis using a multiresidue HPLC procedure, whereas only 12 days were needed for TLC screening followed by HPLC confirmation of positive results.

A characteristic feature of TLC separations employing a single development with capillary controlled flow is the production of a series of zones with an ever increasing width roughly proportional to the migration distance. The available migration distance is fixed by the capillary forces involved in transporting the mobile phase through the layer and at some distance from the solvent entry position these forces become too weak to maintain a useful mobile phase velocity. Thus the separation capacity in the above experiment is limited first of all by the increasing zone dimensions in the direction of zone migration and ultimately by the total migration distance available for the separation. In practice this results in a capacity of about 10–14 baseline-separated zones and is largely independent of the normal experimental variables. This means that the separation of more than six to eight components with baseline resolution is always difficult providing the impetus to seek alternative ways of developing the chromatogram to yield a higher zone capacity. This can be achieved by two multidimensional techniques. Unidimensional multiple development provides a mechanism to counteract zone broadening making better use of the development distance afforded by capillary controlled flow and two-dimensional development provides a mechanism to make better use of the selectivity space available under the same conditions. These two complementary approaches are not difficult to implement in TLC and add considerably to the problem solving capacity of the method while retaining some, if not always all, of the attributes defined in Table 1.

The success of all multidimensional methods in chromatography is dependent on the creation of complementary separation mechanisms, applied in a sequential manner, to enhance the separation capacity of the system. The separation mechanisms do not have to exist within a common separation mode and by the use of a suitable interface, generally, different separation modes can be combined. For TLC this includes the common column separation methods of gas, liquid and supercritical fluid chromatography and the common sample preparation techniques of solid-phase extraction and supercritical fluid extraction. The relative merits of this approach will be discussed in this review as well as predictions for the directions this field might take in the future. The important area of spectroscopic methods to improve the information content of thin-layer chromatograms is treated in a companion paper [7], and therefore specifically excluded from this review.

2. Theoretical aspects of planar separations

The migration of the mobile phase through the sorbent layer in TLC is induced by capillary forces and it is an observed empirical fact that the position of the solvent front after some time t is given by the simple quadratic relationship $(Z_f)^2 = \kappa t$ where Z_f is the position reached by the solvent front above the solvent entry position and κ is the velocity constant. The velocity constant depends on external parameters such as the saturation level of the vapor phase in contact with the layer as indicated in Fig. 1 [8]. Evaporation of mobile phase from the layer in an unsaturated chamber tends to reduce the solvent front velocity and adsorption of vapors by the layer in a saturated chamber increases the solvent front velocity. The velocity constant is not easy to determine accurately, but for a given set of experimental conditions it can be related to the properties of the mobile and stationary phases by Eq. 1

$$\kappa = 2K_0 d_p(\gamma/\eta) \cos \phi \quad (1)$$

where K_0 is the permeability constant of the layer, d_p the average particle size, γ the surface tension of the mobile phase, η the mobile phase viscosity, and ϕ the contact angle between the

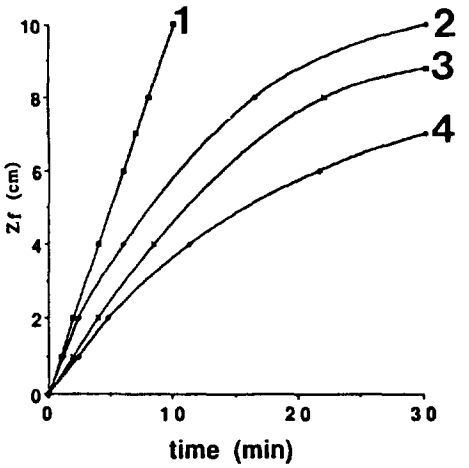

Fig. 1. Relationship between the solvent front migration distance and time for an enclosed layer using forced flow development (1), an exposed layer in a saturated chamber (2), a covered layer (sandwich configuration) (3), and an exposed layer in an unsaturated chamber (4). From Ref. [8]; © Research Institute for Medicinal Plants.

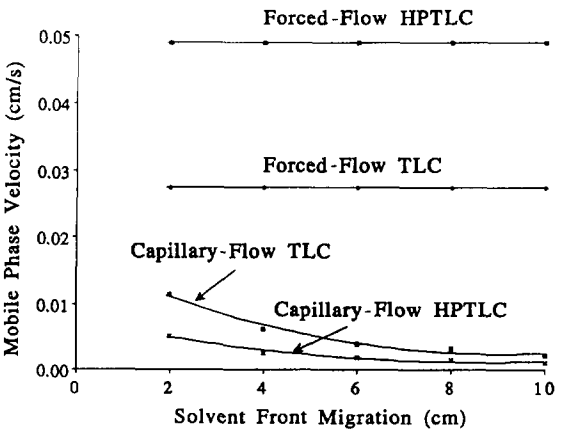

Fig. 2. Variation of the mobile phase velocity as a function of the solvent front migration distance for conventional and high-performance silica gel TLC plates at the optimum mobile phase velocity for minimum zone broadening (forced flow development) and under capillary controlled flow conditions with hexane as the mobile phase.

mobile phase and the layer. Higher velocities are reached by using homogeneous layers of a relatively large particle size with a mobile phase that fully wets the layer and has a favorable ratio of surface tension to viscosity. Almost all solvents wet silica gel adequately but this is not the case for reversed-phase chemically bonded layers developed with predominantly aqueous mobile phases. To obtain practically useful mobile phase velocities in this case layers of a larger particle size, 10–15 μm, and a defined, but lower level of modification of the silica surface by the silanizing reagent are used. When the mobile phase velocity is controlled by external means, such as in forced-flow development, the parameters of Eq. 1 become unimportant provided that sufficient pressure is available to provide the desired velocity. This is the situation in HPLC as well.

The layer homogeneity, average particle size, and mobile phase velocity are parameters that affect the kinetic performance of a layer. Not addressed by Eq. 1 is whether capillary forces are sufficient to provide mobile phase velocities sufficient to minimize zone broadening. The optimum mobile phase velocity to minimize zone broadening can be determined by forced-flow development and is a system constant. As shown in Fig. 2, capillary forces are inadequate to achieve the required velocity. Also, the velocity declines as the solvent front migration distance increases and is increasingly less favorable for maintaining acceptable kinetic performance as the solvent front migrates further along the layer. The consequence of the inadequate mobile phase velocity under capillary flow controlled conditions is that zone broadening is largely dominated by diffusion and the useful development distance for a separation is defined by the range of acceptable mobile phase velocities [9].

A further consequence of the quadratic decrease in mobile phase velocity in capillary flow controlled development is that solutes are forced to migrate through regions of different local efficiency and the plate height for the layer must be specified by an average value. A plot of the average plate height as a function of the solvent front migration distance (Fig. 3) reveals several important features of the optimum kinetic separation conditions. Firstly, there is a dominant relationship between the average particle size, development length, and zone capacity under capillary flow controlled conditions. Layers of a small average particle size (nominally 5 μm)

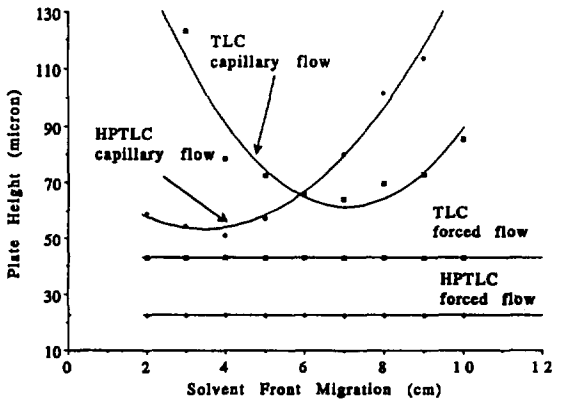

Fig. 3. Variation of the average plate height as a function of the solvent front migration distance for conventional and high-performance silica gel thin-layer plates with capillary controlled and forced flow development.

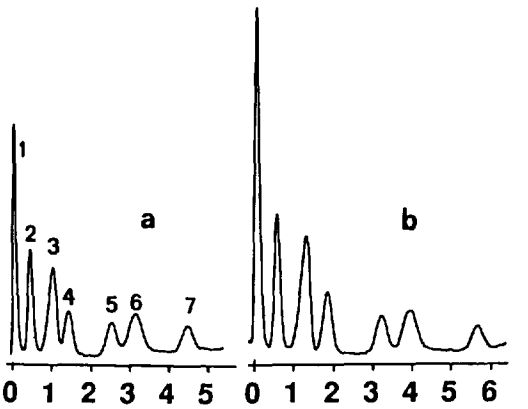

Fig. 4. Separation of the polar aromatic flavor compounds found in cinnamon on a high-performance (a) and a conventional (b) silica gel thin-layer plate with the mobile phase hexane–chloroform–triethylamine (90:6:4). Peaks: 1 = cinnamic acid; 2 = cinnamyl alcohol; 3 = eugenol; 4 = coumarin; 5 = 2-methoxycinnamaldehyde; 6 = cinnamaldehyde; 7 = cinnamyl acetate.

provide more compact zones provided that the solvent front migration distance does not exceed about 5–6 cm. It is futile to use solvent migration distances greater than this for high-performance TLC (HPTLC) plates since the mobile phase velocity declines to the point where zone broadening exceeds the rate of zone center separation. The minimum in the average plate height for the high-performance layer is more favorable than that of the conventional TLC layer, but not by very much. More significant is that the minimum is shifted to a longer development length for the conventional TLC layer; a direct result of the higher mobile phase velocity range for the conventional TLC plate for the same development length. When the development length is optimized the separation performance of conventional and high-performance TLC are not very different (Fig. 4) [10], the virtue of HPTLC being that it requires a shorter migration distance to achieve a given efficiency resulting in faster separations and more compact zones which are easier to detect by scanning densitometry.

Also revealed in Fig. 3 is the fact that the minimum in the average plate height under capillary controlled flow is always greater than the minimum obtained using forced-flow development for the same layer. The limited range of mobile phase velocities under capillary controlled flow prevents the optimum performance of the layers being realized. This combined with shorter separation times is the compelling theoretical reason to prefer forced-flow development in TLC. As we shall see later, there are some practical problems which have limited general interest in this technique.

2.1. Kinetic properties of precoated layers

Thin-layer plates have a heterogeneous structure consisting of the sorbent particles held together by the addition of a binder as well as other possible additives such as a fluorescence indicator for visualization of UV-absorbing compounds. The binder for modern precoated TLC plates, typically a salt of poly(acrylic acid) or less commonly gypsum, imparts the desired mechanical strength, durability and abrasion resistance to the sorbent layer. Typical values for the kinetic properties of commercially available precoated silica gel plates and reference values for HPLC column sorbents are summarized in Table 3 [11–14]. The similar values for the interparticle porosity of columns and layers suggest that their packing density is similar. The significant difference in the total porosity and intraparticle po-

Table 3
Characteristic properties of precoated silica gel layers and columns

Parameter	HPTLC layers	TLC layers	HPLC column
Total porosity	0.65–0.70	0.65–0.75	0.8–0.9
Interparticle porosity	0.35–0.45	0.35–0.45	0.4–0.5
Intraparticle porosity	0.28	0.28	0.4–0.5
Flow resistance parameter	875–1500	600–1200	500–1000
Apparent particle size (μm)	5–7	8–10	
Minimum plate height (μm)	22–25	35–45	2–3 d_p [a]
Optimum velocity (mm/s)	0.3–0.5	0.2–0.5	2
Minimum reduced plate height	3.5–4.5	3.5–4.5	1.5–3.0
Optimum reduced velocity	0.7–1.0	0.6–1.2	3–5
Knox coefficients			
(i) flow anisotropy	0.4–0.8	1.7–2.8	0.5–1.0
(ii) longitudinal diffusion	1.2–1.6	1.2–2.0	1–4
(iii) resistance to mass transfer	1.4–2.4	0.70–0.85	0.05
Separation impedance	10 000–20 000	11 000–13 000	2000–9000

[a] d_p = Average particle size.

rosity indicates that the intraparticle volume of the layers is substantially smaller. A significant amount of the binder used in stabilizing the layers must be contained within the pores. The mean pore size and pore size distribution of the precoated layers determined by size-exclusion chromatography are in accordance with expected values for the raw silica gel, tending to confirm that the binder resides largely within the pores and does not specifically block the pore entrances [13].

Fig. 5 illustrates the variation of the reduced plate height as a function of the reduced velocity for a conventional and a HPTLC plate superimposed upon the results predicted for an ideal column [12,14]. The results for the layers are not only moved to a lower optimum reduced velocity compared to the ideal column but the minimum in the reduced plate height is significantly larger. Also, at increasing values of the reduced velocity, the reduced plate height for the layers increases more rapidly than is predicted for an ideal column. Resistance to mass transfer, therefore, is significantly greater for the precoated layers than for columns. Under capillary-controlled-flow conditions the mobile phase velocities attained are too slow for resistance to mass transfer to be a significant contributor to zone broadening. Under forced-flow conditions the mass transfer term decreases the separation speed compared to columns by dictating that separations are performed at lower velocities to maintain acceptable performance. This is illustrated in Fig. 6 for the separation of polycyclic aromatic hydrocarbons by forced-flow development at close to the optimum velocity indicated in Fig. 5.

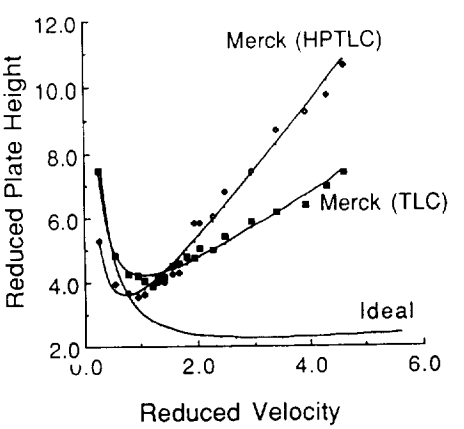

Fig. 5. Plot of the reduced plate height against the reduced mobile phase velocity for a high-performance and a conventional TLC plate superimposed on a curve for an ideal column ($A = 1$, $B = 2$, and $C = 0.05$). From Ref. [12]; © Research Institute for Medicinal Plants.

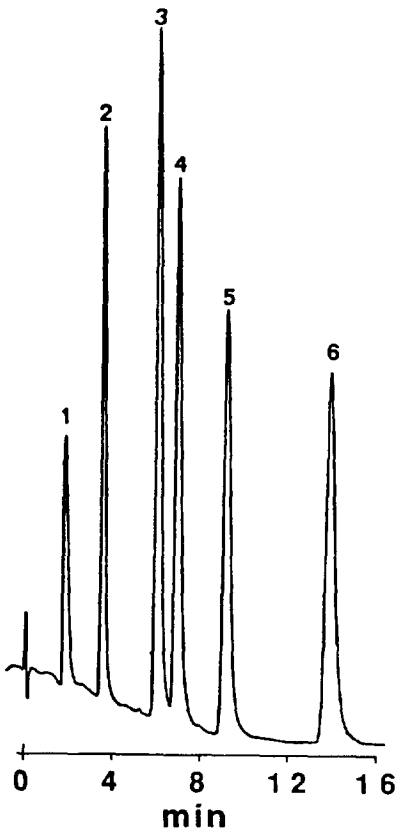

Fig. 6. Separation of polycyclic aromatic hydrocarbons on a high-performance silica gel precoated TLC plate in the elution mode. The development distance was 18 cm with hexane as the mobile phase at a velocity of 0.07 cm/s (u_{opt} = 0.05 cm/s). Peaks: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = phenanthrene; 5 = fluoranthene; 6 = triphenylene. From Ref [1]; © American Chemical Society.

The reasons for the difference between typical columns and layers can be deduced by interrogating the coefficients for the Knox equation ($h = A\nu^{1/3} + B/\nu + C\nu$, where h is the reduced plate height and ν the reduced velocity) giving the best fit to the experimental data [12]. The A term characterizes the flow anisotropy within the streaming part of the mobile phase and is related to the uniformity and packing density of the layer. The high-performance layers (A = 0.4–0.8) are well packed and comparable to the best slurry-packed columns. The conventional layers are not as homogeneously packed (A = 1.7–2.8) probably due to a wider distribution of particle sizes. The B term characterizes the contribution of longitudinal diffusion to the plate height and should have, and does have, similar values for the layers and typical columns. The C term is a measure of the resistance to mass transfer between the components of the stationary phase and the streaming part of the mobile phase. The range of values for the layers is about an order of magnitude larger than those typical of a column. The reason for this large difference cannot be deduced with certainty but one possibility is that it is related to different sorption rates for the silica gel surface and that portion of the surface modified by the binder.

The above observations suggest that there is not much room for improvement in the performance of commercially available precoated TLC layers when capillary-controlled-flow conditions are used for development. The basic problem is that the capillary forces are too weak to maintain a reasonable flow velocity range for optimum kinetic performance during development. One possibility would be to reduce the flow resistance of the layers but this would be difficult to achieve since the flow resistance characteristics of the layers are already close to their anticipated minimum value (Table 3). Reducing the layer thickness from the standard size of about 200 to 100 μm or so would result in an increase in the mobile phase velocity of about 1.1 to 2.5 (depending on the experimental conditions) without an obvious penalty in usability of the layer as a result of a reduction in the sample capacity [15]. A more obvious solution is to take advantage of the zone refocussing mechanism that accompanies unidimensional multiple development [16]. Each time the solvent front traverses the stationary sample it compresses the zone in the direction of development. The compression occurs because the mobile phase first contacts the bottom edge of the zone where the sample molecules start to move forward before those molecules still ahead of the solvent front. Once the solvent front has reached beyond the zone, the refocused zone migrates and is broadened by diffusion in the usual way. If a balance or equivalence can be struck between the zone refocusing and zone broadening mecha-

nisms, it is possible to migrate a zone a considerable distance without significant zone broadening beyond that observed for the first development, as indicated in Fig. 7. A natural consequence of the zone refocusing mechanism is that the original spot, if the sample is applied as a spot, will initially become oval shaped, and if a sufficient number of developments are used, will be refocused to a line. These changes in zone shape are not detrimental to obtaining reliable information of the zone separation or concentration by scanning densitometry. A more complete picture of unidimensional multiple development will be presented in Section 3.

