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OVERPRESSURED MULTI-LAYER CHROMATOGRAPHY

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SUMMARY

A new version of overpressured layer chromatography using two, three or more chromatoplates during one separation was developed. The admission of the eluent to the multi-layer system as a critical step is performed by making a perforation in the chromatoplates at the eluent inlet of a suitable size and shape. The technique, called overpressured multi-layer chromatography, is the most up-to-date version of layer liquid chromatography and is very attractive because a large number of samples (50–100 or more) can be separated during one development. It can be used effectively, *e.g.*, in plant breeding, clinical laboratories and industrial control laboratories and for the sequence analysis of proteins and nucleic acids.

INTRODUCTION

Conventional planar and non-planar and also thin- and thick-layer liquid chromatographic techniques require little equipment and are fairly simple. Among the planar layer liquid chromatographic techniques, paper chromatography (PC) and its variants were first developed in the 1940s by Martin and Synge[1]. Thin-layer chromatography (TLC) discovered by Izmailov and Shraiber[2], improved by Kirchner *et al.*[3] and standardized and extended by Stahl *et al.*[4,5], contributed to the isolation and analysis of many natural and synthetic substances. The combination of the flame ionization detector with TLC resulted in a special non-planar layer liquid chromatographic technique (TLC–FID)[6] with a thin layer of sorbent, *e.g.*, on a glass rod (open column).

Column and planar and non-planar layer liquid chromatographic techniques have developed together and it is not surprising that the rapid development of high-performance liquid chromatography (HPLC) entailed the need for a fundamental reappraisal of TLC as the most popular planar layer liquid chromatographic technique. It is also understandable that the latest efforts aimed at the further development of TLC have been characterized by the desire to introduce sophisticated instrumental techniques similar to those in HPLC. These efforts are in apparent contradiction with the advantages offered by the simplicity of the instruments and the

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flexibility of the operational steps in conventional layer liquid chromatographic techniques.

The first successful attempt was the development of overpressured layer chromatography (OPLC) using a pressurized ultramicro chamber[7–9]. In this chamber system, the sorbent layer is completely covered with a flexible membrane under external pressure. Thus OPLC, which is a collective term for different versions of the technique, corresponds to HPLC on a column having a very thin but wide cross-section[10].

Depending on the application, linear, circular (radial) and anticircular (triangular) development modes can be performed in OPLC and each has its own merits. In the linear development mode, one- and two-directional and two-dimensional developments are possible[11,12]. It should be mentioned that the linear versions of OPLC require precoated chromatoplates whose edges are impregnated with a suitable impregnating liquid.

OPLC is suitable for both on-line and off-line sample application, separation and detection[13–15]. The OPLC system permits both analytical and preparative investigations[16]. The resolution and spot capacity of different versions of OPLC are considerably increased in comparison with conventional layer techniques[17,18].

These advantages of OPLC ensured progress in the field of layer liquid chromatography similarly to the development of HPLC in the field of column liquid chromatography.

However, because OPLC is theoretically and practically a planar layer version of HPLC and, at the same time, has further special advantages as a planar layer system, there are additional development possibilities.

With the apparatus used so far in OPLC, only one chromatoplate could be developed, but the efficiency of this technique could be significantly increased if more than one chromatoplate could be developed simultaneously. In this paper, we outline technical and theoretical aspects of the applicability of overpressured multi-layer chromatography (OPMLC), which seems to be a promising new version of the original technique.

EXPERIMENTAL

OPMLC was carried out with Chrompres 10 and Chrompres 25 OPLC chromatographs from Labor-MIM (Budapest, Hungary). The separations were performed with perforated and/or non-perforated silica gel $60F_{254}$ TLC and HPTLC chromatoplates (Merck, Darmstadt, F.R.G.). Impregnation of the chromatoplates on two, three or four sides was performed with Impres polymer suspension from Labor-MIM. Densitograms were taken with Shimadzu (Kyoto, Japan) CS-920 and CS-930 scanners. Samples were applied with a Nanomat sample applicator (Camag, Muttenz, Switzerland) or with a Hamilton (Bonaduz, Switzerland) syringe.

A standard mixture of PTH-amino acids (Pierce, Rockford, IL, U.S.A.) was prepared by dissolution in the eluent to be used. Choline, betaine and trigonelline were purchased from Fluka (Buchs, Switzerland) and other chemicals from Merck (Darmstadt, F.R.G.) and Serva (Heidelberg, F.R.G.). The solvents used were LiChrosolv (Merck) and compounds of analytical-reagent grade.

RESULTS AND DISCUSSION

Technical aspects of the multi-layer system

The sorbent layer plays a central role in TLC and this is especially valid for OPLC. In order to achieve a linear chromatogram in OPLC, the edges of the sorbent layer on the chromatoplate must be suitably sealed by removing the sorbent from the edges of the sorbent layer and forming a polymer film by impregnation, *e.g.*, with a polymer suspension. Thus the eluent cannot escape as a result of the overpressure. However, this is not sufficient to provide an effective separation. The movement of the eluent with a linear solvent front can be ensured by placing a narrow plastic sheet on the layer or making a narrow channel in the layer ahead of the solvent inlet. In practice, a polythene or PTFE insert sheet with an eluent-leading channel is placed between the sorbent layer and the water cushion to protect the cushion and to direct the eluent as a linear front.