It would seem that improvements in the separation performance related to inadequacies of the mobile phase velocity could be more obviously addressed by using forced-flow development. This is at least partly true. As was discussed above, the separation performance of forced-flow development is not equivalent to that of HPLC because of the difference in the characteristic kinetic properties of commercially available layers and columns. Whereas reducing the contribution from resistance to mass transfer would provide only minor improvements in the separation performance in capillary-controlled-flow systems, it is critical to achieving optimum performance and reducing the separation time in forced-flow development. If this problem is related to the binder then this should be the focus of renewed effort to enhance the performance of forced-flow methods. It is difficult to imagine a commercial product that contained no binder of sorts, since the layers themselves are very unstable in the absence of a mechanism to maintain their integrity when subjected to external forces. On the other hand, very little is known about the influence of the type of binder, its concentration, etc., on the separation performance in forced-flow TLC, since this subject has not been systematically studied before.

In its most common embodiment forced-flow development is performed using the overpres-

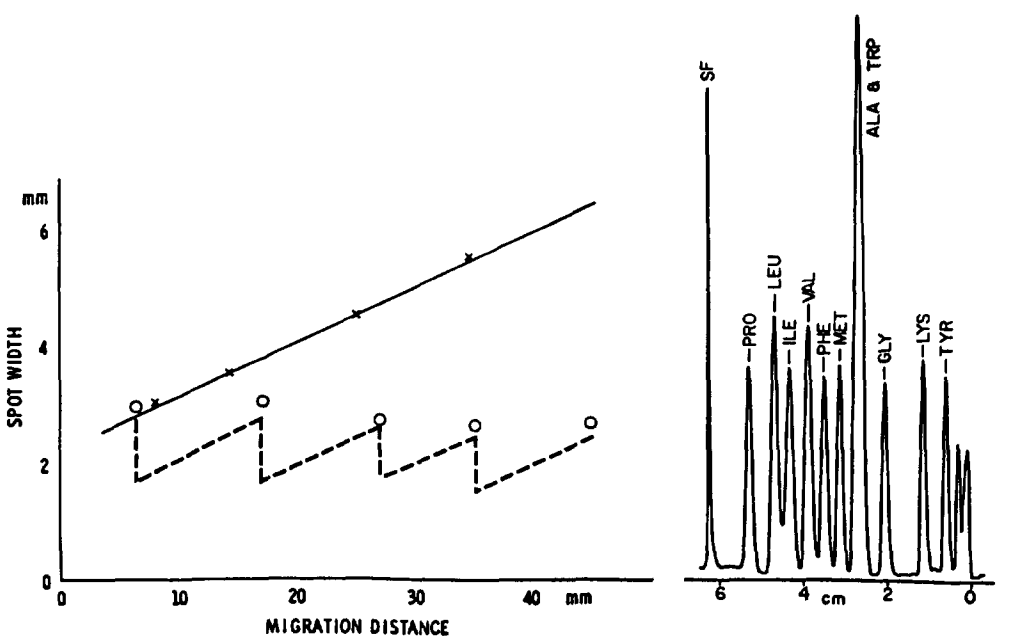

Fig. 7. An illustration of the zone refocusing mechanism (left) and its application to the separation of a mixture of phenylthiohydantoin-amino acids (right). The broken line on the left-hand side of the figure represents the change in spot size due to the expansion and contraction stages in multiple development and the solid line depicts the expected zone width for a zone migrating the same distance in a single development. After Ref. [16]; © Research Institute for Medicinal Plants.

sured development chamber in which the layer is sealed by applying hydraulic pressure to one side of a polymeric membrane in intimate contact with the sorbent surface and the mobile phase is forced through the layer using an independent eluent pump [2,4,17–19]. Some problems remain with this approach: the edges of the layer must be sealed prior to use by impregnation with glue; the formation of a disturbing zone behind the gas–liquid interface as the solvent penetrates the dry layer but fails to instantaneously displace or dissolve the gas trapped in sorbent pores and interparticle spaces can distort the chromatogram; the difficulty of developing a detector with spatial selectivity limits on-line detection to samples separated sequentially while simultaneous sample separation in lanes would be preferred; the influence of the wall effect on chromatographic performance is not well understood; and a lack of evolutionary development of the equipment has not promoted its general use in routine laboratories. A detailed account of these and other issues can be found elsewhere [17–22]. Other approaches to forced-flow development include high pressure planar chromatography [23–25], rotation planar chromatography [26], vacuum planar chromatography [27] and electrochromatography [28–30]. Of these approaches electrochromatography using electroosmosis as the mechanism to move the mobile phase through the layer is the most appealing but also the least explored. Still, from these ideas new methods may appear for forced-flow development that could dramatically improve performance and separation time in planar chromatography and have a considerable impact on how TLC is practiced in the future. Until then, multiple development remains the most straightforward approach for improving the observed kinetic performance of planar chromatography and can be applied without the need for special apparatus (see Section 3).

2.2. Statistical theory of zone overlap in one- and two-dimensional planar chromatography

The separation capacity of a chromatographic system is conveniently represented by its peak capacity, that is the maximum number of component peaks that can be separated at any specified resolution level, in a given separation time or distance [2]. As an analogy, the peak capacity envisages the chromatogram as being similar to a string of pearls, each pearl touching its neighbor with no unoccupied space between the pearls. As such it results in an inflated estimate of the capacity of the system to separate real samples unless combined with some statistical model to account for the random distribution of sample components in the separation space [31,32].

Guiochon and co-workers have developed a model to determine the spot capacity in thin-layer chromatography under different experimental conditions. An exact solution to this problem is more difficult than for columns since the plate height in TLC is a complex function of the characteristics of the chromatographic system (see Section 2). Solving this problem for different sets of conditions indicates that it is very easy to achieve a spot capacity between 10 and 20 for a single development under capillary flow controlled conditions, but it is extremely difficult to reach 25, and practically impossible to exceed 30 [33]. For comparison, it is very easy to achieve a peak capacity between 20 and 40, but extremely difficult to reach 150 to 200 in HPLC. A similar spot capacity should be possible using forced-flow TLC. The available pressure drop and plate length using commercially available equipment would suggest a more practical limit of about 80 might be achievable. The spot capacity for two-dimensional TLC is less than the product of two unidimensional developments but is still considerably greater than the single column value in HPLC [34,35]. At the start of the second development, the separated spots have increased in size due to the first development and are always larger than the initial starting spot size. Also, during the second development the spots broaden laterally, so that they must be separated with a greater resolution at the beginning of the second development if they are to have the specified resolution at the end of the second development. Calculations show that it should be easy to achieve a spot capacity of 100 to 250, but

difficult to reach 400, and nearly impossible to exceed 500 in two-dimensional TLC with capillary controlled flow in both directions. With forced-flow development in two-dimensional TLC it should be easy to generate spot capacities well in excess of 500 with an upper bound of several thousand, depending on the choice of operating conditions.

Two-dimensional separations in planar chromatography are rather trivial to perform, and this aspect combined with the theoretical predictions of greatly increased separation power, are the driving force for this work. Details will be presented shortly. In reviewing the theoretical predictions it should be emphasized that some of the results are probably inflated because of the values assumed for the layer characteristic properties in the model are not in agreement with recent measurements [9,36]. In practice spot capacities of about 10–14 have been observed using capillary-controlled-flow conditions with precoated TLC plates in a single development. It seems reasonable that a spot capacity in the neighborhood of 100 might be realized in a two-dimensional development using precoated plates and capillary controlled flow, although we are unaware of any substantiating experimental data. A spot capacity of 40–50 seems more realistic for a single development in forced-flow overpressured TLC using available equipment and precoated layers. A spot capacity of 1500 or more for two-dimensional forced-flow development using existing equipment and materials is then not too fanciful, but again, to our knowledge has not been substantiated by experiment. It is obvious that the inadequate mobile phase velocity range and its influence on the useful development length restricts the spot capacity of two-dimensional TLC with capillary controlled flow to useful, while modest, gains in separation performance. To really increase the separation capacity of TLC in a major way forced-flow development would be required for both dimensions. Alternatively, if capillary controlled flow coupled with the zone refocusing mechanism of multiple development was used a spot capacity of about 40 for each dimension could be realized and again, a significant increase in the separation capacity of the layer attained. This would be experimentally easier to perform although the separation time would be long compared to the use of forced-flow development.

Statistical models of peak overlap in chromatography predict that the number of single component peaks as a function of the peak capacity is quite low and that most observed peaks in complex mixtures will be multiplets consisting of two or more unresolved species [31,32]. Davis and co-workers [32,37–39] have developed several statistical models to predict spot overlap in two-dimensional TLC. This work showed that the spot capacity of a two-dimensional separation is utilized less effectively than the spot capacity of a single dimension but the probability of obtaining single component spots is significantly greater because of the larger spot capacity of the two-dimensional separation. This theory also predicts that only a low peak capacity is required in the second dimension to obtain well-resolved two-dimensional separations. These conclusions are based on computer simulations and await experimental verification, but intuitively seem sensible and not obviously at variance with practical observations.

3. Unidimensional multiple development

Multiple development is a complementary approach to forced-flow development for increasing the separation performance of TLC and simplifying the separation of complex mixtures utilizing mobile phase composition gradients [2,16,40–43]. All unidimensional multiple development techniques employ successive repeated development of the layer in the same direction with removal of mobile phase between developments. The main variants are multiple chromatography and incremental multiple development. In multiple chromatography the plate is developed repeatedly over the same distance with the same mobile phase and removed from the developing chamber between developments to evaporate the mobile phase adsorbed by the layer, the dry layer being returned to the development chamber for rede-

velopment in the same direction as in the previous step. Multiple chromatography can also be performed with a variable solvent entry position by simultaneously incrementing the solvent entry position and the solvent front position so as to maintain a fixed development distance for each development [44,45]. The solvent entry position is easily repositioned to a higher level on the plate (but still below the slowest of the migrating zones) by incrementing the volume of mobile phase in a fixed volume development chamber or by cutting off a portion of the lower edge of the plate between developments.

In incremental multiple development the first development distance is the shortest and each subsequent development is incremented by, usually, an equal distance or time, or less frequently, by variable distances or times arrived at by trial and error to provide a desirable final resolution for the sample components. The last development is usually the longest and, in most cases, corresponds to the maximum useful development length of the layer. Incremental multiple development employing a decreasing solvent strength gradient is the method employed for separations in the automated multiple development chamber [41].

Two additional features of multiple development are worthy of comment. The use of a fixed migration distance is rarely the optimum approach for spreading the sample components across the whole chromatogram and it is fairly common practice to increase the development distance (or time) for later development steps to avoid pushing some of the least retained components into the solvent front [44]. The longest practical development distance is dictated by the range of mobile phase velocities, which eventually fall below a useful value when capillary controlled flow is responsible for solvent migration. This would not be a restriction for forced-flow multiple development, but this approach remains virtually unexplored in spite of its attractive features [46]. Secondly, on silica gel layers, zones of similar polarity tend to migrate together from the origin and become separated higher up the plate. For difficult separations long migration distances are required, and at each of the subsequent development steps substantial time is wasted while the advancing solvent front reaches the level of the lowest spot in the chromatogram. In addition, this process results in critical separations being attempted in those regions of the layer where the mobile phase velocity is inadequate to maintain acceptable performance. This problem can be circumvented by incrementing the position of the solvent entry position to maintain a fixed distance below the slowest moving zone in the chromatogram at each development step, as illustrated in Fig. 8 [44]. Incremental multiple development provides a superior separation compared to multiple chromatography in this case by minimizing zone broadening and enhancing the zone center separations by migrating the sample components over a longer distance while maintaining a mobile phase velocity range closer to the best value for the separation. Multiple chromatography is better reserved for those examples where only a modest increase in resolution is required over a single development to achieve the desired separation and the sample components are fairly evenly distributed throughout the final chromatogram [47].

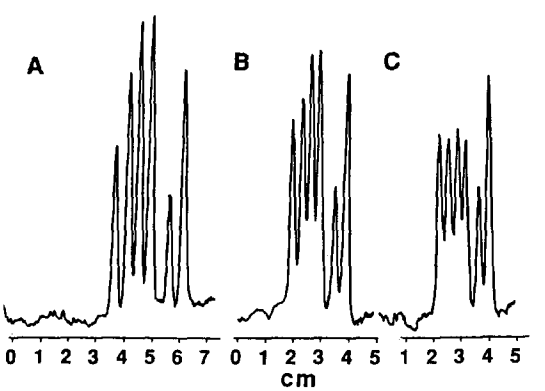

Fig. 8. Separation of a mixture of estrogens by incremental multiple development with a variable (A) or fixed (B) solvent entry position, and by multiple chromatography (C). A 9-step sequence with the mobile phase cyclohexane–ethyl acetate (3:1) was used for (A) and (B) and seven 7-cm developments for (C). The estrogens in order of migration are 17β-dihydroequilenin, 17α-dihydroequilenin, 17β-estradiol, 17α-estradiol, equilenin and estrone. From Ref. [44]; © Research Institute for Medicinal Plants.

The versatility of the multiple development process can be increased by employing changes in the solvent composition for some or all of the development steps [41]. For mixtures of a wide polarity range, gradient multiple development is invariably required as demonstrated by the separation of poly(ethylene glycol) oligomers as their 3,5-dinitrobenzoyl esters (Fig. 9) [41,48]. The distribution of oligomers and their polarity range prevents a complete separation being achieved with any single development system. The mobile phase can be optimized to resolve either the low oligomer number or high oligomer number components but not both simultaneously. Using multiple development with a stepwise mobile phase gradient all of the oligomers are separated in a single chromatogram.

Gradients of increasing solvent strength are used to fractionate complex mixtures by separating just a few components in each step [3,16,49,50]. Individual compounds are usually identified and quantified by scanning intermediate steps at which the sample components of interest are separated. In this way the zone capacity is not limited by the useful development length for the layer established by the mobile phase velocity under capillary controlled flow and can be much larger than that predicted for a complete separation recorded as a single chromatogram. On the other hand, the approach can be tedious when many compounds are of interest and it is difficult to automate in any convenient manner.

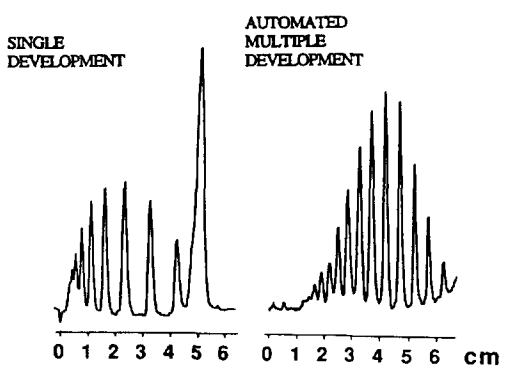

Fig. 9. Comparison of the separation of the 3,5-dinitrobenzoyl esters of poly(ethylene glycol) 400 by normal and automated multiple development (AMD) on a silica gel HPTLC plate. The AMD separation employed a 15-step incremental multiple development program with methanol, acetonitrile and dichloromethane as solvents. From Ref. [42]; © Research Institute for Medicinal Plants.

Soczewinski's group [51–55] has described a modified version of the horizontal sandwich development chamber for performing stepwise gradient separations. The special design of the solvent delivery system enables the solvent in the reservoir to be absorbed to the last drop and, as a result, successive volumes of mobile phase of increasing solvent strength can be introduced after complete absorption of the previous fraction by the layer. If each fraction of mobile phase is restricted to some fraction of the void volume of the layer and the sum of the volumes of all the solvent fractions is equal to the total void volume of the layer, the zone separation subsequently obtained can be described mathematically and optimized [56,57]. Any gradient program, including continuous or multiple component ones, can be generated in this way. The actual gradient profile in the layer, however, may be distorted in comparison with that programmed due to solvent demixing and the low rate of exchange of the stagnant mobile phase in the sorbent pores with the mobile phase of greater solvent strength. These effects are likely to lower the predictive accuracy of any mathematical model used to optimize the separation. Applications of the above approach to the separation of pharmaceutically useful natural products from plants have been described [52,58–60].

In contemporary practice incremental multiple development with a solvent gradient of decreasing solvent strength has proven more popular. In this case all the components must be fitted between the position of the sample origin and the final solvent front. The zone capacity is limited but is significantly greater than that for a single development due to the zone refocusing mechanism [1,2,41,61–63].

Not all compounds are suitable for separation by multiple development [41,64]. Compounds with significant vapor pressure may be lost during the repeated solvent evaporation steps. Certain solvents of low volatility and/or high polari-

ty such as acetic acid, triethylamine, dimethyl sulfoxide, etc., are unsuitable selections for mobile phases because of the difficulty of removing them from the layer by vacuum or forced-flow evaporation between development steps. Water can be used but the drying steps will then be lengthy. Solvent residues remaining after the drying step can modify the selectivity of mobile phases used in later steps resulting in irreproducible separations. Solvent impurities can be the source of ghost peaks or irregular baselines in scanning densitometry. Artifact peaks from chemically unstable compounds may be mistaken for sample components. This is an occasional problem generally observed with inorganic oxide sorbents and compounds that are either air sensitive and readily oxidized or hydrolyzed, or compounds catalytically transformed on the layer. For example, β-carotene is readily oxidized on silica gel plates to a more polar product [65]. During development unreacted β-carotene moves to a higher position on the layer leaving an immobilized peak at the origin. This process is repeated for each development in multiple development with the artifact peak corresponding to the position of β-carotene in the previous development step. The final chromatogram contains a series of artifact peaks corresponding to the number of developments. Derivatizing reagents are another source of artifact peaks [66]. Dansyl chloride, for example, is hydrolyzed on silica gel layers to the acid form. This hydrolysis is slow and incomplete, resulting in multiple peaks in the chromatogram at uniform intervals corresponding to the number of developments. Although precautions can be taken to minimize the production of artifact peaks in multiple development the separation of light and/or air sensitive compounds is probably better handled by other techniques [64].