We have found that OPLC instruments are suitable for the development of several chromatoplates simultaneously if the plates are specially modified, *i.e.*, a hole of a suitable shape and size is made adjoining the eluent inlet. The eluent can travel almost unhindered among the sorbent layers, one on top of another, so the chromatograms can be developed simultaneously.

In practice, a hole has to be made in each plate except the lower one (as shown in Fig. 1a). This modification is made near the edge of the plate if linear one-directional development is to be carried out. The spreading of the eluent on the different plates, which is a condition of linear development, can be solved in different manners, *e.g.*, (i)

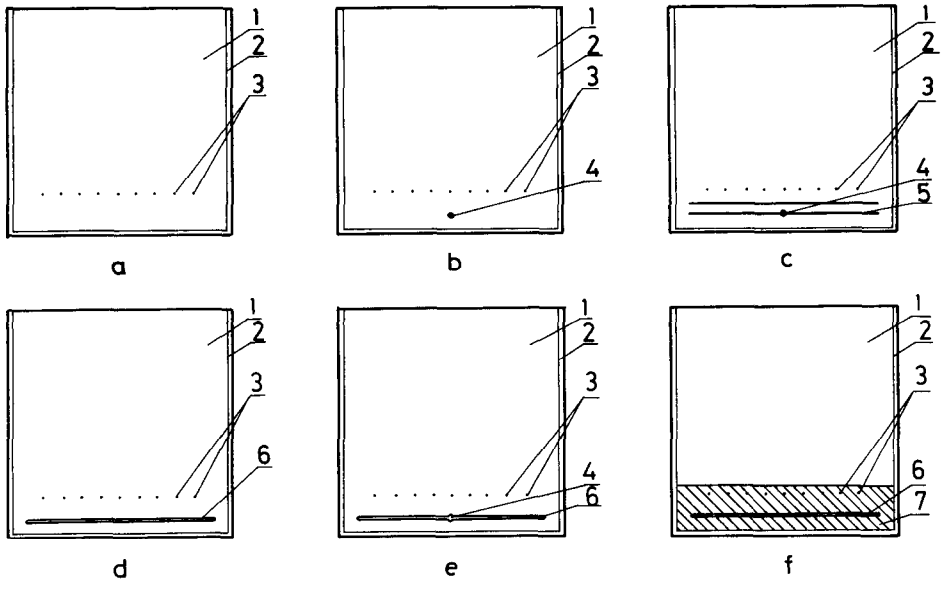

Fig. 1. Chromatoplates for one-directional linear OPMLC. (a) Basic chromatoplate; (b–e) with various perforations; (f) perforated chromatoplate with concentrating zone. 1, Sorbent layer; 2, impregnated edge; 3, sample application sites; 4, hole-like perforation; 5, directing channels in sorbent layer; 6, slit-like perforation; 7, inactive sorbent.

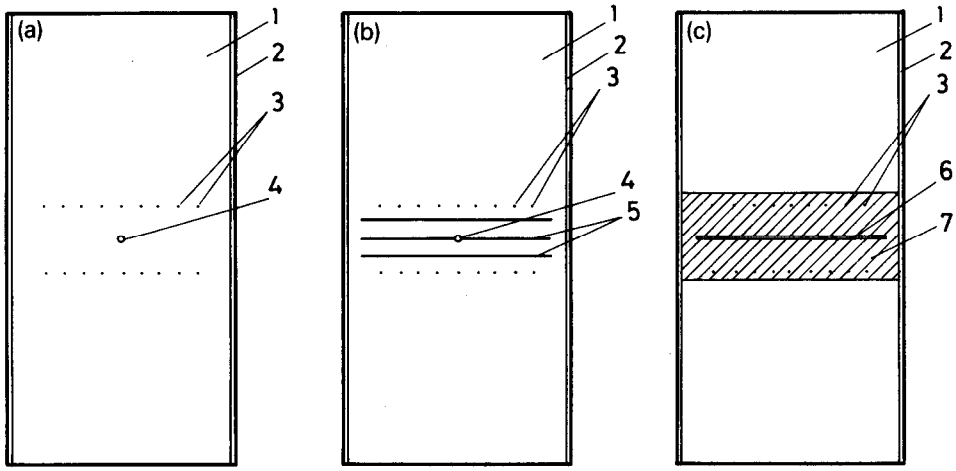

Fig. 2. Chromatoplates for two-directional linear OPMLC. 1, Sorbent layer; 2, impregnated edge; 3, sample application sites; 4, hole-like perforation; 5, directing channels in sorbent layer; 6, slit-like perforation; 7, inactive sorbent.

each sorbent layer can be covered with a perforated insert sheet supplied with leading channel; this is necessary in the situations shown in Fig. 1a and b; (ii) one or several channels leading the eluent can be cut into the sorbent layer (Fig. 1c); or (iii) the perforation is made at right-angles to the direction of the migration of the eluent and its shape is a longitudinal slit (Fig. 1d and e). One can also use chromatoplates having a concentrating zone (Fig. 1f) consisting of inactive sorbent.

If the hole was made in the middle of the plates after several have been placed on top of one another, these plates are suitable for circular multi-layer development. In this instance it is not necessary to impregnate the edges of the plate and to use channels to direct eluent.

If the development is two-directional, the hole is also made in the middle of the plate (as shown in Fig. 2a, b and c), but in this instance each sorbent layer can be covered with a perforated insert sheet supplied with a leading channel (Fig. 2a). Of course, the lower plate is not holed. Several channels leading the eluent can be also cut into the sorbent layer (Fig. 2b). Fig. 2c shows a combination of a longitudinal slit in the plate and a concentrating zone.

In practice one can place the holed plates with eluent-leading channels on the sorbent-free side on top of one another in the OPLC apparatus. The upper layer can be covered with a closure plate supplied with leading channels, while a conventional, hole-free plate as generally used in OPLC is fitted as the lower layer (as shown in Fig. 3). Therefore, although the cushion of the OPLC apparatus is applied to the topmost layer only, each plate serves as a cushion for the sorbent layer below it. Hence each sorbent layer is under pressure.

One can fasten a suitable number of holed covering plates with leading channels together, and several such units can be joined to each other (Fig. 4).

The eluent can be led out from the chromatoplates (sealed at all edges) similarly to the manner in which it is led in, through a hole on the opposite side to that for admission of the eluent. This gives the possibility of continuous development.

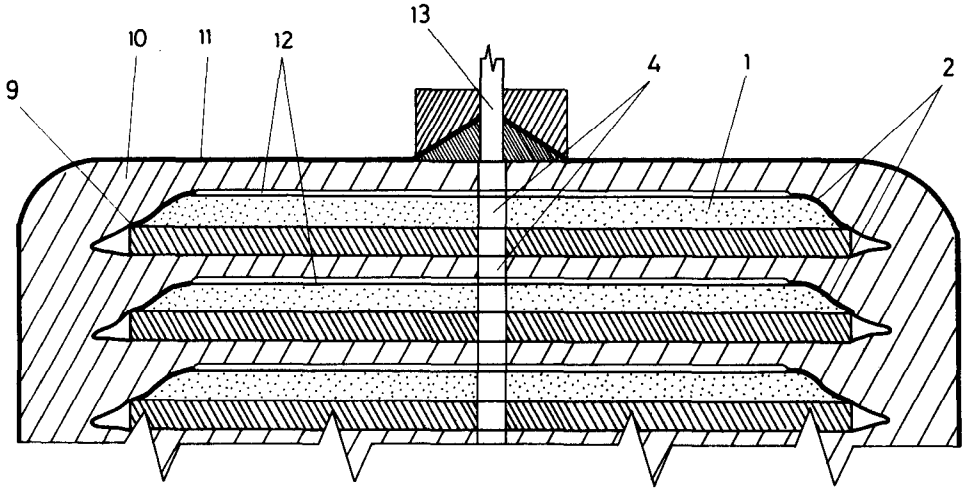

Fig. 3. Cover-plate system for OPMLC. 1, Sorbent layer; 2, impregnated edge; 4, hole-like perforation; 9, support plate; 10, cover-plate system for several plates; 11, cushion of Chrompres instrument; 12, eluent directing channel in cover-plate; 13, eluent inlet.

Theoretical aspects of OPMLC

In conventional layer chromatography, the relationship between the distance of visible eluent front (Z_f) and the development time (t) can be described by a quadratic equation[19]:

$$Z_f^2 = kt$$

where k is the velocity constant.

In OPLC, the eluent can be forced through the sorbent bed by means of a pump

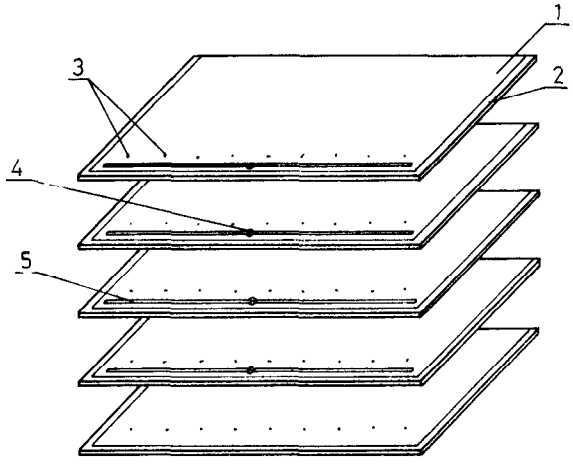

Fig. 4. Combination of plates. 1, Sorbent layer; 2, impregnated edge; 3, sample application sites; 4, hole-like perforation; 5, slit-like perforation (this latter perforation is also suitable for eluent direction).

system at a chosen flow-rate[7]. On feeding the eluent at constant velocity, the speed of the front depends on the cross-sectional area of the sorbent layer in the direction of development. It is obvious that in multi-layer system the cross-sectional area is correlated with the number of chromatoplates used or, more exactly, with the actual thickness of the sorbent layers.