3.1. Theoretical considerations

From the general theory of multiple chromatography it is predicted that the final location of a sample zone after n successive developments of the same length with the same mobile phase, its apparent R_F value, $R_{F,n}$, can be predicted from its R_F value in the first development according to Eq. 2 [67,68]

$$R_{F,n} = 1 - (1 - R_F)^n \quad (2)$$

Given the general uncertainties in the calculation of R_F values [43,69,70], Eq. 2 shows reasonable agreement with experimental results [44]. It follows that the zone center separation, ZC, in multiple chromatography can be expressed by the difference in apparent R_F values for the two zones in any particular development, Eq. 3

$$ZC = (Z_f - Z_0)[(1 - R_{F1})^n - (1 - R_{F2})^n] \quad (3)$$

where Z_f is the distance moved by the solvent front measured from the solvent entry position and Z_0 is the distance between the solvent entry position and the center of the sample application zone. Independent of the R_F value the zone center separation should reach a maximum when the zones have migrated about two-thirds of the development distance. The number of successive developments required to maximize the zone center separation, n_{max}, is given by $n_{max} = -1/\ln(1 - R_{F2})$ and, consequently, for zones with a small R_F value a large number of successive developments will be needed to reach the maximum zone center separation. Once the zones have moved passed the position for maximum separation the compression of the zone center separation distance is shallow, at least for zones migrating close together. As the zones approach the solvent front position the length of each effective development step is shortened (Z_f is fixed and Z_0 corresponds to the position of the lower of the two zones in the previous development). The optimum conditions for separation by multiple chromatography can be summarized by the comments in Table 4.

The general theory of multiple chromatography predicts that the final zone width will be $(1 - R_F)^n$ times the initial zone width after n successive developments, but this is not what is observed in practice [41,44]. This relationship does not take into account the contribution of zone broadening that occurs during the migration of the zones between successive developments. It creates an over optimistic assessment

Table 4
Optimum conditions for isocratic multiple development

(a) Multiple chromatography
(1) Resolution is controlled primarily by the zone center separation since zone widths are relatively constant after the first few developments
(2) The maximum zone center separation for two solutes of similar migration properties occurs when the zones have migrated 0.632 of the development distance
(3) The maximum resolution observed is largely independent of the average R_F value for the zones although if the average R_F value is low, a large number of developments will be needed to reach the maximum resolution value
(4) Difficult to separate compounds should be repeatedly developed with solvents that produce low R_F values corresponding to the most selective mobile phase for the separation

(b) Incremental multiple development
(1) Increment the solvent entry position to maintain a fixed distance below the lowest zone
  (i) zone separation is increased due to the longer migration lengths achieved
  (ii) zone widths are reasonably constant throughout the chromatogram
(2) Increment the solvent front migration distance
  (i) avoids moving portions of the chromatogram into the solvent front region
  (ii) enables resolution to be optimized throughout the chromatogram. The resolution reaches a plateau value and is not reduced by increasing the number of developments
  (iii) the number of developments can be selected based on the requirements of the most difficult zones to separate

of the importance of the zone refocussing mechanism and provides a false connection between zone widths and the number of successive developments. More refined relationships for the dependence of zone widths on the number of successive developments tend to be too pessimistic resulting from a poor understanding of zone broadening in general in planar chromatography [9,42]. In practice, for multiple chromatography it is observed that the zone widths increases in a quasi-linear fashion until they have traveled about two-thirds of the development length and are then compressed as they move closer to the solvent front. In incremental multiple development, if the solvent front migration distance and solvent entry position are incremented simultaneously so as to maintain a fixed development distance in each successive development, the zone widths increase for the first few developments, eventually reaching a constant size virtually independent of the R_F value for the zone in the first development [44].

As shown by Fig. 10, multiple chromatography with a variable solvent entry position always yields improved resolution over multiple chromatography with a fixed solvent entry position [44]. For zone pairs with intermediate average R_F values and the solvent entry position varied for each development, the resolution reaches a plateau after a few developments and then remains unchanged. For zones with low average R_F values there is a considerable improvement in the resolution obtained using the variable solvent

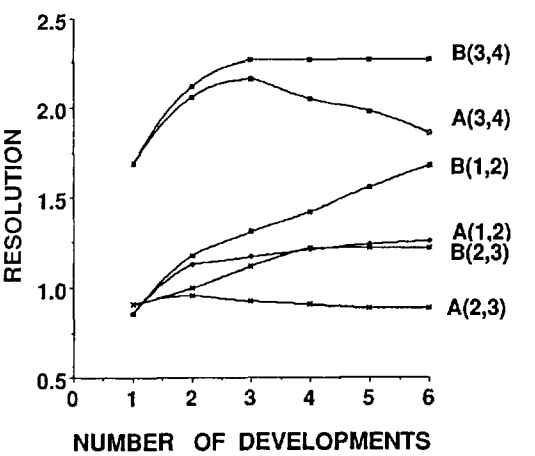

Fig. 10. Plot of resolution between neighboring zones as a function of the number of successive developments in multiple chromatography with a fixed (A) and variable (B) solvent entry position. The R_F values for each zone are 0.14, 0.25, 0.31 and 0.43. From Ref. [44]; © Research Institute for Medicinal Plants.

entry position in comparison with the resolution obtained either from a single development of that obtained in multiple chromatography with a fixed solvent entry position.

3.2. Mobile phase selection

The full resolving power of unidimensional multiple development is realized only when the process variables, described above, and chromatographic selectivity are optimized simultaneously. The chromatographic selectivity is optimized by an intuitive selection of a stationary phase accompanied by a systematic selection of a suitable mobile phase [2,71,72]. Guided trial-and-error methods such as the PRISMA model are generally most suitable for identifying solvents with good selectivity for a particular separation in TLC [73–76].

Solvent selection using the PRISMA model commences with the selection of 10 solvents, chosen from the different Snyder selectivity groups, which are evaluated in parallel to quickly identify appropriate separation conditions. The strength of each solvent is adjusted individually so that the substance zones are distributed in the R_F range 0.2–0.8. Other solvents than those indicated as preferred solvents can be evaluated at this time to identify candidate mobile phases for further optimization. The three solvents indicated as providing the best separation are then selected for optimization using a three-dimensional geometric design which correlates the solvent strength with the selectivity of the mobile phase. The solvent compositions used for optimization are defined by three-coordinate selectivity points which are then systematically evaluated according to a statistical mixture design. Different solvents can be evaluated much faster than is possible for HPLC, since a significant number of experiments can be run at the same time. By using a larger number of solvents than is normally considered because of time constraints in HPLC, it is more likely that solvents of higher selectivity for a particular separation will be identified.

For samples containing components of a wide polarity range it is likely that different solvents will be identified as optimum for separating different regions of the chromatogram. These different solvents then form the basis of the selection criteria used to formulate multisolvent gradients. One general difference between selecting a solvent for multiple development compared to using the same solvent for normal development is that in the former case the mobile phase will be weaker so as not to force the chromatogram into the solvent front with the first few developments. This is achieved by diluting the mobile phase with a strength adjusting solvent, that is, with a solvent in which the components of the chromatogram show minimal migration.

Markowski and co-workers [77–79] have described a model to predict retention in incremental multiple development employing solvent gradients. The model can be applied to single or binary solvents in the adsorption mode where the relationship between retention and solvent composition can be represented by either Eq. 4 or Eq. 5

$$\log k = \log k_0 - m \log C \tag{4}$$

$$\log k = A_0 + A_1 \log C + A_2(\log C)^2 \tag{5}$$

with k representing the solute capacity factor in the solvent system and C the volume fraction of the modifier. The equation constants are evaluated from plots of retention against mobile phase composition. If a two-step gradient is considered for simplicity (Fig. 11), the solute migrates some distance $R_{F(1)}$ that is a fraction of the distance migrated by the solvent front $z_{(1)}$. Since the second development step begins with the same solvent entry position as the first the solute remains immobile in the second development until the second solvent front reaches its position, indicated by the horizontal line in Fig. 11. At this time chromatography begins again and the solute migrates to a new position behind the second solvent front that is a function of its migration properties in the second mobile phase. The position of the solute in the chromatogram is the sum of the migration distance in each development, which is expressed in R_F units in the model (consequently the final development

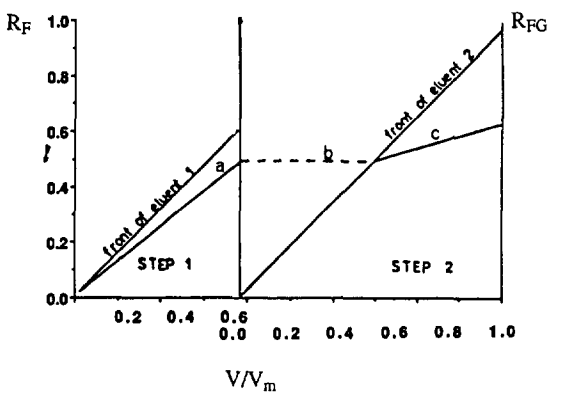

Fig. 11. Schematic diagram of the migration of a solute in two-stage development. a = Migration of the solute during the first development; b = the time the solute remains immobile in the second development until the second solvent front reaches its position; c = migration of the zone in the second development. After Ref. [77]; © Elsevier.

distance, in this case $z_{(2)}$, is always 1.0). The apparent R_F value under gradient incremental multiple development conditions, $R_{F(g)}$, is expressed by

$$R_{F(g)} = z_{(1)}R_{F(1)} + [1 - R_{F(1)}z_{(1)}]R_{F(2)} \quad (6)$$

and the R_F value for any individual segment is calculated from

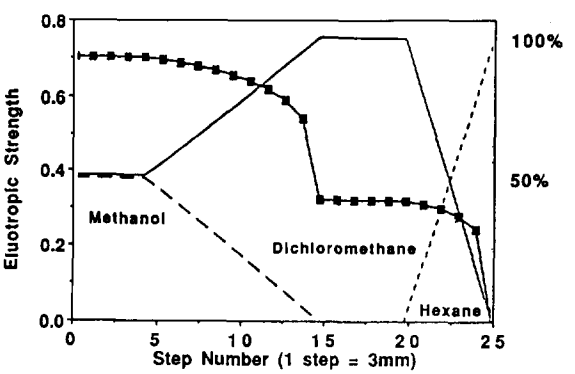

Fig. 12. An example of an idealized universal solvent gradient for the AMD. The thin lines represent the normal depiction of the gradient based on the composition of the selected solvents delivered to the mixer. The bold line through the boxes represents the change in solvent strength for the idealized gradient. From Ref. [84]; © Research Institute for Medicinal Plants.

$$R_{F(i)} = 1/(1 + k_{(i)}) \quad (7)$$

with the capacity factor for the ith development step, $k_{(i)}$, obtained from Eqs. 4 or 5 using the appropriate fraction of the layer's void volume for the initial position of the zone in the chromatogram with respect to the solvent entry position. Eq. 6 is easily extended to n development steps by considering the development process as the sum of a series of two step developments in which the final position is the sum of the migration distances in earlier development steps plus the additional migration distance for the nth development,

$$R_{F(g)} = s_{(n-1)} + [z_{(n)} - s_{(n-1)}]R_{F(n)} \quad (8)$$

where $s_{(n-1)}$ is the position of the zone in the $(n-1)$ development and $z_{(n)}$ is the final development distance. The above calculations are easily performed by computer and separations can be simulated by adjusting the experimental conditions to arrive at the optimum conditions for a particular separation, at least in terms of zone center separations, and with certain assumptions about zone widths, in terms of resolution. The agreement between theory and experiment is quite good when n is small. The main limitations of the theory are caused by solvent demixing and variations in the individual experimental conditions which affect the reliability of Eqs. 4 and 5 to predict the retention properties of different solutes. This model is a useful beginning towards a general theory for the computer-aided optimization of gradient separations by automated multiple development (AMD), which can be quite time consuming to achieve by trial-and-error experimental procedures.

In the absence of a reliable theoretical model to optimize the separation conditions in AMD two general approaches have been adopted for guided trial and error procedures [41]. The first is based on the use of a universal gradient which commences with methanol, ends with hexane, and uses dichloromethane or methyl *tert.*-butyl ether as the intermediate or base solvent [61]. A typical universal gradient of 25 steps is shown in Fig. 12. By scaling and superimposing the chro-

matogram of the separation above the theoretical gradient profile, those regions of the chromatogram affecting the separation are easily identified. The initial strong solvent composition for the first development and the strength of the terminal solvent for the last development are easily identified, thus eliminating steps in the program that are not contributing to the separation. The gradient shape can then be modified to enhance resolution in those regions of the chromatogram that are poorly separated or to make better use of the zone capacity by minimizing regions devoid of sample zones. Thus, for example, the program can be modified to provide a shallow gradient over those regions of the chromatogram where peak separation is inadequate and steeper gradients in regions where the separated zones are well displaced from each other. For relatively simple mixtures this approach is often satisfactory. However, in those cases where the resolution remains inadequate after adjusting the gradient shape it is necessary to identify a different gradient composition for the separation. At this point the PRISMA solvent optimization model can be useful for identifying appropriate solvents with high selectivity for the problem at hand. These solvents can be introduced as the base solvent or incorporated in that segment of the program that affects the separation of the poorly resolved sample components in the universal gradient.

Alternatively, if the composition of the sample is known and standards are available, isocratic plots of log k against the composition of binary solvent mixtures can be used to infer optimum gradient conditions [80–83]. The initial solvent is chosen such that it possesses sufficient strength to cause migration of the most retained substances in the mixture. The final solvent is selected to provide an acceptable separation of the least retained components without migrating them too close to the solvent front. The base solvent is selected based on its ability to provide optimum band spacing throughout the chromatogram.

There are three features which should be kept in mind when adjusting the gradient composition based on assumptions from the idealized solvent gradient constructed by considering the composition of the selected solvents at each step. The AMD apparatus prepares the solvent composition for each successive development by serial dilution. The solvent mixture is prepared in a mixing chamber with a volume of about 22 ml and about 8 ml is withdrawn for the development step. New solvent equivalent in volume to the solvent withdrawn in the previous development step is added to the mixer and homogenized ready for use in the next development step. Compared to the ideal gradient depicted in Fig. 12 there are several consequences of this arrangement. Firstly, a stepped gradient is generated in which the stepped changes in mobile phase composition are not identical (Fig. 13). Secondly, the initial strong solvent appears in nearly all, if not all, of the steps of a typical gradient, albeit in low concentration after a large number of steps. This is not apparent from the idealized gradient constructed from the solvent composition delivered to the mixer and represented by Fig. 12. This problem can be circumvented by emptying the mixer between all or a few developments at the expense of increasing solvent consumption for the separation. Lastly, typical universal gradients do not correspond to linear solvent strength gradients as demonstrated by Colthup et al. [84]. The bold line in Fig. 12

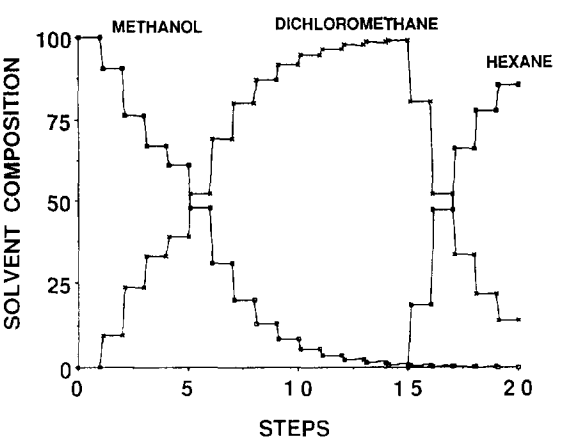

Fig. 13. Actual mobile phase composition for a similar idealized gradient profile to Fig. 12 employing 20 steps. From Ref. [41]; © Research Institute for Medicinal Plants.

depicts the change in solvent strength for the idealized universal gradient. The abrupt change in solvent strength occurring during the gradient can cause a grouping together of sample components and poor resolution in this region of the chromatogram. A method is suggested that enables the AMD to be programmed to produce linear solvent strength gradients by incorporating short phantom steps to achieve the necessary solvent compositions between the desired development segments.

3.3. Automated multiple development

The basis of AMD is the automation of unidimensional, incremental, multiple development with a reverse solvent strength gradient [41,61–63]. The associated theory and methods of solvent selection were discussed in the previous two sections in the general context of unidimensional multiple development. The purpose of this section is to highlight some of the practical details and applications of the AMD approach to planar chromatography.