In linear OPMLC, the basic flow equation is[17]

$$z_i = u_i t$$

where z_i is the migration distance of the ith component or the eluent front, u_i is the linear migration velocity of the ith component or the eluent and t is the time of development. In our experiences, there is almost no difference between the plates with regard to the retention data measured. Therefore, this basic flow equation is also suitable for describing the migration of eluent fronts in the multilayer system (or "layered sorbent column") in general. It should be pointed out that in linear OPMLC, similarly to linear OPLC, the velocity of the eluent front is constant along the plate, in contrast to the circular version of OPMLC, where the velocity of the front decreases along the radius with the developing time.

In classical fully off-line OPLC, in the zone which follows the α front (F_α) the

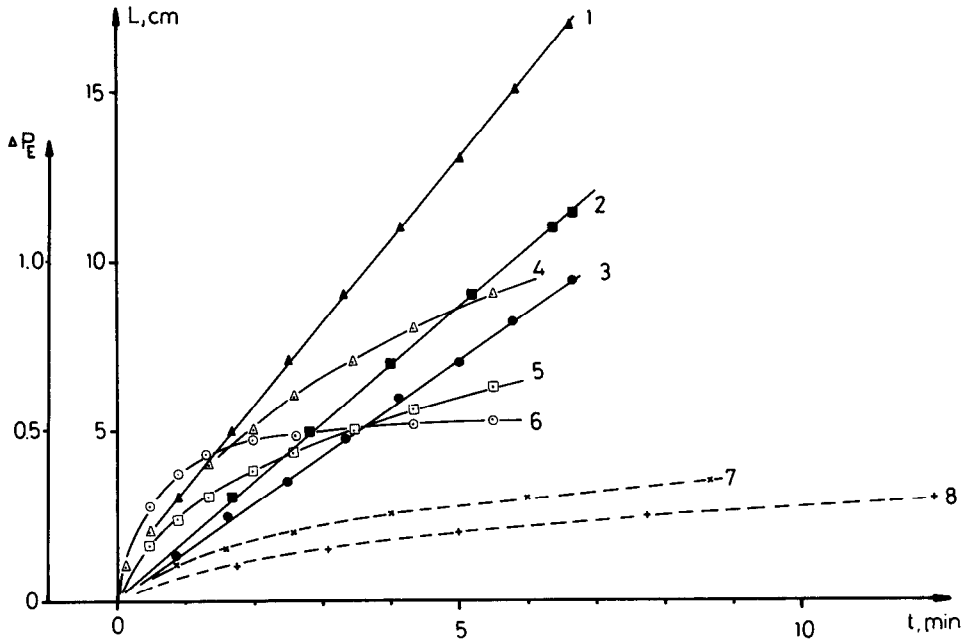

Fig. 5. Comparison of visual eluent front migration (F_α^v), front of total wettness (F_{tw}) and eluent inlet pressure value (ΔP_E) in linear one-directional OPLC, circular OPLC and normal unsaturated (N_{us}) TLC chamber. Chromatographic conditions: Chrompres 10 OPLC instrument; HPTLC silica gel 60, 200 × 200 × 0.17 mm; $P_{ext.}$, 1.5 MPa; eluent, carbon tetrachloride; flow-rate, 0.645 cm^3/min; temperature, 21°C. Linear one-directional OPLC: 1, F_α^v; 2, F_{tw}; 3, ΔP_E; circular OPLC: 4, F_α^v; 5, F_{tw}; 6, ΔP_E; normal TLC: 7, F_α^v, N_{us}; 8, F_{tw}, N_{us}.

spaces among the particles and within the pores are partially filled with both air and eluent and it was called the partially wetted zone (z_{pw})[13]. The next zone towards the eluent inlet is a totally wetted zone (z_{tw}), which is completely filled with eluent. The border between them is the front of total wetness (F_{tw}), which in most instances is not straight. On applying a constant flow-rate, F_α and F_{tw} are independent of the eluent (homologous alkyl alcohols), while the bed pressure drop or eluent inlet pressure increases with increasing homologue number, within experimental error[20]. The pressure drop depends on the viscosity of the eluent, the particle size of the sorbent layer and the external pressure on the layer surface. Within exprimental error, the "incompressible model" is in agreement with experiment and the velocities of the fronts are[20] $u_{F_\alpha} = (1 + \alpha)u_{F_{tw}}$ and $\alpha = \varepsilon_p/\varepsilon_i$, where ε_i is the interstitial and ε_p the interparticulate porosity per total volume of the bed.