Automated multiple development was conceived by Burger [62] and subsequently commercialized by Camag Scientific. A schematic diagram of the apparatus used for AMD is shown in Fig. 14. Samples are applied to the layer as bands or spots in the usual way and the layer mounted vertically in the developing chamber (1). The operating sequence begins with a drying step involving evacuation of the sealed developing chamber, followed by a conditioning step to control the activity of the layer. A wash bottle (5) is used to condition a stream of nitrogen gas which is stored in a flexible reservoir (6) and then admitted to the developing chamber. Any volatile substance can be used for conditioning provided that it can be introduced into the developing chamber by entrainment in a stream of nitrogen, and subsequently removed under vacuum. Typically, solutions of sulfuric acid are used to control relative humidity and aqueous solutions of ammonia or formic acid to control tailing. To commence development, the solvent composition is selected by mixing valve (3) from the solvents available in the reservoir bottles (2) and homogeneously mixed in the mixing chamber (4). A fixed volume of this solvent is forced into the developing chamber and allowed to ascend the layer for a preset time. The mobile phase is then sucked from the developing chamber to a waste solvent bottle (8) and the solvent trapped in the layer removed by vacuum (7). The above sequence represents a single cycle with a complete development program consisting of about 10 to 30 cycles in which the time for development and the solvent composition is varied in all or most of the cycles. All processes are automated and time-sequenced so that once a program is entered into the control

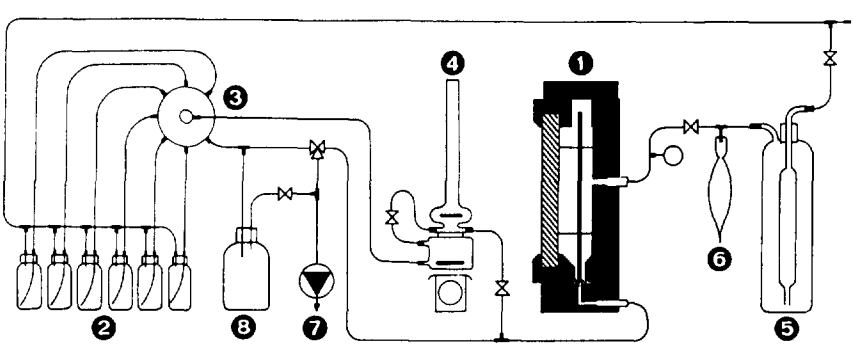

Fig. 14. Schematic diagram of the automated multiple development apparatus. 1 = Enclosed developing chamber; 2 = solvent reservoir bottles; 3 = switching valve for selecting the solvent composition; 4 = gradient mixing chamber; 5 = wash bottle to prepare gas phase; 6 = reservoir for gas phase; 7 = vacuum pump; 8 = solvent waste bottle. From Ref. [41]; © Research Institute for Medicinal Plants.

unit it can be executed in its entirety without further operator intervention. Practical considerations for optimizing the process variables and chromatographic variables in AMD are detailed in Ref. [41]. An optimized AMD separation will contain the sample components adequately resolved from each other and evenly spaced throughout the chromatogram with approximately equal peak widths.

The AMD is a relatively new technique in planar chromatography and it is desirable that a firm foundation be established to understand its operational aspects to ease the bottle neck of methods development. This has not diminished interest in the AMD so far, and the technique has prospered because of its proven applications and the convenience of having a well-controlled, automated device to work with. A partial list of some of the diverse applications of AMD is provided in Table 5, and some further comments follow below.

The German drinking water regulations recognize about 300 crop-protecting agents and their metabolites as potential contaminants of drinking water and ground water sources. AMD was shown to be suitable for screening extracts isolated by solid-phase extraction, to identify possible contaminants. A typical AMD separation is shown in Fig. 15 [87]. A solvent gradient from acetonitrile to hexane, with dichloromethane as the base solvent, is generally used for preliminary screening; this is supported by a second gradient, using methyl *tert.*-butyl ether as the base solvent, for confirmation. Multiple wavelength scanning of the chromatogram is also commonly used to enhance the information content of the chromatogram. Postchromatographic application of specific derivatizing agents

Table 5
Survey of applications employing automated multiple development (AMD)

Application	Ref.
(1) Crop-protecting agents in water	
Organochlorine pesticides	81, 82, 85, 86, 88
Organophosphorus pesticides	86
Carbamates	87
Ureas	87
Triazines	85, 86, 89
(2) Pharmaceutical and clinical applications	
Estrogens in tablet formulations	90
Drugs and metabolites	84
Protein phenylthiohydantoin-amino acid derivatives	91
Ecdysones (insect steroid hormones)	92
Gangliosides in biological fluids	47
(3) Natural products and foods	
Isobutylamides and polyacetylenes in plant extracts	93
Phenols in plant extracts	80, 82
Alkaloids in plant extracts	94
Phospholipids in emulsifiers	63
Vanillin and related flavor compounds in foods	95
Sugars in beverages	83
(4) Industrial chemicals	
Poly(ethylene glycol) oligomer distribution	48
Industrial waste water	62
Explosive residues in soil and water	98

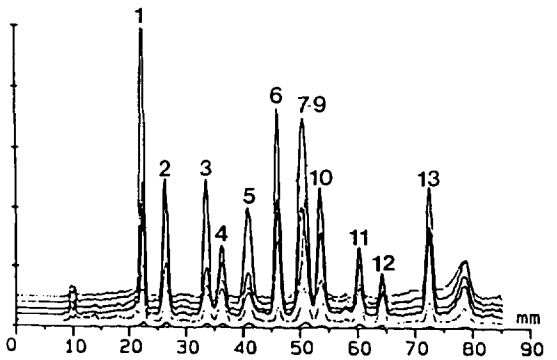

Fig. 15. AMD separation of 13 pesticides and herbicides using a 30-step gradient with methanol and dichloromethane as developing solvents. From Ref. [87]; © Research Institute for Medicinal Plants.

and enzymatic reagents are further techniques used to identify candidate compounds. By the above techniques it is possible to arrive at a small number of substances that could be present in a sample and to eliminate several hundred others from further consideration in just a few hours. More specific analytical procedures can then be used for the final determination.

The botanical origin and authenticity of natural vanilla extracts can be determined from the ratio of the principal polar aromatic flavor compounds 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid and vanillic acid normalized to the major component vanillin [95–97]. These components can be separated from each other and the sample matrix by AMD on silica gel layers. The large peak at the origin in Fig. 16A represents strongly retained matrix components and microparticulates common to vanilla extracts. The TLC method provides high sample throughput because it allows simultaneous sample cleanup and separation. Since natural vanilla is expensive there is an economic incentive to produce counterfeit extracts prepared from other plant materials and synthetic flavor compounds, etc. The counterfeit vanilla extract shown in Fig. 16B is easily distinguished from the authentic extract by the presence of ethyl vanillin, a synthetic flavor compound not found in natural vanilla, by the presence of coumarin, a plant

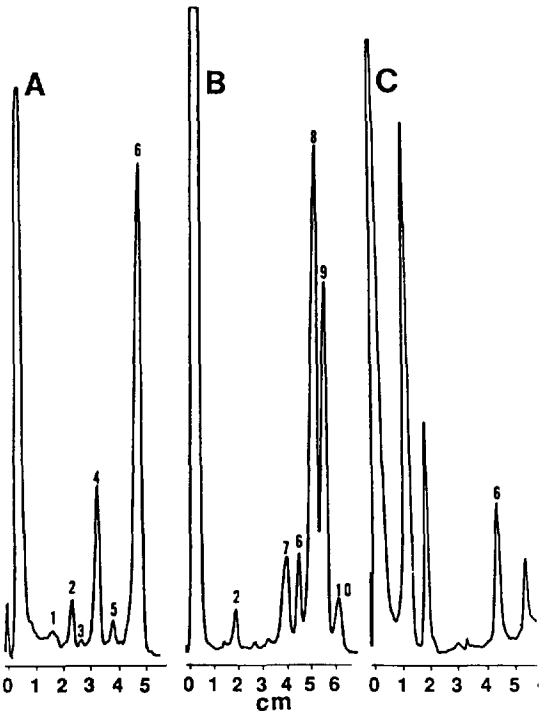

Fig. 16. Separation of vanillin and related flavor compounds by AMD in an authentic vanilla extract (A), counterfeit vanilla extract (B) and an extract from chocolate (C). Peaks: 1 = 4-hydroxybenzyl alcohol and 3,4-dihydroxybenzaldehyde; 2 = 5-(hydroxymethyl)-2-furfural; 3 = 4-hydroxybenzoic acid; 4 = 4-hydroxybenzaldehyde; 5 = vanillic acid; 6 = vanillin; 7 = anisic acid; 8 = ethyl vanillin; 9 = coumarin; 10 = anisaldehyde. From Ref. [1]; © American Chemical Society.

product not found in significant amounts in natural vanilla, and the low concentration of vanillin and the absence of other polar aromatic flavor compounds characteristic of natural vanilla. The chromatogram in Fig. 16C illustrates the separation of vanillin in chocolate. The chocolate sample was slurried in 95% (v/v) ethanol and filtered to remove insoluble cocoa particles prior to application to the layer. The final position of vanillin in the chromatogram is not influenced by the complexity of the sample matrix and TLC with AMD separation has been used to determine natural and synthetic vanilla flavors in a wide range of beverages, dairy products and bakery goods.

Fig. 17 illustrates the separation of phenolic

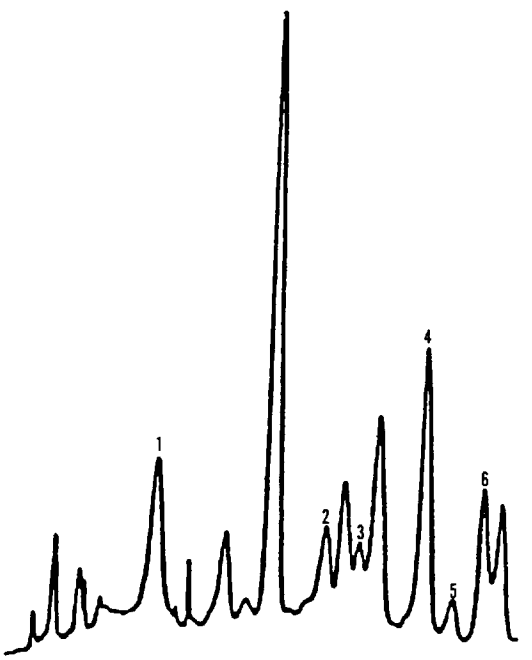

Fig. 17. Separation of phenolic compounds from a sample of chamomile by AMD. Peaks: 1 = apigenin-7-O-glucoside; 2 = caffeic acid; 3 = apiegenin; 4 = umbelliferone; 5 = ferulic acid; 6 = herniarin. From Ref. [80]: © Elsevier.

compounds in a solvent extract from an acidified aqueous suspension of the herb chamomile [80]. A methanol–dichloromethane gradient containing water and formic acid as additives to suppress ionization of the weakly acidic compounds, was used for the separation. The oligomer distribution and average molecular mass (up to 1000) for poly(ethylene glycol) 3,5-dinitrobenzoyl esters was determined by AMD on silica gel layers [48]. An example of a typical separation was presented in Fig. 9. The ethylene oxide oligomer units are transparent at the measuring wavelength and the peak area responses are derived entirely from the 3,5-dinitrobenzoyl groups. Provided that the sample amount applied to the layer is known the average molecular mass values can be computed directly from the integrated report of the chromatogram obtained by scanning densitometry.

4. Two-dimensional development

Two-dimensional TLC enjoyed a renaissance in the mid-1980s after the publications of Guiochon and co-workers demonstrating the possibility that high spot capacities, exceeding those for HPLC as normally practiced, easily could be obtained (see Section 2.2). However, the practical difficulties of realizing the necessary experimental conditions to achieve a high spot capacity and the difficulty of performing quantitative analysis in the two-dimensional separation mode finally caused a loss of interest in the technique, at least among analytical chemists. For a long time biochemists and clinical chemists have used two-dimensional TLC to identify indigenous compounds associated with metabolic disorders [4,99–101]. Two-dimensional TLC is the backbone of ^{32}P-postlabeling assay for DNA adducts thought to be the putative initiating event in carcinogenesis [102,103]. High selectivity is required in these studies, illustrating the virtues of two-dimensional TLC, but the methods are labor and time intensive and usually semi-quantitative at best. A comprehensive review of two-dimensional TLC was given by Zakaria et al. [104] and shorter topical reviews are also available [16,40,71,105]. Rather than duplicate this material we will attempt to update and emphasize those aspects of two-dimensional TLC that could result in a rebirth of interest in the technique by analytical chemists.

4.1. Capillary-controlled-flow conditions

In two-dimensional TLC the sample is spotted at the corner of the layer and developed along one edge of the plate. The plate is then removed from the first mobile phase, dried, rotated through 90°, and redeveloped in the orthogonal direction. If the same solvent is used for both developments the spots will be aligned along the diagonal from the corner at which the plate was spotted to the opposite edge at the position of the second solvent front. The increase in resolution will be modest; no more than 1.41 times the resolution in the first development (corre-

sponding to the increase in the number of theoretical plates associated with doubling the migration distance). This improvement in resolution is too small to be useful for most applications.

The realization of a more efficient separation system implies that the resolved sample should be distributed over the entire plate surface. This can be achieved only if the selectivity of the separation mechanism is complementary in the orthogonal directions. In practice this is not so easy to achieve. Some potential methods for generating two different retention mechanisms in orthogonal directions are summarized in Table 6 [16]. Using a single sorbent and two solvent systems with complementary selectivity is the simplest approach to implement in practice but is often only partially successful [106–109]. In many cases the two solvent systems differ only in their intensity for a given set of intermolecular interactions and are not truly orthogonal in properties. The sample components become distributed about the diagonal and a significant portion of the separation space remains unused. Also, the solvent used in the first development modifies the sorbent, and unless it can be easily and quantitatively removed during the intermediate drying step, or alternatively the modification can be performed reproducibly, this can result in inadequate reproducibility of the separation system from sample to sample. These problems are more noticeable using silica gel layers and polar mobile phases. Chemically bonded layers are compatible with the use of reversed-phase and normal-phase solvent systems as well as permitting the use of additives and buffers as a further means of adjusting selectivity [105,110,111]. Fig. 18 provides an example of the separation of cholesterol and its bile acid metabolites by two-dimensional TLC using a combination of normal-phase and reversed-phase solvent systems for the orthogonal developments on a cyanopropylsilanized silica gel layer [110]. Aminopropylsilanized silica gel layers can be used as ion-exchange media with an acidic buffered mobile phase and as a reversed-phase or normal-phase separation media under non-acidic conditions. Although few of the described systems may be truly optimized in terms of obtaining the maximum separation capacity theoretically predicted, in a number of cases as many as 20 to 30 components have been successfully separated using different mobile phases for the orthogonal developments, indicating the potential of this approach for obtaining a useful increase in the separation capacity in TLC. The fact that a further increase in the separation performance might be obtained through the use of multiple development [90,112] or AMD [113] for either or both ortho-

Table 6
Possible methods for generating two different retention mechanisms in orthogonal directions

(1) A single sorbent layer is used and sequentially developed in orthogonal directions by two mobile phases with different selectivities for the sample components.
(2) A bilayer plate prepared from two sorbents with different selectivities can be used. The sorbent layer for the first development is a narrow strip that abuts the much larger area used for the second development. Commercially available plates have silica gel and reversed-phase layers as adjacent zones.
(3) A layer containing an intimate mixture of two complementary sorbents can be used with different mobile phases such that the retention mechanism for the two, orthogonal developments is dominated by the properties of one of the sorbents in each direction. This is not a common approach; for an example using polyethyleneimine–cellulose for the separation of oligonucleotides see Ref. [103].
(4) Between developments the characteristic properties of the layer are modified by impregnation with a chemical reagent or immiscible solvent prior to the second development.
(5) Between developments the properties of the sample are modified by chemical reaction or derivatization prior to the second development.

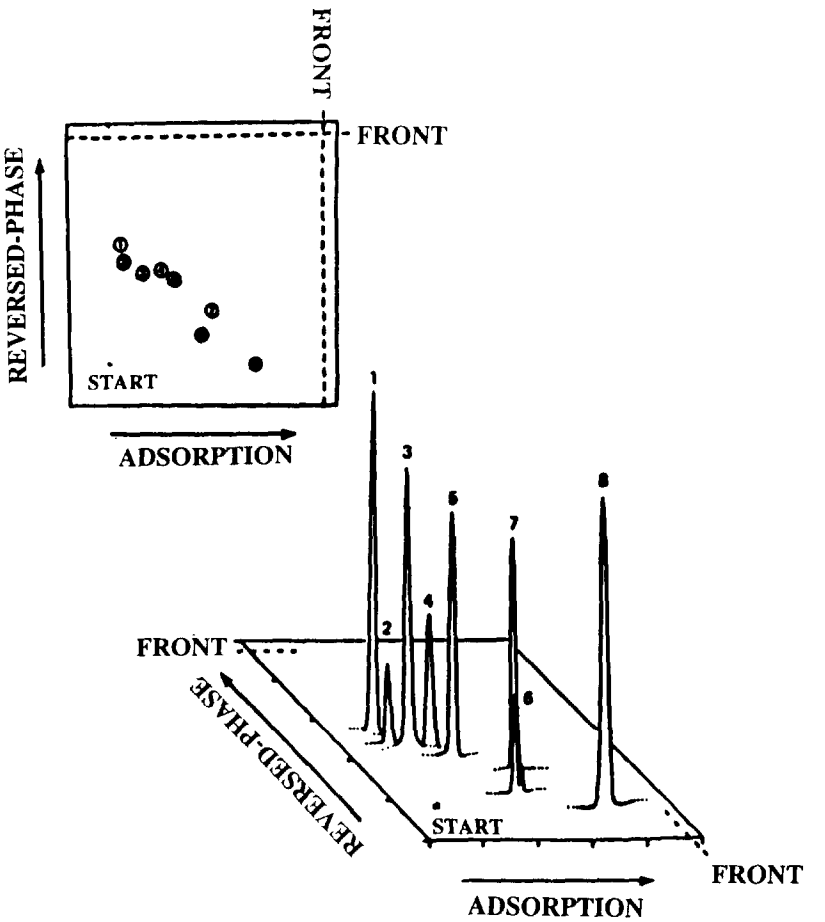

Fig. 18. Two-dimensional separation of cholesterol and its bile acid metabolites on a cyanopropylsilanized silica gel layer using acetone–water (1:1) for the first development and light petroleum–acetone (7:3) for the second orthogonal development. Peaks: 1 = cholic acid; 2 = dehydrocholic acid; 3 = cholic acid methyl ester; 4 = chenodesoxycholic acid; 5 = desoxycholic acid; 6 = 7-hydroxycholesterol; 7 = lithocholic acid; 8 = cholesterol. From Ref. [110]. © Marcel Dekker.

gonal developments seem to have been rarely recognized. Fig. 19 is an example of the separation of pharmaceutically important estrogens obtained by two-dimensional TLC employing multiple development for the separations in both orthogonal directions [90].

The use of bilayer plates is an attractive means of creating complementary retention mechanisms in two-dimensional TLC but has received less attention than mobile phase optimization [105,114–116]. Commercially available layers have a 3-cm strip of silica gel (or octadecylsilanized silica gel) coated side-by-side with a 17-cm strip of octadecylsilanized silica gel (or silica gel). An alternative approach to using a bilayer plate is to make a normal-phase separation on silica gel with simultaneous paraffin impregnation for a subsequent reversed-phase separation on the paraffin impregnated layer as the second orthogonal development [117].

Impregnating the sorbent layer with a selective reagent between developments is also easily carried out [118,119]. For example, Grinberg and Weinstein [120] separated dansyl amino acids into their enantiomeric forms employing a reversed-phase separation in the first direction,

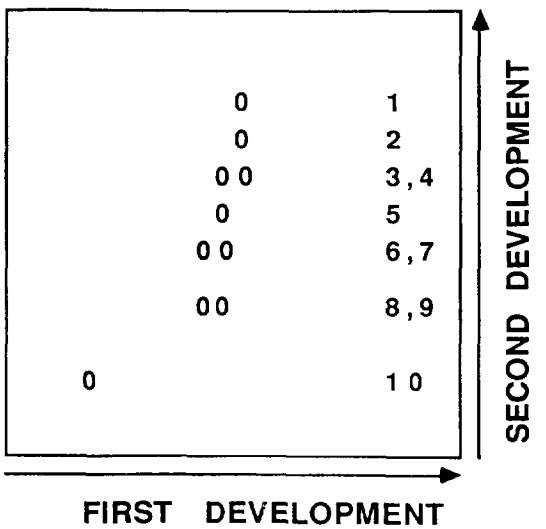

Fig. 19. Separation of pharmaceutically important estrogens on silica gel by two-dimensional TLC employing multiple development for the orthogonal separations. The solvent system for the first development was cyclohexane–ethyl acetate (3:1) with sequential developments of 5, 6, 7, 8, 9, 10 and 12 min. The solvent system for the second development was cyclohexane–ethyl acetate–2-propanol–triethylamine (74:18:2.5:5.5) for 6 developments of 10 min each. Identification: 1 = estrone; 2 = equilin; 3 = 17α-estradiol; 4 = 17β-estradiol; 5 = equilenin; 6 = 17α-dihydroequilin; 7 = 17β-dihydroequilin; 8 = 17α-dihydroequilenin; 9 = 19β-dihydroequilenin; 10 = estriol. From Ref. [90]; © Research Institute for Medicinal Plants.

impregnation of the layer with the chiral resolving agent copper N,N-di-*n*-propyl-L-alanine between developments, followed by a reversed-phase separation in the second orthogonal development. Similarly, silver nitrate impregnation of silica gel has been used to affect a separation of saturated and unsaturated lipids after a reversed-phase separation on a bilayer plate [121]. Wilson [122] employed boronic acids as mobile phase additives for the selective separation of steroids containing vicinal diol groups. The same mobile phases were used in both developments with the boronic acid added for the second development to complex with the steroids containing suitable functional groups. As an example of the use of derivatization to change the properties of the solutes between developments, Bakavolina et al. [123] partially separated some pharmaceutically important benzodiazepines by normal-phase chromatography, hydrolyzed in situ the benzodiazepines with acid between developments forming the benzophenones, and then obtained complete separation of the benzophenones in the second orthogonal development.

4.2. Forced-flow conditions

The maximum spot capacity in two-dimensional TLC can only be achieved if the kinetic properties of the layer are optimized and the retention mechanisms for the two orthogonal developments are complementary. The former condition can be met using forced-flow development, the latter, as discussed above, is often difficult to satisfy completely. Two-dimensional TLC with forced-flow development is possible using the overpressured development chamber; the plate being removed from the chamber between developments to evaporate the solvent used for the first development [124–126]. Recording the final separation in the presence of the sorbent is not straightforward (see Section 4.4). On-line detection with elution as the second displacement mode would be more convenient as well as increasing the separation potential of the system [35,127–129]. This is possible if the separated spots are eluted through a pair of quartz plates running along the complete length of the layer opposite to the solvent entry position for the second displacement. The quartz plates form the detection cell which is evenly illuminated with monochromatic light and focused onto a photodiode array. The signals from the array are recorded as a three-dimensional space–time–concentration chromatogram.

The advantages of forced-flow two-dimensional TLC have to be judged against the practical difficulties in implementing the technique. The detection problem is generally considered the Achilles heel of the system, whether on-line with elution or in situ using scanning densitometry. The on-line approach is obviously complex and requires considerable computational power and data storage capacity. Problems which over time are likely to be less of an inhibition to im-

plementation. A disturbing zone results whenever the sealed dry layer first contacts the mobile phase and the trapped air is displaced as a mixture of microbubbles and partially dissolved by the solvent. As the disturbing zone traverses the chromatogram it can cause distortion of the zone profiles unless precautions are taken to prevent its formation. Several remedies are available, but may not be convenient in all circumstances [2,22,130]. The intermediate drying step between development presents problems when using a sealed system. Removal of the layer between the orthogonal developments is always required when the overpressured development chamber is used since it has a single, fixed solvent entry position. All the seals must be broken and then remade with the plate installed in a different position (rotated through 90° compared to the original position). Leaks and other problems may interfere in the second development. The layer need not be removed in the equipment described for bidirectional column chromatography since two orthogonal solvent entry positions are available. Complete removal of the first mobile phase prior to the start of the second development is achieved by forcing inert gas through the overpressured layer or by pumping a third solvent through the layer, with the proviso that the third solvent is miscible with the mobile phases used for both chromatographic displacements as well as being a weak solvent with respect to the retention mechanism employed in the second chromatographic displacement. Inadequate removal of the first mobile phase prior to the second orthogonal chromatographic displacement can lead to distortion of the chromatogram and an increase in zone broadening. To the above problems must be added the difficulty of finding two complementary retention mechanisms for the orthogonal chromatographic displacements for many samples. There is little activity in two-dimensional forced-flow TLC at the moment, and therefore it must be judged that the problems for the present outweigh the benefits. This situation can change quickly as the pace with which new technology is introduced is unpredictable and so two-dimensional forced-flow TLC should be judged as dormant for now but not dead.

4.3. Computer-aided simulations

Several methods of computer simulation of two-dimensional chromatograms employing unidimensional chromatographic experimental data and either a mathematical function for ranking of chromatograms or visual interpretation using contour diagrams have been developed [71,72,131]. Since any spot in a two-dimensional chromatogram can be defined by a pair of x and y coordinates, the quality of a separation can be established by comparing the separation distance between all pairs of components in the chromatogram for any given combination of solvent systems and/or stationary phases. Resolution functions are absented by the difficulty of determining the true peak profiles in two-dimensional chromatograms as usually recorded. The simplest computational approach is to seek the lowest correlation between unidimensional R_F values in each of two sequential solvent systems [108,124,132]. The lowest correlation is a measure of the extent of the deviation of the data from a diagonal relationship and does not respect individual spot center separations except as they contribute to the overall correlation coefficient. It is effective at easily identifying poor separation systems which show a high correlation, but less useful at identifying the optimum separation system from a number of acceptable systems. Gonnord et al. [133] defined two mathematical functions, termed D_A and D_B, to judge the quality of simulated two-dimensional separations using the R_F values for each solute in two sequential solvent systems as the x and y coordinates. D_A was defined as the sum of the squares of the distance between all spot pairs and D_B as the inverse of the squares of the inter-spot distances after the elimination of unresolved pairs or by defining an arbitrary minimum separation distance for all unresolved pairs. The function D_A gives equal weighting to all spot pairs whereas D_B gives a higher weighting to poorly separated pairs, and consequently, D_B tends to select fewer optimum separations with overlapping spots than D_A. These functions were modified by Steinbrunner and co-workers [131,134] who, rather than summing the squares of the distances with D_A, summed the distances

(the distance function); a similar modification was made to D_B to produce the inverse distance function, with overlapping spots arbitrary assigned a distance of 1 mm. Nurok and co-workers [115,134,135] have since extended the above approach and introduced a planar chromatographic response function, PRF, defined by Eq. 9.

$$\mathrm{PRF} = \sum_{i=1}^{k_s-1} \sum_{j=i+1}^{k_s} \ln(S_{D,ij}/S_{D,spec}) \quad (9)$$

where k_s is the number of spots in the mixture, $S_{D,ij}$ is the distance between spot centers and $S_{D,spec}$ is a specified separation distance. Pairs of spots with $S_{D,ij} > S_{D,spec}$ are assigned separations of $S_{D,spec}$ thereby limiting their contribution to the PRF. Thus the PRF considers only poorly separated spot pairs unlike the inverse distance function which considers all spot pairs. Howard and Bonicke [136] have defined an alternative response function based on the distance between the mid-points of each pair of spots. With this function even small changes in the separations cause a rapid variation in the response value obtained, but once separated, two spots would contribute no further. Heimler and Boddi [137] used cluster analysis as a method to compare separations obtained by two-dimensional TLC. In this case optimization was not the object of the comparison. The method was used to establish the similarity of samples separated by two-dimensional TLC based on the number and position of common compounds in the chromatograms.

None of the above criteria can be considered ideal for evaluating all two-dimensional chromatograms. Those functions based on the use of a critical pair are useful when the only requirement is that all spots be separated by a defined minimum distance. It only provides information about the poorest separated spot pairs in a mixture. The PRF functions should provide a better indication of which two-dimensional system will provide the best overall spot separation. The distance function is insensitive to the presence of poorly separated spots and, therefore, is a poor criterion for judging the success of a particular separation. It is, however, a good judge of how well dispersed the spot centers are in the separation space and may be of value for evaluating the separation of complex mixtures where spot overlap is inevitable and where utilizing all the separation space of the system is important.

The agreement between computer simulated two-dimensional chromatograms and experimental results vary from very good to poor. A large number of factors can affect the agreement such as variations in the sorption properties of the plate due to changes in humidity, modification of the sorbent characteristics by the first solvent system in the second orthogonal development, variations in the solvent velocity constant between chromatographic systems, and solvent demixing. In common with all computer-aided methods development strategies in chromatography a weak point remains the selection of a single-valued function to rank chromatograms. Perhaps the simple truth is that no such function will ever prove adequate in all situations and a different approach to characterizing the information content of a chromatogram is required. It is not unusual for different chromatographic response functions to rank separations differently and to identify different sets of optimum conditions adding to the confusion in making a final selection. However, since computer simulations require only a fraction of the time and materials required by experiments they are useful for aiding the optimization of two-dimensional planar separations.

4.4. Imaging two-dimensional chromatograms

Certainly one of the principal reasons two-dimensional TLC is considered by many analytical chemists a qualitative technique is the lack of convenient methods for displaying two-dimensional chromatograms and of obtaining in situ quantitative information from individual zones. Scanning of two-dimensional chromatograms using laser-based indirect fluorometric detection [138] and photoacoustic detection [139], and fast atom bombardment and liquid secondary ion mass spectrometry [140,141], has been demonstrated. It is also possible to scan two-dimensional chromatograms when the analytes contain

radioactive elements using modern radioimaging devices [142,143]. These approaches require either special equipment that is not found in most analytical laboratories or, in the case of radioimaging, is limited to samples that are not commonly encountered in everyday work. The common method of detection in TLC is slit scanning densitometry [2,4,144]. It is only recently that this technique has been adapted to recording two-dimensional chromatograms. Image analysis devices are an emerging detection technique in TLC and are compatible with the requirements of a system for recording two-dimensional TLC separations. The latter two approaches would seem capable of eliminating the detection/recording bottleneck restricting the greater utilization of two-dimensional TLC and are the techniques that will be discussed in this section.

Slit-scanning densitometers were designed to locate and quantify sample zones organized into lanes and were not considered suitable for imaging surfaces. Taube and Neuhoff [145] used a high-precision microscope–photomultiplier and scanning stage with associated software to demonstrate the feasibility of imaging two-dimensional TLC separations with a slit-scanning device. Prosek and co-workers [146,147] have developed a software package for imaging two-dimensional TLC separations with a Camag slit-scanning densitometer. The TLC plate is scanned in the normal way with a slit slightly wider than the spot diameters (Fig. 20). Scanning is then repeated by making a small displacement in the x direction such that the displacement is a small fraction of the slit height, for example, 10%. Integration of the analog signal is done in the x direction during each scan and after all scans are completed, the positions of the spots are located, and integration in the y direction is then computed by analysis of the stored scan data. The correct position of each peak on the two-dimensional plate is located according to the peak height maxima by searching the individual scans for the largest values and establishing the zone dimensions in the x and y direction. The chromatogram is displayed in two-dimensional format as circles on a plane or in three-dimensions as a surface with peaks rising from a plane.

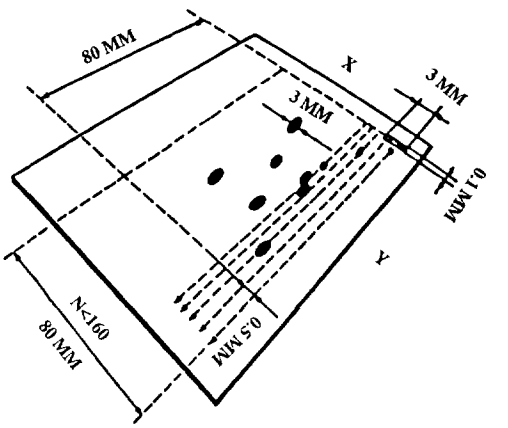

Fig. 20. Representation of the principle used to scan a two-dimensional TLC separation with a linear slit-scanning densitometer. From Ref. [146]; © Research Institute for Medicinal Plants.

An alternative approach is made available with the Shimadzu flying spot scanning densitometer [148–150]. A combination of a fixed slit and a spiral slit on a rotating disk located at the exit of the monochromator is used to form a minute beam (0.4×0.4 mm) which can be moved across the surface of the layer in a zigzag manner (Fig. 21). The reciprocating motion of the rotating disk displaces the light beam across

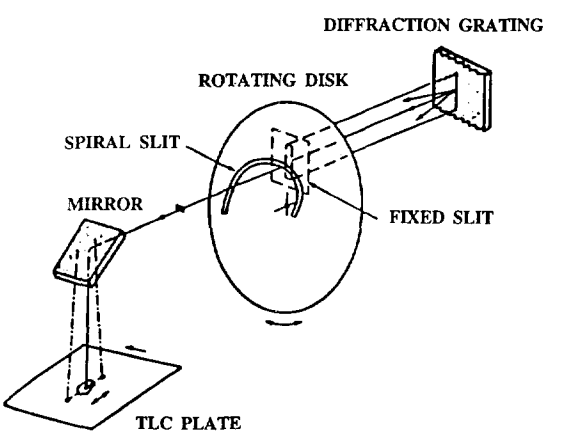

Fig. 21. Principle of zigzag scanning using a flying spot scanning densitometer for imaging two-dimensional TLC separations. From Ref. [150]; © Elsevier.

a segment of the layer (x direction) while the simultaneous movement of the scan stage by 0.2 mm in the orthogonal direction (y direction) at the end of each reciprocating motion causes the measuring beam to move across the layer in a raster fashion. To scan the whole TLC plate each raster scan of 10 mm (x direction) is repeated sequentially by moving the sample stage by 10-mm increments to cover the width of the layer with full scans in the y direction for each segment. It requires about 15 min to scan a 10×10 cm TLC plate using the above process and a few minutes for computation of the representation of the surface as a contour map or three-dimensional image.

Mechanical scanning of the surface of a TLC plate is slow by whatever approach is taken compared to the alternative of electronic scanning. On the other hand the mechanical scanners take advantage of existing instrumentation for lane scanning using new software for data analysis. Only a single sample can be separated on each layer in two-dimensional TLC so calibration requires that standards and samples are run on different plates. The methods employed for integrating the absorbance of each zone, defining zone boundaries, and subtracting the background contribution from the layer become increasingly important if meaningful quantitative data is to be obtained. We are not aware of any published independent evaluation of the above products from which we could make some general comments on the reliability and ease of use in a typical analytical laboratory.

In TLC the separation is static after the completion of the development step and it should be simple to image such a system using a video camera or similar device [144,151–156]. Unlike mechanical scanning techniques, electronic scanning enables images of a surface to be obtained rapidly and individual images to be summed to improve signal-to-noise ratios. Equipment requirements for image analysis are an imaging detector such as a vidicon tube or charged-coupled video camera, a computer with video digitizer, and associated light source and optical system. A suitable arrangement for image analysis in the reflectance mode is shown in Fig. 22 [151]. The plate is evenly illuminated with monochromatic light and the reflected light focused as a scaled image of the plate directly onto the active element of the vidicon. The vidicon functions as a two-dimensional array of unit detectors continuously scanned by an electron beam. Each detector is periodically discharged by the electron beam and the signal digitized for computer analysis. The captured images are initialized, stored, and transformed by computer into chromatographic data. In only a few cases are exact details of the procedures used to convert images into x and y coordinates and optical density given. Common to all techniques of data handling is background subtraction in which the accumulated images of a blank plate are subtracted from the analytical plate on a pixel-by-pixel basis. Thresholding is also routinely used to ensure that negative values for the plate luminescence do not occur. Image analysis is a data-intensive technique and to a large extent the number of images that can be stored and the type of calculation algorithms used are a function of the capacity of the computer.