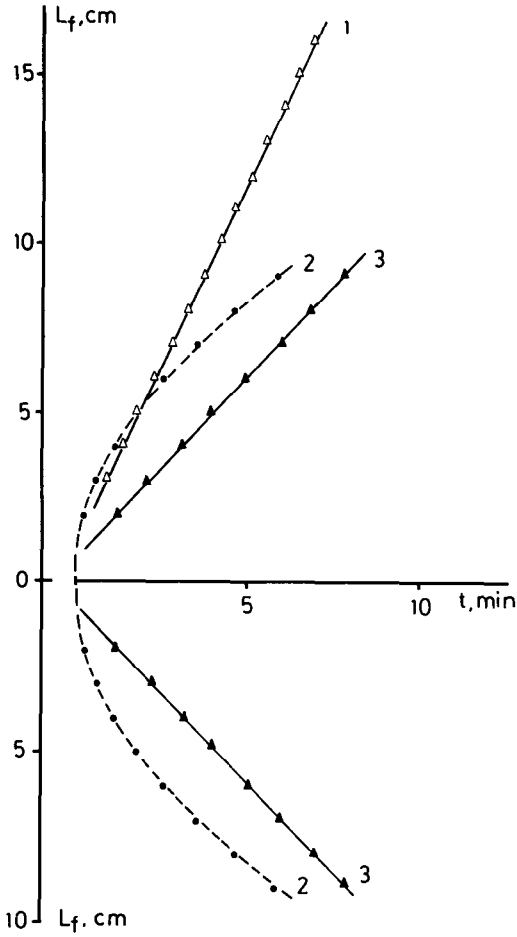

Fig. 6. Migration of visual eluent fronts measured on the top sorbent layer using different fully off-line overpressured three-layer chromatographic methods and a constant flow-rate. Chromatographic conditions: Chrompres 10 OPLC instrument; HPTLC silica gel 60; eluent, chloroform; temperature, 22.3°C; flow-rate on three sorbent layers, 1.575 cm^3/min (*i.e.*, 0.525 cm^3/min on a single layer); $P_{ext.}$, 1.3 MPa. 1, Linear one-directional OPMLC; 2, circular OPMLC; 3, linear two-directional OPMLC.

Fig. 5 compares the changes in the migration of the fronts and the eluent inlet pressure in TLC and in OPLC. It can be seen that the pressure drop varies linearly with time of development and there is a fundamental difference among the conditions of eluent flow in conventional layer chromatography and linear and circular OPLC at a constant flow-rate.

Fig. 6 shows the migration of the eluent fronts measured on the top sorbent layer using different fully off-line OPLC on three sorbent layers and at a constant flow-rate. It can be seen that in the linear versions of OPMLC there is really a constant eluent front velocity, but in circular OPMLC this relationship is not valid. It is assumed that the migrations of the eluent fronts are the same on the second and third plates, but their measurement is technically impossible.

It is known that in conventional TLC the components of the eluent which are sorbed strongly by the sorbent sites can cause secondary fronts (F_β, F_γ, etc.)[21]. These fronts are independent of F_{tw} and can occur during adsorption and also during reversed-phase developments when the eluent consists of solvents of different strengths. The effect of this solvent demixing is stronger in a fully off-line OPLC system, owing to the total elimination of the vapour space, than in chambers with a small vapour space, *e.g.*, in sandwich chambers[5,19]. These fronts divide the sorbent layer into zones of different eluting strength, within which the solvent polarity is almost the same, whereas at the fronts themselves there is a sudden increasing in eluent strength giving rise to "polarity steps". This phenomenon also occurs in circular and linear OPMLC.

The R_F of secondary fronts depends on the eluent composition. The eluent strength of a mixture can be calculated according to Snyder[22] and it was correlated with the R_F, in the case of fully off-line OPLC, using silica gel 60 and different apolar and polar mixtures[23].

The location of F_{tw} can be altered by changing the flow-rate and the R_F value of F_{tw} can be increased or decreased by increasing or decreasing the flow-rate[20]. Complete elimination of F_{tw} can be achieved by applying a pre-run prior to the separation in which the components to be analysed do not migrate and the air is removed from the sorbent layer. Eluents used in either TLC or HPLC can be applied in OPLC or OPMLC if the concentration of the polar constituent in the mixture is higher than 20%. On changing the apolar constituent to a more polar material both the $R_{F\beta}$ value and the eluent strength of Z_β will increase.

In HPLC there is a characteristic relationship between average theoretical plate height (H) and the eluent front velocity (u). It follows from the basic principles of OPLC that such a relationship can likewise be established[24,25]. In conventional layer chromatography the height equivalent to a theoretical plate (HETP) can be calculated according to Guiochon and Siouffi[26] and it is also applicable to off-line OPLC systems[8]. For a given substance (i) the HETP (H) is given by

$$H_i = \frac{\sigma_i^2}{(L_f - s_0)R_{Fi}}$$

where σ_i is one-fourth of the width of the spot, L_f is the front distance, s_0 is the distance between the position of spotting and the trough of the eluent inlet and R_F is the retention factor.

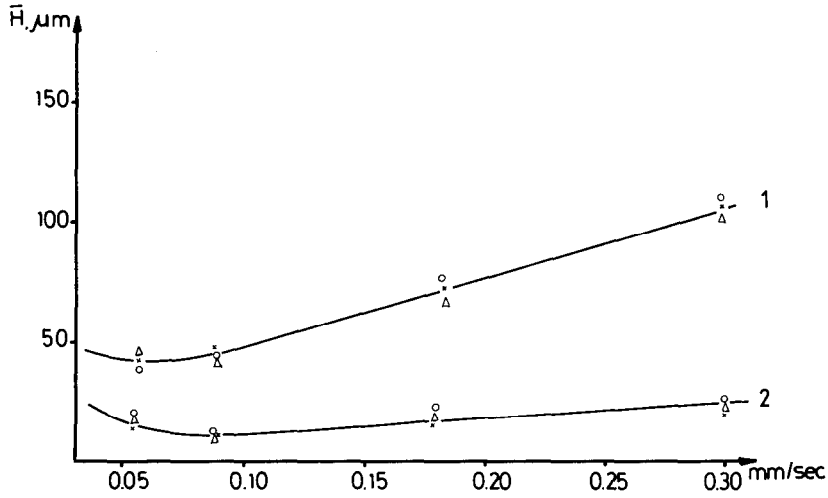

Fig. 7. Relationship between average theoretical plate height (H) and eluent front velocity (u) in OPMLC on three sorbent layers for quaternary ammonium compounds. Chromatographic conditions: Chrompres 25 instrument; $P_{ext.}$, 2.0 MPa; sorbent, HPTLC silica gel 60 F_{254} with impregnated edges; eluent, isopropyl alcohol–methanol–0.9 M sodium acetate (20:3:30, v/v/v); reagent, Dragendorff. 1, Choline; 2, betaine. △, Upper plate; ×, second plate; ○, lower plate.

Owing to the effect of focusing, an initial (starting) spot width may be defined that is different from the spot width deposited. The initial spot variance (σ_{0i}^{*}) of a given compound (i) is

$$\sigma_{0i}^{*} = \sigma_{0}^{s}(1 - R_{Fi}^{E})R_{Fi}^{s}$$

where σ_0^s is the spot variance of the solvent deposited and R_{Fi}^{s} and R_{Fi}^{E} are the retention factors in the solvent and eluent, respectively.

In off-line OPMLC, the HETP may vary with the linear front velocity similarly to off-line OPLC (Fig. 7). It can be seen that the relationship between the HETP values and eluent front velocity does not differ significantly on the three sorbent layers in the case of two quaternary ammonium compounds.

It follows from these results that in OPMLC the maximum value of the spot capacity (n_M), which is the maximum number of compounds that can be separated with the system, is given separately for each plate by

$$n_M = \frac{1}{2} \cdot \frac{L}{H}$$

where L is the distance of development and H is the average HETP value of a compound under given conditions[27,28].

Applications of OPMLC

The application of several chromatoplates during one separation in same chamber is known in TLC[5,19] and generates special advantages (*e.g.*, a large number of

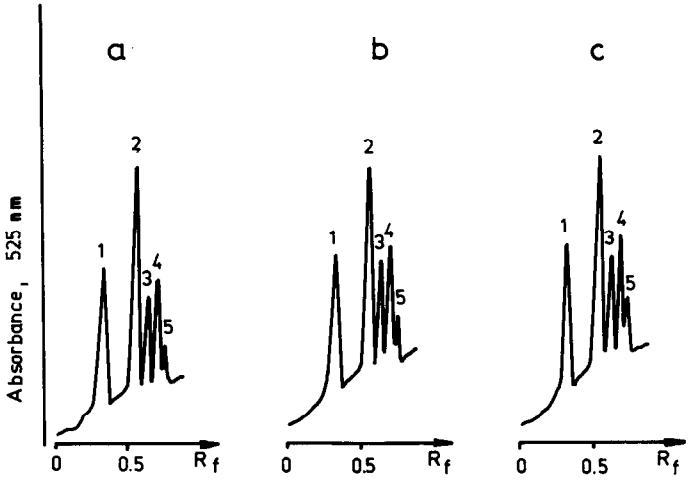

Fig. 8. Separation of quaternary ammonium compounds by circular OPMLC on three sorbent layers. Chromatographic conditions as in Fig. 7. Quantitative evaluation with Shimadzu CS-930 scanner. (a) Upper plate; (b) second plate; (c) lower plate. 1, N^{ε}-Trimethyl-L-lysine (TML); 2, choline; 3, carnitine; 4, trigonelline; 5, betaine.

samples can be used). However, the integration of a multilayer system in OPLC also exploits the advantages of a forced flow. OPMLC is qualitatively a new layer liquid chromatographic technique.

At the present stage of development of OPLC, 2–8 plates can be developed simultaneously, depending on the type of apparatus. The size of the plates and the direction of migration of the eluent (one-directional, two-directional, circular) can be chosen appropriately.