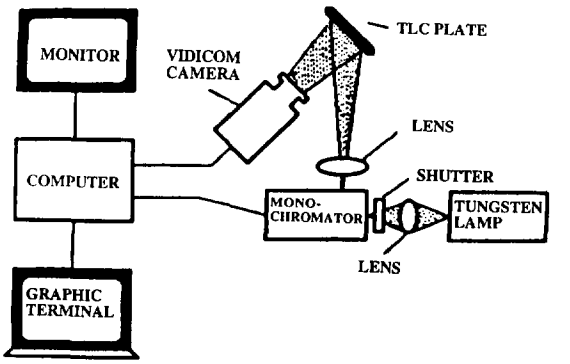

Fig. 22. Optical layout for an image analyzer used to record two-dimensional TLC separations in the reflectance mode. From Ref. [151]; © Elsevier.

The main attractions of image analysis for detection in TLC are fast data acquisition, simple instrument design, and the absence of moving parts. A portable image analyzer for field work has already been described [157]. However, there are several limitations with systems based on today's technology [153]. The spatial

resolution of a scanning system is determined by the number of pixels per unit image area and is typically 512×512 pixels for commercial cameras. This is barely adequate for some applications using HPTLC. Silicon-based area sensors and line sensors are fairly insensitive to UV radiation. This can be partially rectified using phosphor coatings and intensifiers, but most low cost systems are limited to the visible region. Non-linearity of the grey scale and unequal resolution between the x and y directions is a further drawback of most standard devices. Compared to slit-scanning densitometers the available wavelength measuring range, sample detectability, and dynamic signal range are significantly restricted for comparable system costs.

Image analysis has many applications beyond densitometry and the associated technology is in a research-intensive, evolutionary phase. Significant developments can be expected and image analysis will likely become the preferred method of densitometric evaluation for one- and two-dimensional TLC separations at some time in the future.

5. Coupled chromatographic systems with planar chromatography as one component

There are several reasons for coupling chromatographic systems in which planar chromatography is employed as one of the separation dimensions [16,40]. The obvious reason is to improve the separation capacity of the separation system by using two sequential complementary retention mechanism for the separation. This is the reason common to all the multimodal separation techniques. The unique feature of TLC is the possibility of using the layer as a storage device. After development in TLC the separated sample components are stationary and can be further investigated free of time constraints. In terms of detection possibilities, chemical modification of the separated zones is facilitated, the chromatogram can be scanned multiple times to enhance signal to noise ratios, the chromatogram can be scanned sequentially using different detection principles to enhance the information content of the separation, the separation can be easily transported to different locations for detection, and some detection techniques that are enhanced by sorption onto a solid surface, such as room temperature phosphorescence, fluorescence line narrowing at low temperature, and surface enhanced Raman scattering, are facilitated [144,158–161]. Other detection techniques such as infrared and mass spectrometry may be facilitated by the absence of solvent during the detection process [7,140,162,163]. A further reason for using planar chromatography in a coupled chromatographic system is that the TLC layer is disposable after a single use. Consequently, it can be used to provide sample cleanup simultaneously with separation, obfuscating the problems and time associated with regenerating column systems between separations.

The common approaches to coupling chromatographic systems are generally characterized as on-line or off-line. Off-line coupling does not normally require any special apparatus for an interface and is free of limitations of flow and solvent mismatches between the two separation dimensions. It is almost always applicable, such as collecting the effluent from a column with a fraction collector, removing the mobile phase, and redissolving the sample in a second solvent for application to the layer for separation in the second dimension. On-line coupling usually demands more technical ingenuity in setup, but once established is generally time and labor saving, minimizes the risk of contamination, and possibly can be completely automated. Since no special considerations are involved in the off-line coupling of other chromatographic techniques to planar chromatography we will describe largely the on-line approach to coupled systems employing planar chromatography as one separation dimension.

5.1. Gas chromatography

Gas chromatography (GC)–TLC is not particularly difficult to carryout but has not been widely practiced since the late 1960s when several working instruments were described [164–

166]. In most instances TLC was used to either confirm the identity of a GC peak or as a test of peak homogeneity. These problems are generally more conveniently solved by GC–mass spectrometry today. The GC effluent was generally spit between a flame ionization detector and a heated capillary transfer line that carried the major portion of the effluent to the layer. The capillary orifice was positioned close to the layer which was supported on a translation table that enabled its position to be moved with respect to the detection of components in the effluent by the flame ionization detector. The distance between the capillary orifice and the layer was the most critical parameter in maintaining a high recovery of the analytes as reasonably compact zones.

The coupling of TLC to GC (TLC–GC) is possible using laser desorption to vaporize the sample zones separated by TLC [167]. The TLC plate is placed in a copper chamber sealed by a quartz window. Carrier gas enters the chamber from three corners, passes over the sorbent surface, and exits through the fourth corner where a heated capillary transfer line directs it into a standard gas chromatographic inlet. The laser beam is focused onto the sorbent surface through the quartz window and scanned across the sorbent surface by a mirror driven by a stepping motor. Using a low-powered, pulsed laser results in rapid heating and vaporization of the sample without significant ionization. To obtain high sample recovery with minimum pyrolysis the laser power, laser pulse repetition rate, space between the sorbent surface and the quartz window, and carrier gas flow-rate have to be optimized. Also, low recovery can be caused by adsorption of desorbed compounds on the cool surfaces of the chamber or by readsorption by the sorbent during their passage to the capillary transfer line. The composition of individual zones was then determined by the separations obtained by GC. The usefulness of this approach is limited by background peaks from the sorbent coupled with pyrolysis products generated from the analytes. In related studies pyrolysis–GC has been used to identify and quantify analytes isolated by TLC after scrapping off appropriate zones from the layer and introducing them into a standard pyrolyzer inlet [168,169].

5.2. Supercritical fluid extraction and chromatography

Supercritical fluids have characteristic properties somewhat intermediate between those of gases and liquids. Their low viscosity and absence of surface tension increases the speed of fluid penetration into porous matrices and promotes faster extractions than observed with liquids. Higher diffusion coefficients provide better mass transfer characteristics favorable for both chromatography and extraction. Higher densities than gases provide liquid-like solubilizing properties. In addition, since densities can be changed by adjusting either pressure or temperature, the solubilizing properties of a fluid are easily varied over a useful range by adjusting these parameters. Alternatively, the solubilizing power of a fluid can be changed by adding small amounts of a solvent modifier to the fluid. The past decade has seen many developments in the application of supercritical fluids to chromatography and extraction including the production of laboratory instrumentation to carry out the above processes under optimized conditions [170–172].

Little of the current enthusiasm for supercritical fluids in chromatography and extraction has spilled over to TLC [173,174]. In the 1970s, Stahl and Schild [175,176] developed an apparatus for supercritical fluid extraction with deposition of the fluid extracts on a moving TLC plate. The apparatus consisted of a thermostated fluid delivery system, a micro-extraction autoclave that could be sealed at its exit by a shut-off valve, and a narrow capillary of 25–50 μm internal diameter positioned a few millimeters above the TLC plate located on a translation table. The fine capillary was required to accommodate the large pressure drop between the high-pressure fluid used for extraction and the low-pressure gas applied to the TLC plate. Decompression of the fluid occurs with rapid cooling, favoring the deposition process while not inhibiting the removal of the fluid as a gas. Samples were

extracted sequentially by incrementing the density between individual extractions and the intensity of the zones observed after separation by TLC was used to identify the optimum conditions for the extraction of target compounds. The advantage of TLC for this purpose was both its capacity as a storage device and the fact that all extracted material was available for inspection so that the selectivity of the extraction process could be studied.

Keller and co-workers [177,178] have described an automated apparatus for packed column supercritical fluid chromatography (SFC) with direct coupling to TLC. The interface (Fig. 23) consists of a splitter at the column exit which directs part of the fluid to a detector, the remainder flows to a thermostated pinched capillary held a few millimeters above the TLC plate, which is moved on rollers driven by an electric motor. Compared to collecting the effluent from the SFC in decompression vessels the direct deposition of the effluent on the TLC plate leads to significant losses of analytes. The recovery of deposited analyte is strongly dependent on the presence of solvent modifier and the shape of the jet and its temperature, which in turn must be optimized for the flow-rate employed. The highest recoveries are obtained when the conditions are adjusted such that the decompressed fluid jet leaves a sharp wet line of modifier on the TLC plate. In the absence of a modifier recovery of analytes is usually less efficient. Analyte losses result from the difficulty of retaining by the layer effluent particles expelled at high speed from the restrictor. Hence, the transfer is enhanced by allowing the solute to be transported to the layer

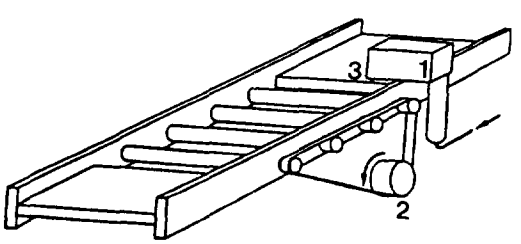

Fig. 23. Interface for packed-column SFC–TLC. 1 = Heating block; 2 = electric motor; 3 = pinched stainless-steel capillary. From Ref. [177]; © Vieweg.

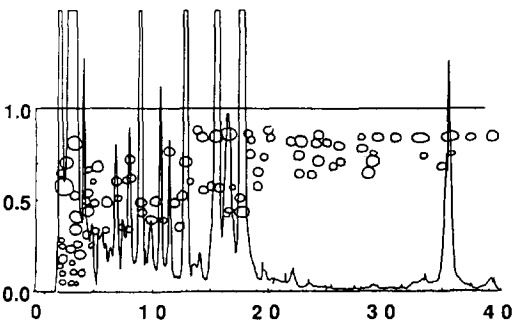

Fig. 24. Bidimensional recording of the separation of an extract of cloves. The peaks represent the recording of the column chromatogram and the spots the separation of the deposited column chromatogram by TLC. From Ref. [177]; © Vieweg.

in liquid droplets and deposited as such on the TLC plate. Fig. 24 is an example of a bidimensional recording of the column chromatogram superimposed over the TLC chromatogram of the deposited column effluent recorded in such a way that the peaks in the column chromatogram are aligned with the spots resulting from their separation in the TLC chromatogram. Most of the above studies were performed in a semi-preparative mode and yielded sufficient quantities of separated material for unknown compounds to be identified spectroscopically after scrapping the spots from the layer and recovering the analyte with an appropriate solvent.

5.3. Liquid chromatography

The combination of liquid chromatography in its various forms and planar chromatography has received the most attention. Diallo et al. [179] used a flow splitter and a commercially available spray jet interface to couple high-speed countercurrent chromatography (HSCCC) to TLC. The fractions stored and then separated on the TLC plate were used to optimize the isolation conditions for natural products by HSCCC. Bladek designed a simple apparatus (Fig. 25) for the cleanup of extracts isolated by solid-phase extraction (SPE) [180]. The apparatus can be inserted into a standard SPE vacuum manifold and consists of an L-shaped PTFE block with a

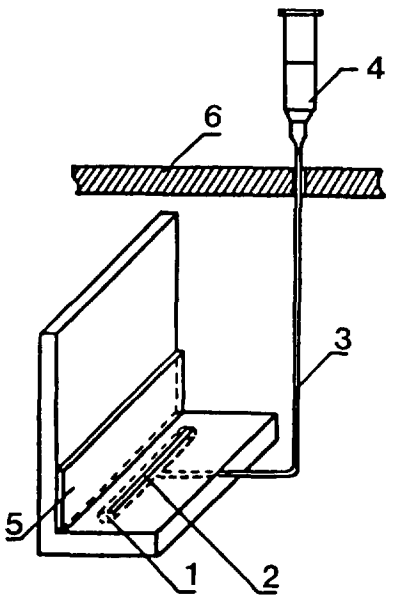

Fig. 25. Vaccum manifold insert and TLC plate holder for SPE–TLC. 1 = Channel; 2 = trough; 3 = metal capillary; 4 = SPE cartridge; 5 = heater; 6 = cover plate of vacuum manifold. From Ref. [180]; © Research Institute for Medicinal Plants.

channel (1) and trough (2) cut into it connected by a metal capillary (3) to the SPE cartridge. A heater (5) is mounted on the vertical face of the L-shaped block. Aliquots of eluent are passed through the cartridge and ascend the TLC plate housed in the trough. The purpose of the heater is to aid in the vacuum evaporation of the solvent so as to obtain a narrow band of sample for subsequent separation by TLC. Mincsovics et al. [181] used forced-flow TLC coupled on-line to column liquid chromatography (HPLC) for sample cleanup by SPE using a layer instead of a cartridge. The sample is applied to the layer as a band and then eluted from the layer by forced-flow development in an overpressured development chamber, the outlet of which was connected to the HPLC column injector. The main limitation of this cleanup procedure is the sample loadability of the layer, since the sample must be applied as a narrow band to ensure acceptable chromatography in the cleanup step. However, this approach should offer higher resolving power for the sample and matrix in the cleanup step compared to a standard SPE cartridge. The TLC plate, like a cartridge, can be disposed of after each sample application.

In 1969 Van Dijk [182] described a direct coupling between gravity flow column liquid chromatography and TLC. The interface consisted of a variable splitter with the major portion of the column effluent going to a fraction collector and the minor portion being simultaneously applied to a TLC plate. The plate was moved incrementally beneath the applicator capillary tube and synchronized with the changes in the fraction collector tubes. A shroud mounted around the applicator capillary allowed concentration of the effluent by forced-flow convection forming a series of uniform spots on the layer corresponding in composition to individual tubes in the fraction collector. Boshoff et al. [183] were the first to describe a direct coupling of HPLC–TLC. The interface included a splitter to match the column flow to the absorption capacity of the layer (ca. 0.6 ml/min). The eluent was transferred to the layer by a fine steel capillary (0.25 mm I.D.) located above the layer in such a way that a liquid bridge was maintained at all times. The TLC plate was mounted on a block heater and shrouded by a vacuum manifold to aid solvent evaporation. A worm gear moved the TLC plate at a constant speed under the applicator during the deposition of the column eluent. The TLC plate was used in these studies as a storage detector with compounds subsequently identified by fluorescence induction. No chromatography was involved after deposition of the column eluent but this would have been possible with the proposed interface design. Karmen et al. [184,185] devised a convenient system for fractionating the effluent from an HPLC column and concentrating the fractions prior to radioassay using a TLC contact spotter. A succession of aliquots of the HPLC effluent were deposited into wells formed in a non-wetting fluorocarbon film and evaporated to near dryness where the aliquots form droplets of uniform size. The droplets were then transferred by pressure contact to filter papers impregnated with a scintillator as uniform spots of 2-3 mm

diameter for detection by autoradiography. The purpose of these studies was the detection of weak activity radiolabeled analytes in samples fractionated by HPLC, but the interface used is a standard transfer applicator for TLC and should function just as well for applying samples for separation by TLC. The design of the contact spotter allows aliquot sizes up to 100 to 350 μl to be collected and evaporated to a residue.

Fig. 26 is an example of a spray jet interface used to couple narrow bore HPLC columns with TLC [186–188]. A similar device based on a modification of the Linomat band applicator for TLC is commercially available [61,94,189]. At flow-rates characteristic of narrow bore columns, 5–100 μl/min, the total column effluent can be applied to the layer; a splitter before the applicator must be used at higher flow-rates. The critical parameters for obtaining a narrow application zone on the layer are the flow-rate and temperature of the nitrogen support gas, the column flow-rate and mobile phase composition, the distance between the needle of the spray jet and the layer surface, and the speed at which the layer is translated beneath the spray jet. When fully optimized the column chromatogram can be transferred and deposited on the layer without additional zone broadening resulting from the transfer. The deposited zones, however, have a characteristic horseshoe shape distribution of material in the direction of application as a consequence of the distribution of the analytes in the column eluent [186,187]. Refocusing of the application band, as occurs in multiple development, is an easy solution to this problem. The high resolving power of LC–TLC is demonstrated for the separation of a mixture of 56 pesticides in Fig. 27 [61]. The mixture is separated into 17 fractions on the reversed-phase column. Each fraction can be transferred successively to the silica gel TLC plate for separation by automated multiple development. The bottom part of Fig. 27 illustrates the TLC separation of coeluted components in fraction 13.