Inorganic (*e.g.*, silica gel, alumina, talc) or organic (*e.g.*, cellulose, polyamide) sorbents can be used on the plate. Siliceous marl and Celite can be employed as inactive sorbents.

Circular OPMLC is the simplest version of this attractive technique. In this mode we can use the plates without edge impregnation and eluent directing channels, but except for the lower plate we have to perforate the other plates in the middle where the eluent inlet point is also. Fig. 8 illustrates the separation of quaternary ammonium compounds by circular OPMLC on three silica gel layers, demonstrating only one densitogram from each chromatogram. It can be seen that the separation efficiency is very similar on each chromatogram.

Fig. 9 shows the separation of quaternary ammonium compounds by two-directional linear OPMLC, illustrating schematically only one densitogram from the two-directional three-layer chromatograms. Fig. 9 illustrates well the attraction of this operating mode.

Fig. 10 shows the determination of trigonelline with different NO_3-N levels on tomato plants using repeated application of the samples in overpressured two-layer chromatography.

The Chrompres 10 OPLC instrument is also suitable for development over longer distances. Using a 30 × 20 cm fine-particle silica gel 60 layer (a gift from

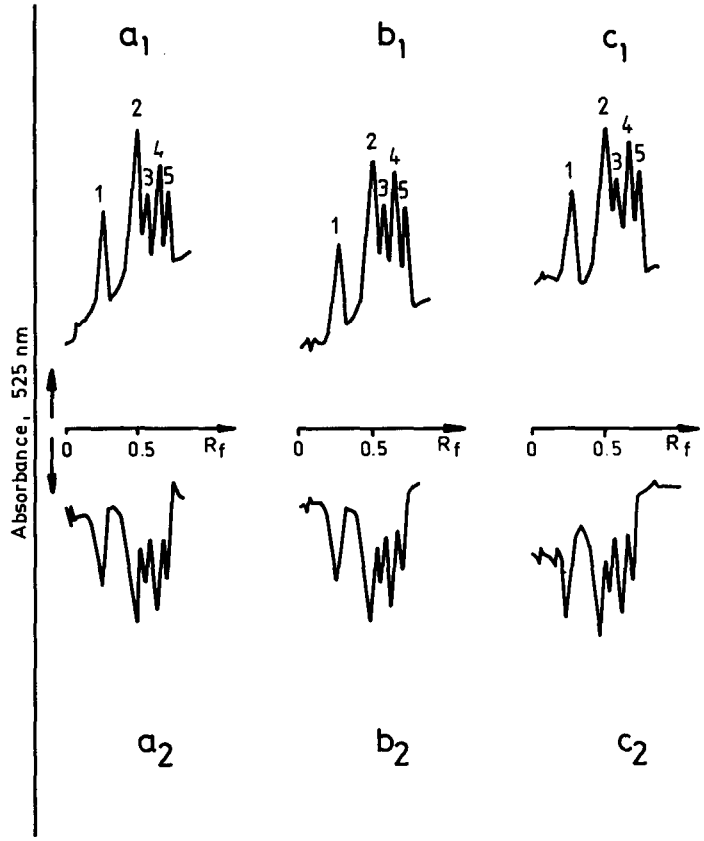

Fig. 9. Separation of quaternary ammonium compounds by two-directional linear OPMLC on three sorbent layers. Chromatographic conditions as in Fig. 7. (a_1 and a_2) Upper plate; (b_1 and b_2) second plate; (c_1 and c_2) lower plate. 1, TML; 2, choline; 3, carnitine; 4, trigonelline; 5, betaine.

Merck), the efficient separation of PTH-amino acids can be achieved by overpressured two-layer chromatography (Fig. 11).

Some special advantages of OPMLC are the following. Plates with different kinds of sorbent layers can be employed in a multi-plate system, so an eluent can be tested on several sorbent layers simultaneously and quickly. Conventional OPLC, as classical TLC or HPTLC, gives the possibility of using different specific colour reactions. In OPMLC there is a good possibility of using different colour reactions on the various plates, using the same sample on each plate.

To summarize, OPMLC is an attractive version of OPLC and 50–100 or more samples can be separated during one development. OPMLC can be used effectively, *e.g.*, in plant breeding, clinical laboratories and industrial control laboratories, and for the sequence analysis of proteins and nucleic acids.