The two obvious limitations of the spray jet interface are its restricted flow capacity as far as conventional diameter columns are concerned and the need to ensure that the column mobile phase is compatible with the requirements of the TLC separation. For example, it is very difficult

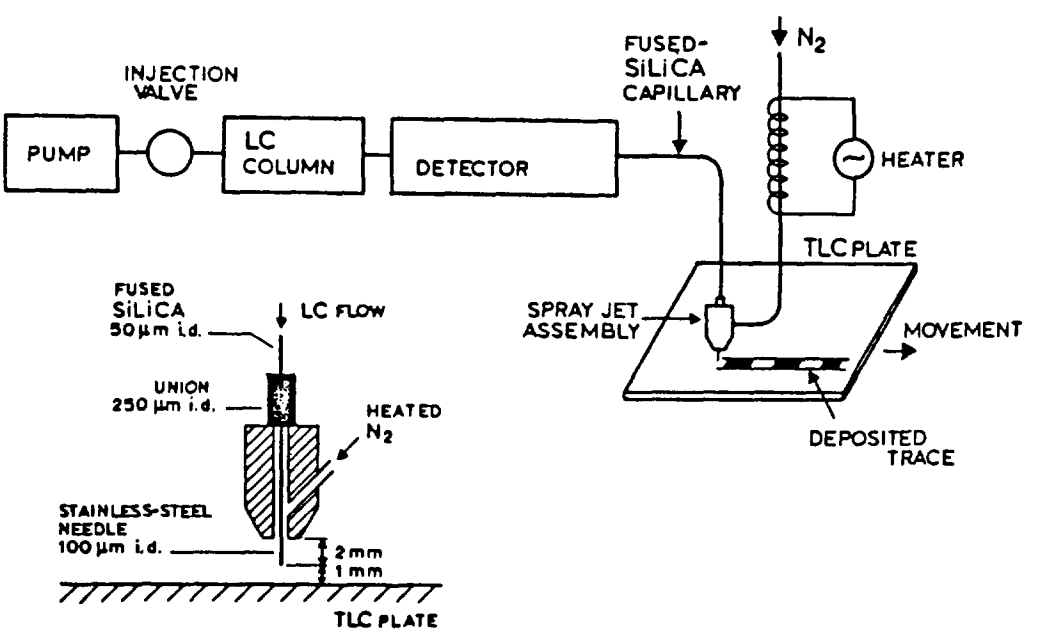

Fig. 26. Diagram of a spray jet interface for narrow-bore LC–TLC with an enlarged view of the spray jet. From Ref. [188]; © Elsevier.

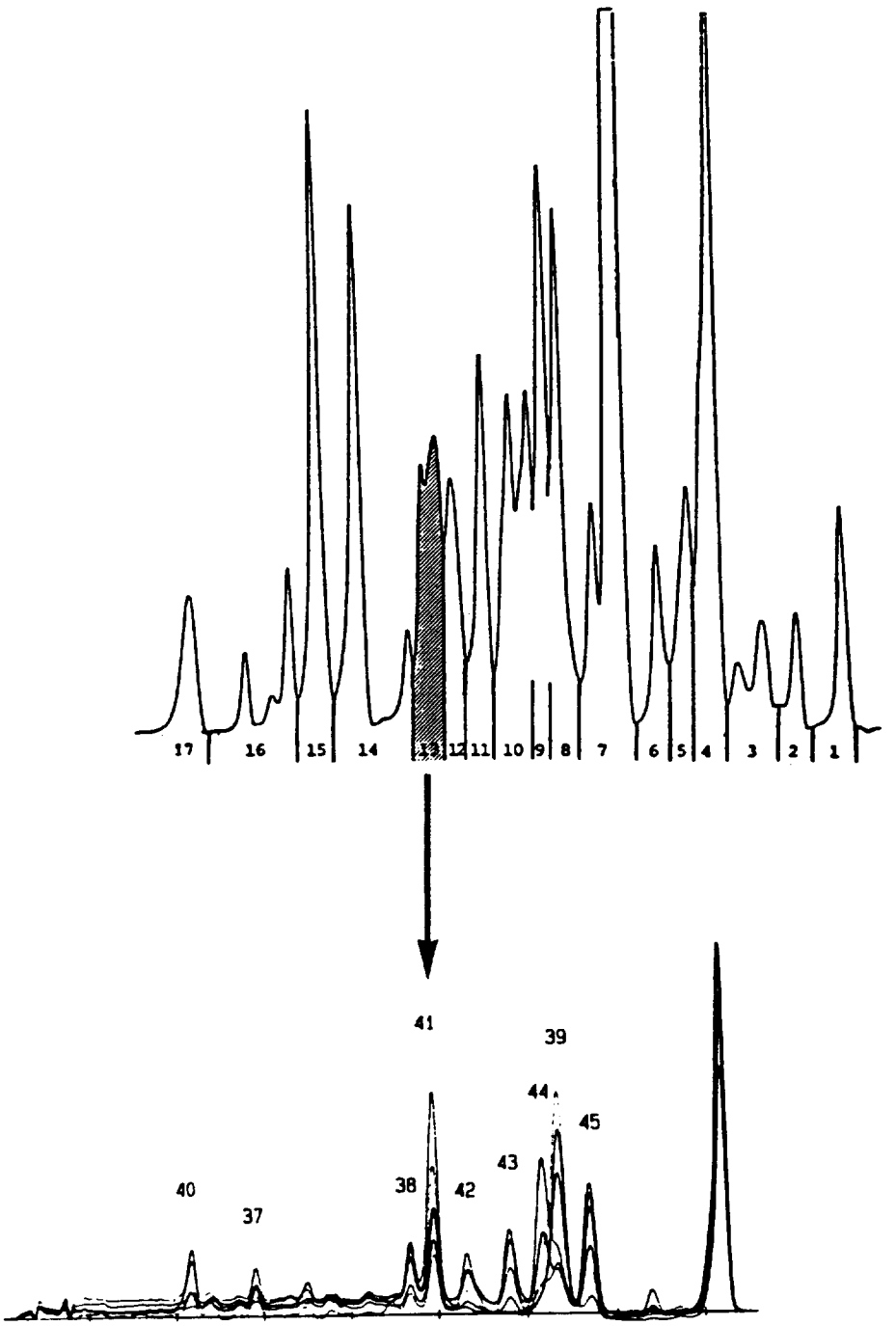

Fig. 27. Application of LC–TLC to the separation of a mixture of 56 pesticides. The upper chromatogram represents the 17 fractions obtained by narrow-bore reversed-phase liquid chromatography, the other the separation of the components coeluting in fraction 13 by TLC on silica gel with automated multiple development. From Ref [61]; © Marcel Dekker.

to handle mobile phases containing inorganic salts that are commonly used as buffers and competing ions in some column separation mechanisms, such as ion exchange, ion-pair chromatography, reversed-phase chromatography, etc. Such problems can be overcome by adding an on-line SPE step to concentrate column fractions and to exchange the solvent composition prior to spray jet application to the TLC layer. Muller and Jork [190] have described a suitable apparatus for this purpose (Fig. 28) and its application to the determination of 4(5)-methylimidazole in caramel, a common coloring agent for foodstuffs. 4(5)-Methylimidazole was isolated from high-molecular-mass ionic interfering compounds by separation on a hydrophobic size-exclusion column with an ionic mobile phase. The fraction containing 4(5)-methylimidazole was concentrated by SPE using an ion-exchange packing, desalted, and eluted to the spray-jet applicator in a small volume of organic solvent containing ammonia. The SPE device is fully automated so that cartridge exchange, conditioning, fractionation, and elution can be carried out in a sequential fashion coordinated with the operation of the HPLC and TLC sample applicator. An example of the determination of 4(5)-methylimidazole in a cola beverage using this system is shown in Fig. 29 [190].

LC–TLC has also received some attention as a solvent elimination interface for spectroscopic identification using diffuse reflectance Fourier transform IR [191,192], fluorescence [158,193], or surface enhanced resonance Raman spectroscopy [194]. In these studies the column was used for the separation and the eluent applied to the layer as a continuous trace or as multiple spots. Separation by TLC was not involved, with one exception [192]. Application of the column eluent to the TLC plate preserved the original column chromatogram, allowed removal of the solvent to avoid spectral interferences, allowed spectral techniques to be used that were im-

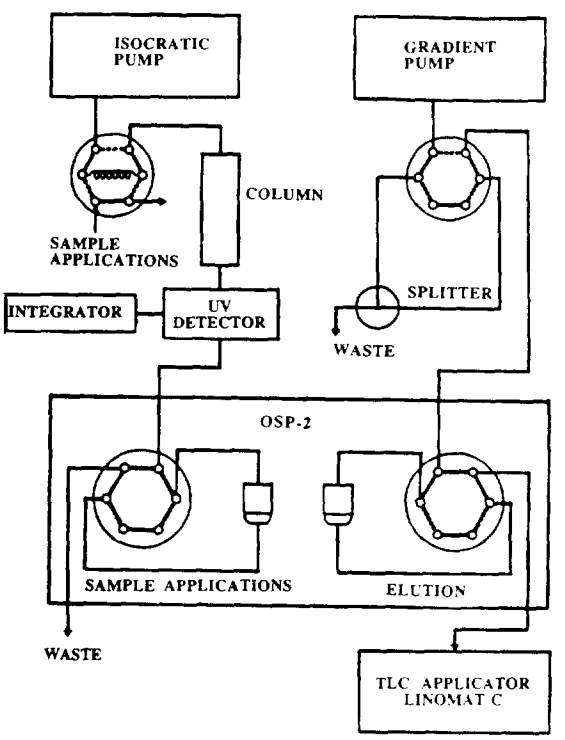

Fig. 28. Instrumental configuration for automated LC–SPE–TLC. From Ref. [190]; © Research Institute for Medicinal Plants.

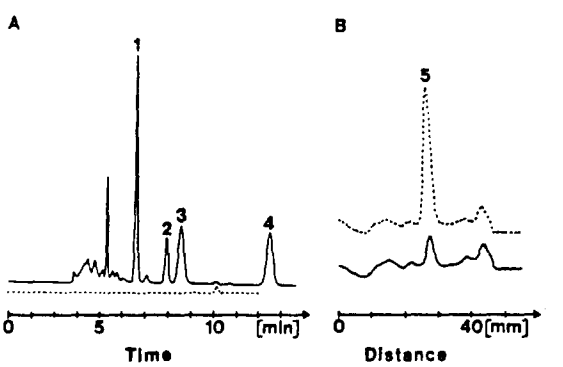

Fig. 29. On-line LC–SPE–TLC determination of 4(5)-methylimidazole in a cola beverage. (A) Separation by LC on a TSK gel G2500PW$_{XL}$ size-exclusion column; the peak in the dotted line indicates the position of 4(5)-methylimidazole in the chromatogram. (B) TLC separation of 4(5)-methylimidazole in the final step of the LC–SPE–TLC separation; the dotted line is the detector response for a standard of 4(5)-methylimidazole. Peaks: 1 = sodium benzoate; 2 = sodium saccharin; 3 = aspartame; 4 = cafeine; 5 = 4(5)-methylimidazole. From Ref. [190]; © Research Institute for Medicinal Plants.

practical in a continuous flowing system, and allowed advantage to be taken of surface enhancement of certain spectral features.

Although the total amount of research in coupled chromatographic systems with TLC as one separation dimension is disappointingly small at present, the impediment to success is not very great, and increased use can be expected in the future. It is likely that a lack of access to equipment for modern TLC in many chromatographic laboratories, coupled with a misunderstanding of the separation capacity of TLC and unfounded uncertainty in the reliability of quantitative TLC measurements, may conspire to slow the growth of coupled chromatographic systems incorporating TLC as one separation component. It is clear to us that the advantages outweigh the disadvantages and this research area is deserving of more attention than it receives at present.

References

[1] C.F. Poole and S.K. Poole, *Anal. Chem.*, 66 (1994) 27A.

[2] C.F. Poole and S.K. Poole, *Chromatography Today*, Elsevier, Amsterdam, 1991, pp. 649–735.

[3] B. Fried and J. Sherma, *Thin-Layer Chromatography —Techniques and Applications*, Marcel Dekker, New York, 1994.

[4] J. Sherma and B. Fried, *Handbook of Thin-Layer Chromatography*, Marcel Dekker, New York, 1991.

[5] A.M. Siouffi, E. Mincsovics and E. Tyihak, *J. Chromatogr.*, 492 (1989) 471.

[6] J.P. Abjean, *J. Planar Chromatogr.*, 6 (1993) 147.

[7] I.D. Wilson and G.W. Somsen, *J. Chromatogr. A*, 703 (1995) 613.

[8] C.F. Poole, *J. Planar Chromatogr.*, 2 (1989) 95.

[9] C.F. Poole and W.P.N. Fernando, *J. Planar Chromatogr.*, 5 (1992) 323.

[10] S.K. Poole and C.F. Poole, *Analyst*, 119 (1994) 113.

[11] W.P.N. Fernando and C.F. Poole, *J. Planar Chromatogr.*, 3 (1990) 389.

[12] W.P.N. Fernando and C.F. Poole, *J. Planar Chromatogr.*, 4 (1991) 278.

[13] W.P.N. Fernando and C.F. Poole, *J. Planar Chromatogr.*, 5 (1992) 50.

[14] C.F. Poole and W.P.N. Fernando, *J. Planar Chromatogr.*, 6 (1993) 357.

[15] S.K. Poole, H.D. Ahmed, M.T. Belay, W.P.N. Fernando and C.F. Poole, *J. Planar Chromatogr.*, 3 (1990) 133.

[16] C.F. Poole, S.K. Poole, W.P.N. Fernando, T.A. Dean, H.D. Ahmed and J.A. Berndt, *J. Planar Chromatogr.*, 2 (1989) 336.

[17] Z. Witkiewicz, M. Mazurek and J. Bladek, *J. Planar Chromatogr.*, 6 (1993) 407.

[18] Z. Witkiewicz and J. Bladek, *J. Chromatogr.*, 373 (1986) 111.

[19] E. Tyihak and E. Mincsovics, *J. Planar Chromatogr.*, 1 (1988) 6.

[20] M.-F. Gonnord and A.M. Siouffi, *J. Planar Chromatogr.*, 3 (1990) 206.

[21] L. Botz, E. Wehrli and O. Sticher, *J. Planar Chromatogr.*, 6 (1993) 13.

[22] Sz. Nyiredy, S.Y. Meszaros, K. Dallenbach-Tolke, K. Nyiredy-Mikita and O. Sticher, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 352.

[23] R.E. Kaiser and R.I. Reider, *J. Assoc. Off. Anal. Chem.*, 66 (1989) 79.

[24] R.E. Kaiser, *Einfuhrung in die HPPLC*, Hüthig, Heidelberg, 1987.

[25] G. Flodberg and J. Roeraade, *J. Planar Chromatogr.*, 6 (1993) 252.

[26] Sz. Nyiredy, L. Botz and O. Sticher, *J. Planar Chromatogr.*, 2 (1989) 53.

[27] P. Delvorde, C. Regnault and E. Postaire, *J. Liq. Chromatogr.*, 15 (1992) 1673.

[28] D.L. Mould and R.L.M. Synge, *Analyst*, 77 (1952) 964.

[29] V. Pretorius, B.J. Hopkins and J.D. Schieke, *J. Chromatogr.*, 99 (1974) 23.

[30] M. Prosek and M. Pukl, *Abstracts of the 7th International Symposium on Instrumental Planar Chromatography*, The Chromatographic Society, Nottingham, 1993, p.9.

[31] J.C. Giddings, in H.J. Cortes (Editor), *Multidimensional Chromatography —Techniques and Applications*, Marcel Dekker, New York, 1990, pp. 1–28.

[32] J.M. Davis, *Adv. Chromatogr.*, 34 (1994) 109.

[33] G. Guiochon and A.M. Siouffi, *J. Chromatogr.*, 245 (1982) 1.

[34] G. Guiochon, M.F. Gonnord, A.M. Siouffi and M. Zakaria, *J. Chromatogr.*, 250 (1982) 1.

[35] G. Guiochon, L.A. Beaver, M.F. Gonnord, A.M. Siouffi and M. Zakaria, *J. Chromatogr.*, 255 (1983) 415.

[36] E.J. Cavalli and C. Guinchard, *Chromatographia*, 37 (1993) 107.

[37] J.M. Davis, *Anal. Chem.*, 63 (1991) 2141.

[38] F.J. Oros and J.M. Davis, *J. Chromatogr.*, 591 (1992) 1.

[39] W. Shi and J.M. Davis, *Anal. Chem.*, 65 (1993) 482.

[40] C.F. Poole and S.K. Poole, in H.J. Cortes (Editor), *Multidimensional Chromatography —Techniques and Applications*, Marcel Dekker, New York, 1990, pp. 29–73.

[41] C.F. Poole and M.T. Belay, *J. Planar Chromatogr.*, 4 (1991) 345.