CONCLUSIONS

Preliminary experiences with OPMLC show that the combination of a multi-

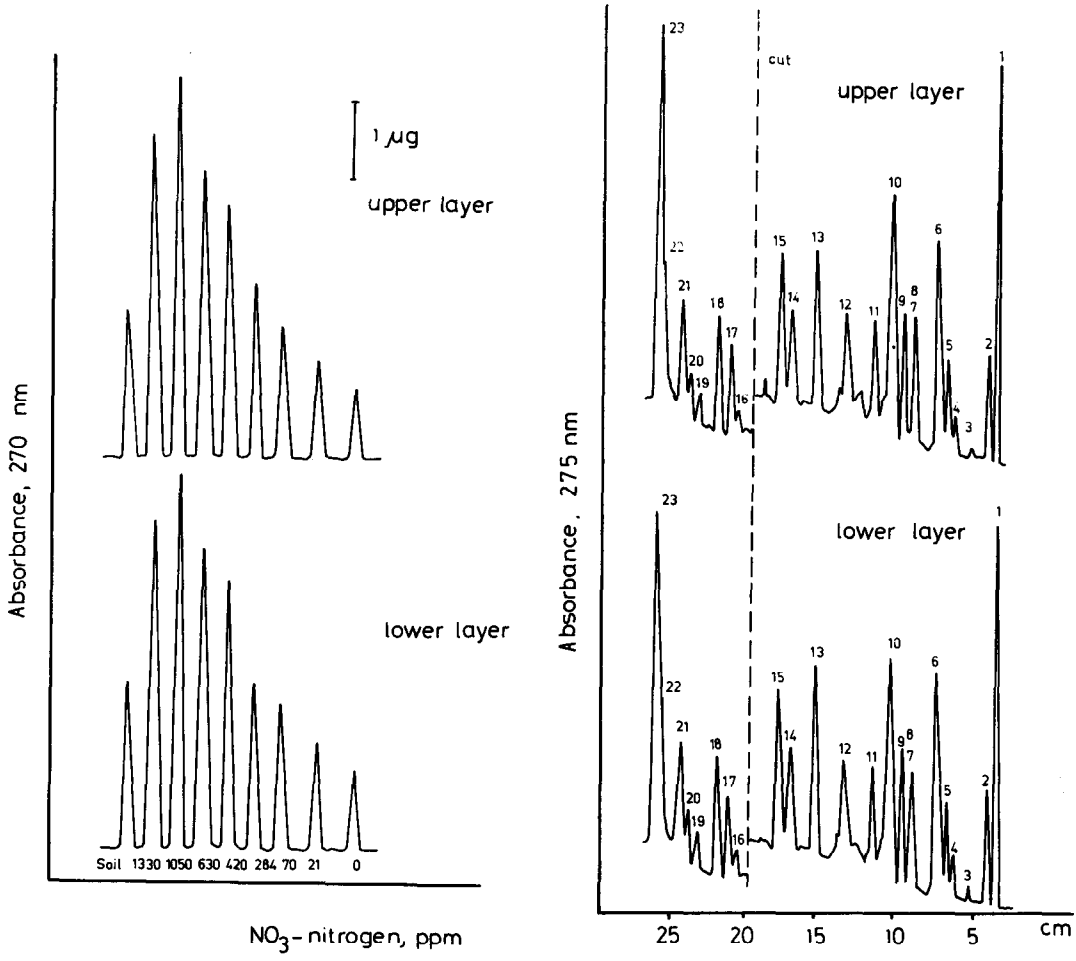

Fig. 10. Determination of trigonelline in tomato leaf extracts with different NO_3-N levels by OPMLC on two sorbent layers. Chromatographic conditions as in Fig. 7, except quantitative evaluation at 270 nm with Shimadzu CS-930 scanner.

Fig. 11. Separation of PTH-amino acids by OPMLC on two sorbent layers simultaneously. Chromatographic conditions: Chrompres 10; HPTLC silica gel 60 F_{254} (20 × 30 cm, experimental plate) with impregnated edges; 1st eluent, chloroform–methanol–acetic acid (90:10:3, v/v/v); 2nd eluent, dichloromethane–ethyl acetate (90:10, v/v); sample, 400 ng of each PTH-amino acid; external pressure on the membrane in Chrompres 10 chamber, 1.4 MPa; absorbance, 275 nm using Shimadzu CS-920 scanner; 1st development time, 25 min at 19 cm; 2nd development time, 43 min (continuous development). The plate is cut for quantitative evaluation. 1, $CySO_3K$; 2, His; 3, CH_3SO_2; 4, Asn; 5, Gln; 6, Asp; 7, Ser; 8, Glu; 9, Thr; 10, Lys; 11, Tyr; 12, Gly; 13, Trp; 14, Ala; 15, Met; 16, Phe; 17, Val; 18, Nle; 19, Ile; 20, Leu; 21, Pro; 22–23, eluent fronts.

layer system with a forced eluent flow complicates really to a certain extent the original simple and flexible TLC technique and also partly conventional ΘPLC. However, the result is an efficient and promising technique in the field of layer liquid chromatography which is applicable to analytical and preparative separations in various types of laboratories.

The development of OPMLC exploits unique possibilities of the layer liquid system which are absent from column liquid systems. The exploitation of the multi-layer system is carried out with the use of the modern column liquid chromatographic instrumentation, *e.g.*, a pump system for the admission of the eluent into the multi-layer system. The development of OPMLC makes a fundamental reassessment of densitometry desirable, with special emphasis on acceleration of measurement, sensitivity and resolution.

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