[42] C.F. Poole, S.K. Poole and M.T. Belay, *J. Planar Chromatogr.*, 6 (1993) 438.
[43] F. Geiss, *Fundamentals of Thin Layer Chromatography (Planar Chromatography)*, Hüthig, Heidelberg, 1987.
[44] S.K. Poole and C.F. Poole, *J. Planar Chromatogr.*, 5 (1992) 221.
[45] C.F. Poole, H.T. Butler, M.E. Coddens, S. Khatib and R. Vandervennet, *J. Chromatogr.*, 302 (1984) 149.
[46] J. Vajda, L. Leisztner, J. Pick and N. Anh-Tuan, *Chromatographia*, 21 (1986) 152.
[47] J. Muthig, *J. Chromatogr. B*, 657 (1994) 75.
[48] M.T. Belay and C.F. Poole, *J. Planar Chromatogr.*, 4 (1991) 424.
[49] K.Y. Lee, C.F. Poole and A. Zlatkis, in W. Bertsch, S. Hara, R.E. Kaiser and A. Zlatkis (Editors), *Instrumental HPTLC*, Hüthig, Heidelberg, 1980, pp. 245–273.
[50] S.A. Schuette and C.F. Poole, *J. Chromatogr.*, 239 (1982) 251.
[51] E. Soczewinski, in R.E. Kaiser (Editor), *Planar Chromatography*, Vol. 1, Hüthig, Heidelberg, 1986, pp. 79–117.
[52] G. Matysik and L. Jusiak, *J. Chromatogr.*, 518 (1990) 273.
[53] T.H. Dzido, G. Matysik and E. Soczewinski, *J. Planar Chromatogr.*, 4 (1991) 161.
[54] E. Soczewinski and G. Matysik, *J. Planar Chromatogr.*, 5 (1992) 388.
[55] T.H. Dzido, *J. Planar Chromatogr.*, 6 (1993) 78.
[56] W. Markowski, *J. Chromatogr.*, 485 (1989) 517.
[57] W. Markowski, E. Soczewinski and G. Matysik, *J. Liq. Chromatogr.*, 10 (1987) 1261.
[58] G. Matysik, J. Kowalski, H. Strzelecka and E. Soczewinski, *J. Planar Chromatogr.*, 7 (1994) 129.
[59] G. Matysik and E. Wojtasik, *J. Planar Chromatogr.*, 7 (1994) 34.
[60] G. Matysik, *J. Planar Chromatogr.*, 5 (1992) 146.
[61] D.E. Jaenchen and H.J. Issaq, *J. Liq. Chromatogr.*, 11 (1988) 1941.
[62] K. Burger, *Fresenius' Z. Anal. Chem.*, 318 (1984) 228.
[63] U. de la Vigne and D.E. Janchen, *Inform.*, 1 (1990) 477.
[64] C.F. Poole, S.K. Poole, T.A. Dean and N.M. Chirco, *J. Planar Chromatogr.*, 2 (1989) 180.
[65] M.J. Cikalo, S.K. Poole and C.F. Poole, *J. Planar Chromatogr.*, 5 (1992) 200.
[66] H.D. Ahmed and C.F. Poole, *J. Planar Chromatogr.*, 4 (1991) 218.
[67] J.A. Thoma, *Anal. Chem.*, 35 (1963) 214.
[68] T.H. Jupille and J.A. Perry, *J. Chromatogr.*, 99 (1974) 231.
[69] Sz. Nyiredy, Z. Fater, L. Botz and O. Sticher, *J. Planar Chromatogr.*, 5 (1992) 308.
[70] K. Dross, C. Sonntag and R. Mannhold, *J. Chromatogr.*, 639 (1993) 287.
[71] D. Nurok, *Chem. Rev.*, 89 (1989) 363.
[72] A.M. Sioufi, *J. Chromatogr.*, 556 (1991) 81.
[73] Sz. Nyiredy, K. Dallenbach-Tolke and O. Sticher, *J. Planar Chromatogr.*, 1 (1988) 336.
[74] P. Harmala, *J. Planar Chromatogr.*, 4 (1991) 460.
[75] P. Harmala, H. Vuorela, E.-L. Rahko and R. Hiltunen, *J. Chromatogr.*, 593 (1992) 329.
[76] P. Vuorela, E.-L. Rahko, R. Hiltunen and H. Vuorela, *J. Chromatogr. A*, 670 (1994) 191.
[77] W. Markowski and E. Soczewinski, *J. Chromatogr.*, 623 (1992) 139.
[78] W. Markowski, *J. Chromatogr.*, 635 (1993) 283.
[79] W. Markowski and G. Matysik, *J. Chromatogr.*, 646 (1993) 434.
[80] E. Menziani, B. Tosi, A. Bonora, P. Reschiglian and G. Lodi, *J. Chromatogr.*, 511 (1990) 396.
[81] G. Lodi, A. Betti, Y.D. Kahie and A.M. Mahamed, *J. Chromatogr.*, 545 (1991) 214.
[82] G. Lodi, A. Betti, E. Menziani, V. Brandolini and B. Tosi, *J. Planar Chromatogr.*, 4 (1991) 106.
[83] G. Lodi, A. Betti, V. Brandolini, E. Menziani and B. Tosi, *J. Planar Chromatogr.*, 7 (1994) 29.
[84] P.V. Colthup, J.A. Bell and D.L. Gadsdon, *J. Planar Chromatogr.*, 6 (1993) 386.
[85] K. Burger, J. Kohler and H. Jork, in G. Angeletti and B. Bjorseth (Editors), *Organic Micropollutants in the Aquatic Environment*, Kluwer, Dordrecht, 1990, pp. 115–129.
[86] U. de la Vigne and D. Janchen, *J. Planar Chromatogr.*, 4 (1990) 6.
[87] E. Zietz and I. Ricker, *J. Planar Chromatogr.*, 2 (1989) 262.
[88] U. de la Vigne, D. Janchen and W.H. Weber, *J. Chromatogr.*, 553 (1991) 489.
[89] H. Jork, G. Keller and U. Kocher, *J. Planar Chromatogr.*, 5 (1992) 246.
[90] S.K. Poole, M.T. Belay and C.F. Poole, *J. Planar Chromatogr.*, 5 (1992) 16.
[91] M.T. Belay and C.F. Poole, *J. Planar Chromatogr.*, 6 (1993) 43.
[92] I.D. Wilson and S. Lewis, *J. Chromatogr.*, 408 (1987) 445.
[93] M.F.M. Trypsteen, R.G.E. van Steversen and B.M.J. De Spiegeleer, *Analyst*, 114 (1989) 1021.
[94] O.R. Queckenberg and A.W. Fraham, *J. Planar Chromatogr.*, 6 (1993) 55.
[95] M.T. Belay and C.F. Poole, *Chromatographia*, 37 (1993) 365.
[96] S.K. Poole, S.L. Daly and C.F. Poole, *J. Planar Chromatogr.*, 6 (1993) 129.
[97] W. Kiridina, S.K. Poole and C.F. Poole, *J. Planar Chromatogr.*, 7 (1994) 273.
[98] C. Steuckart, E. Berger-Preiss and K. Levesen, *Anal. Chem.*, 66 (1994) 2570.
[99] M.C. Hsieh and H.K. Berry, *J. Planar Chromatogr.*, 5 (1992) 118.
[100] K.T. Mitchell, J.E. Ferrell and W.H. Huestis, *Anal. Biochem.*, 158 (1986) 447.
[101] E. Heftmann (Editor), *Chromatography, 5th Edition, Part B: Applications*, Elsevier, Amsterdam, 1992.

[102] A.P. Reddy, D. Pruess-Schwartz and L.J. Marnet, *Chem. Res. Toxicol.*, 5 (1992) 19.
[103] G.G. Spencer, A.C. Beach and R.C. Gupta, *J. Chromatogr.*, 612 (1993) 295.
[104] M. Zakaria, M.-F. Gonnord and G. Guiochon, *J. Chromatogr.*, 271 (1983) 127.
[105] H.J. Issaq, *Trends Anal. Chem.*, 9 (1990) 36.
[106] F. Smets, H.F. De Brabander, P.J. Bloom and G. Pottie, *J. Planar Chromatogr.*, 4 (1991) 207.
[107] H.D. Smolarz and M. Waksmund-Hajnos, *J. Planar Chromatogr.*, 6 (1993) 278.
[108] E. Talas, L. Botz, J. Margitfalvi, O. Sticher and A. Baiker, *J. Planar Chromatogr.*, 5 (1992) 28.
[109] H. Peck, A.W. Stott and J.B. Turner, *J. Chromatogr.*, 367 (1986) 289.
[110] W. Jost and H.E. Hauck, *Adv. Chromatogr.*, 27 (1987) 129.
[111] M.W. Stasko, K.M. Witherup, T.J. Ghiorzi, T.C. McCloud, S. Look, G.M. Muschik and H.J. Issaq, *J. Liq. Chromatogr.*, 12 (1989) 2138.
[112] G. Matysik and E. Soczewinski, *J. Chromatogr.*, 369 (1986) 19.
[113] L. Van Poucke, D. Rousseau, C. Van Peteghem and B.M.J. De Spiegeleer, *J. Planar Chromatogr.*, 2 (1989) 395.
[114] H.J. Issaq, K.E. Seburn, P. Andrews and D. Schaufelberger, *J. Liq. Chromatogr.*, 12 (1989) 3129.
[115] S. Habibi-Goudarzi, K.J. Ruterbories, J.E. Steinbrunner and D. Nurok, *J. Planar Chromatogr.*, 1 (1988) 161.
[116] J.G. Alvarez and J.C. Touchstone, *J. Chromatogr.*, 436 (1988) 515.
[117] I.D. Wilson, *J. Chromatogr.*, 287 (1984) 183.
[118] G. Grassini-Strazza, V. Carunchio and A.M. Girelli, *J. Chromatogr.*, 466 (1989) 1.
[119] J. Gasparic, *Adv. Chromatogr.*, 31 (1992) 153.
[120] N. Grinberg and S. Weinstein, *J. Chromatogr.*, 303 (1984) 251.
[121] M.H. Jee and A.S. Richie, *J. Chromatogr.*, 299 (1984) 460.
[122] I.D. Wilson, *J. Planar Chromatogr.*, 5 (1992) 316.
[123] M. Bakavolina, V. Navarathan and N.K. Nair, *J. Chromatogr.*, 299 (1984) 465.
[124] P. Harmala, L. Botz, O. Sticher and R. Hiltunen, *J. Planar Chromatogr.*, 3 (1990) 515.
[125] H. Kalasz, M. Bathori, L.S. Ettre and B. Polyak, *J. Planar Chromatogr.*, 6 (1993) 481.
[126] M. Mazurek and Z. Witkiewiez, *J. Planar Chromatogr.*, 4 (1991) 379.
[127] G. Guiochon, M.-F. Gonnord, M. Zakaria, L.A. Beaver and A.-M. Siouffi, *Chromatographia*, 17 (1983) 121.
[128] G. Guiochon, A. Krstulovic and H. Colin, *J. Chromatogr.*, 265 (1983) 159.
[129] M.-F. Gonnord and A.-M. Siouffi, *J. Planar Chromatogr.*, 3 (1990) 206.
[130] A. Velayudhan, B. Lillig and Cs. Horváth, *J. Chromatogr.*, 435 (1988) 397.
[131] J.E. Steinbrunner, E.K. Johnson, S. Habibi-Goudariz and D. Nurok, in R.E. Kaiser (Editor), *Planar Chromatography*, Vol. 1, Hüthig, Heidelberg, 1986, pp. 239–235.
[132] B.M.J. De Spiegeleer, W. Van den Bossche, P. De Moerloose and D. Massart, *Chromatographia*, 23 (1987) 407.
[133] M.-F. Gonnord, F. Levi and G. Guiochon, *J. Chromatogr.*, 264 (1983) 1.
[134] J.E. Steinbrunner, D.J. Malik and D. Nurok, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 560.
[135] D. Nurok, S. Habibi-Goudarzi and R. Kleyle, *Anal. Chem.*, 59 (1987) 2424.
[136] A.G. Howard and I.A. Bonicke, *Anal. Chim. Acta*, 223 (1989) 411.
[137] D. Heimler and V. Boddi, *J. Chromatogr.*, 466 (1989) 371.
[138] Y. Ma, L.B. Koutny and E.S. Yeung, *Anal. Chem.*, 61 (1989) 1931.
[139] H. Kawazumi and E.S. Yeung, *Appl. Spec.*, 43 (1989) 249.
[140] K.L. Busch, *J. Planar Chromatogr.*, 5 (1992) 72.
[141] Y. Nakagawa, and K. Iwatani, *J. Chromatogr.*, 562 (1991) 99.
[142] D.M. Wieland, M.C. Tobes and T.J. Mangner (Editors), *Analytical and Chromatographic Techniques in Radiopharmaceutical Chemistry*, Springer, New York, 1986.
[143] H. Filthuth, *J. Planar Chromatogr.*, 2 (1989) 198.
[144] C.F. Poole and S.K. Poole, *J. Chromatogr.*, 492 (1989) 539.
[145] D. Taube and V. Neuhoff, *J. Chromatogr.*, 437 (1988) 411.
[146] M. Prosek, M. Pukl, A. Golc-Wondra and D. Fercej-Temeljotov, *J. Planar Chromatogr.*, 2 (1989) 464.
[147] M. Prosek, I. Drusany and A. Golc-Wondra, *J. Chromatogr.*, 553 (1991) 477.
[148] K.K. Lam and D.E. Sequera, *J. Liq. Chromatogr.*, 13 (1990) 2901.
[149] D.E. Sequera, in J.E. Touchstone (Editor), *Planar Chromatography in the Life Sciences*, Wiley, New York, 1990, pp. 185–196.
[150] H. Yamamoto, K. Nakamura, D. Nakatani and H. Terada, *J. Chromatogr.*, 543 (1991) 201.
[151] D.H. Burns, J.B. Callis and G.D. Christian, *Trends Anal. Chem.*, 5 (1986) 50.
[152] P.B. Oldham, *Anal. Instrum.*, 19 (1990) 49.
[153] V.A. Pollak, A. Doelemeyer, W. Winkler and J. Schulze-Clewing, *J. Chromatogr.*, 596 (1992) 241.
[154] J.A. Cosgrove and R.B. Bilhorn, *J. Planar Chromatogr.*, 2 (1989) 362.
[155] F.G. Sanchez, A.N. Dias and M.R.F. Correa, *J. Chromatogr. A*, 655 (1993) 31.
[156] S.M. Brown and K.L. Busch, *J. Planar Chromatogr.*, 5 (1992) 338.
[157] P.K. Aldridge, J.B. Callis and D.H. Burns, *J. Liq. Chromatogr.*, 13 (1990) 2829.

[158] J.W. Hofstraat, M. Engelsma, R.J. van de Nesse, U.A.Th. Brinkman, C. Gooijer and N.H. Velthorst, *Anal. Chim. Acta*, 193 (1987) 193.
[159] R.S. Cooper, R. Jankowiak, J.M. Hayes, L. Pei-qi and G.J. Small, *Anal. Chem.*, 60 (1988) 2692.
[160] E. Koglin, *J. Planar Chromatogr.*, 3 (1990) 117.
[161] E. Koglin, *J. Planar Chromatogr.*, 6 (1993) 88.
[162] S.P. Bouffard, J.E. Katon, A.J. Sommer and N.D. Danielson, *Anal. Chem.*, 66 (1994) 1937.
[163] P.R. Brown and B.T. Beauchemin, *J. Liq. Chromatogr.*, 11 (1988) 1001.
[164] J. Janak, in A. Niederwieser and G. Patali (Editors), *Progress in Thin-Layer Chromatography and Related Methods*, Vol. 2, Ann Arbor Science, Ann Arbor, MI, 1971, pp. 63–91.
[165] R.E. Kaiser, *Fresenius' Z. Anal Chem.*, 205 (1964) 284.
[166] H.C. Curtius and M. Muller, *J. Chromatogr.*, 32 (1968) 222.
[167] J. Zhu and E.S. Yeung, *Anal. Chem.*, 61 (1989) 1906.
[168] A.J. Al-Sayegh and S.J. Lyle, *J. Anal. Appl. Pyrolysis*, 14 (1989) 323.
[169] M. Nishikawa, M. Tatsuno, S. Suzuki and H. Tsuchih, *Forensic Sci. Int.*, 49 (1991) 113.
[170] C.F. Poole and S.K. Poole, *Chromatography Today*, Elsevier, Amsterdam, 1991, pp. 601–648.
[171] M.L. Lee and K.E. Markides (Editors), *Analytical Supercritical Fluid Chromatography and Extraction*, Chromatography Conferences, Provo, UT, 1990.
[172] B. Wenclawiak (Editor), *Analysis with Supercritical Fluids —Extraction and Chromatography*, Springer, Heidelberg, 1992.
[173] J.L. Veuthey, M. Caude and R. Rosset, *Analusis*, 18 (1990) 103.
[174] R.W. Vannoort, J.-P. Chervet, H. Lingeman, G.J. de Jong and U.A.Th. Brinkman, *J. Chromatogr.*, 505 (1990) 45.
[175] E. Stahl, *J. Chromatogr.*, 142 (1977) 15.
[176] E. Stahl and W. Schild, *Fresenius' Z. Anal. Chem.*, 280 (1976) 99.
[177] U. Keller and I. Flament, *Chromatographia*, 28 (1989) 445.
[178] L. Wunsche, U. Keller and I. Flament, *J. Chromatogr.*, 552 (1991) 539.
[179] B. Diallo, R. Vanhaelen-Fastre and M. Vanhaelen, *J. Chromatogr.*, 558 (1991) 446.
[180] J. Bladek, *J. Planar Chromatogr.*, 6 (1993) 495.
[181] E. Mincsovics, M. Garami and E. Tyihak, *J. Planar Chromatogr.*, 4 (1991) 299.
[182] J.H. van Dijk, *Fresenius' Z. Anal. Chem.*, 247 (1969) 262.
[183] P.R. Boshoff, B.J. Hopkins and V. Pretorious, *J. Chromatogr.*, 126 (1976) 35.
[184] A. Karmen, G. Malikin, L. Freundlich and S. Lam, *J. Chromatogr.*, 349 (1985) 2677.
[185] A. Karmen, G. Malikin and S. Lam, *J. Chromatogr.*, 302 (1984) 31.
[186] J.W. Hofstraat, M. Engelsma, R.J. van de Nesse, C. Gooijer, N.H. Velthorst and U.A.Th. Brinkman, *Anal. Chim. Acta*, 186 (1986) 247.
[187] J.W. Hofstraat, S. Griffioen, R.J. van de Nesse, U.A.Th. Brinkman, C. Gooijer and N.H. Velthorst, *J. Planar Chromatogr.*, 1 (1988) 220.
[188] R.J. van de Nesse, G.J.M. Hoogland, J.J.M. de Moel, C. Gooijer, U.A.Th. Brinkman and N.H. Velthorst, *J. Chromatogr.*, 552 (1991) 613.
[189] K. Burger, *Analusis*, 18 (1990) i113.
[190] E. Muller and H. Jork, *J. Planar Chromatogr.*, 6 (1993) 21.
[191] C. Fujimoto, T. Morita, K. Jinno and K.H. Shafer, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 810.
[192] C. Fujimoto, T. Morita and K. Jinno, *J. Chromatogr.*, 438 (1988) 329.
[193] S.A. Soper and T. Kuwana, *Appl. Spec.*, 43 (1989) 883.
[194] S.A. Soper, K.L. Ratzlaff and T. Kuwana, *Anal. Chem.*, 62 (1990) 1438.