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# OPTIMIZATION OF OPERATING PARAMETERS IN OVERPRESSURED THIN-LAYER CHROMATOGRAPHY*

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## SUMMARY

By adjusting the solvent by means of a pump system in overpressured thin-layer chromatography using a pressurized ultramicro (PUM) chamber it is possible to separate substances with optional development distances. In the PUM chamber the external pressure on the flexible cover membrane must always be higher than the input pressure of the solvent. The input pressure of the solvent increases linearly with increasing solvent migration distance. An increase in the solvent flow velocity always results in higher input pressures, which must be taken into account by choosing an appropriate external pressure on the membrane.

The number of theoretical plates and the separation numbers obtained with a PUM chamber of the linear type on fine-particle sorbent layers are also better with longer solvent migration distances than in a normal TLC chamber. The advantages and the necessity for development with a longer migration distance are demonstrated with the example of the separation of amino acids on a fine-particle silica gel chromatoplate.

## INTRODUCTION

The development of overpressured thin-layer chromatography (OPTLC)[1–3] is an important stage in planar liquid chromatography because this technique combines the advantages of classical thin-layer chromatography (TLC), modern high-performance TLC (HPTLC) using fine-particle sorbent layers and high-performance liquid chromatography (HPLC).

The combination of the various advantages of these three techniques was achieved by use of the so-called pressurized ultramicro (PUM) chamber[2]. The essential

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feature of PUM chambers of circular and linear types is that the sorbent layer is completely covered with a flexible cover membrane under an external pressure so that in the closed chamber a layer of water forms between the Plexiglass cover-plate and the flexible and fixed membrane, and the vapour space above the layer is virtually eliminated. In this chamber system the flexible membrane under external pressure behaves as a stiff wall. In the PUM chamber it is possible to adjust the flow velocity by means of a pump system.

The linear migration of the solvent front in a PUM chamber of the linear type was studied by impregnating the sides of the layer and placing a narrow plastic sheet on the layer or making a narrow channel in the layer before the position of the solvent inlet.

The quadratic law of TLC development[4] is not valid in linear OPTLC using different plates and solvents. The migration of the solvent front in linear OPTLC is described by a simple equation[5]:

$$k = \frac{Z_f}{t}$$

where the velocity constant ($k$) is a function of the flow velocity of mobile phase, the quality of the sorbent and the dimensions of the bed particles. This relationship shows that linear OPTLC technique really approaches column chromatographic conditions.

In circular OPTLC, however, the area to be wetted increases quadratically with the linear movement of the solvent front, and it is therefore obvious that in circular OPTLC the classical quadratic law of TLC development[4] is valid. Therefore, the development of linear OPTLC and the optimization of operating parameters in linear OPTLC are expedient.

## EXPERIMENTAL

### *Apparatus*

A linear PUM chamber was obtained from Labor MIM (Esztergom-Budapest, Hungary).

*In situ* quantitative evaluation of the spots on the developed chromatograms was accomplished with a Zeiss PMQ III chromatogram spectrophotometer. Solvent was admitted into the PUM chamber with an S13 Micropump (Labor MIM).

### *Chromatographic plates*

TLC and HPTLC silica gel 60 $F_{254}$ (Merck, Darmstadt, G.F.R.) both without impregnation of the edges and with impregnated edges (in paraffin candle at 105°C, so the sorbent layer is covered with another glass or plastic plate) and pre-coated silica gel plastic sheets (Reanal, Budapest, Hungary) for classical TLC and with impregnated edges (in an inert plastic dispersion) for OPTLC (Silpres N with silica gel of different particle sizes) were used.

### *Chemicals*

Camag (Muttenz, Switzerland) Test Dye Mixture I was used as a model for separation. Authentic amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). All chemicals were of guaranteed reagent grade and were used without further purification.

Ninhydrin spray reagent was prepared by dissolving 0.5 g of ninhydrin and 0.05 g of copper sulphate in 80 ml of methanol plus 20 ml of acetic acid.

## RESULTS AND DISCUSSION

Fig. 1 demonstrates that in linear OPTLC the external pressure on the membrane (by forming a water layer between the Plexiglass cover-plate and the flexible membrane) is stable during the separation. This external pressure on the membrane must always be higher than the input pressure of the solvent (the overpressure), so that the migration of the solvent is undisturbed and stable for longer migration distances (line 5). However, if the solvent flow velocity is increased the input pressure of the solvent is always higher. This must be taken into account by choosing an appropriate external pressure on the membrane. It can be seen that the input pressure of the solvent increases linearly with the migration distance (line 4).

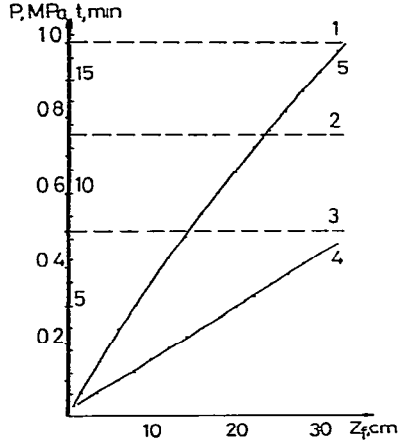


Fig. 1. Variation of input pressure of solvent and of external pressure on membrane and variation of time with the distance travelled by the solvent. Sorbent, Silpres N-1 ($d_i$ = 10–11 $\mu$m); solvent, methylene chloride; flow-rate of solvent, 20 cm$^3$/h. 1–3, External pressure on membrane; 4, input pressure of solvent during the separation; 5, distance travelled by the solvent ($Z_f$) *vs.* time ($t$).

Table I shows that in linear OPTLC the number of theoretical plates increases regularly with migration distance ($Z_f$) but mainly on a fine-particle sorbent layer.

Fig. 2 illustrates that in the linear PUM chamber the separation number increases near linearly with solvent migration distance on normal TLC plates compared with the characteristic curve for a normal saturated TLC chamber.

The advantages and the necessity for development for a longer migration distance will be illustrated with the example of the separation of amino acids on a fine-particle silica gel chromatoplate.

It is generally considered that in the linear TLC and HPTLC development mode the maximal number of components that can be resolved in one development (15 and 4–6 cm, respectively) is approximately ten, and this is valid for amino acids too. However, in current practice it is necessary to separate 20–30 protein amino acids (classical amino acids and methylated basic amino acids and other derivatives)[8].

TABLE I

THE EFFECT OF CHAMBER SYSTEM AND SORBENT QUALITY ON THE AVERAGE PLATE HEIGHT ($\bar{H}$)[6] AND THE NUMBER OF THEORETICAL PLATES ($N$)

Solvent, methylene chloride; temperature, 26°C; flow-rate, 20 cm³/h; external pressure on membrane, 1.0 MPa; substance, butter yellow (1.5 μg/μl in *n*-heptane).

| $Z_f$ (mm) | 1* | | 2* | | 3* | | 4* | | 5* | | 6* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\bar{H}$ (μm) | $N$ | $\bar{H}$ (μm) | $N$ | $\bar{H}$ (μm) | $N$ | $\bar{H}$ (μm) | $N$ | $\bar{H}$ (μm) | $N$ | $\bar{H}$ (μm) | $N$ |
| 20 | 42.3 | 473 | 18.8 | 1063 | 8.7 | 2299 | 31.6 | 633 | 16.4 | 1235 | 8.3 | 2410 |
| 40 | 34.1 | 1173 | 19.0 | 2105 | 10.4 | 3846 | 32.1 | 1246 | 16.5 | 2424 | 8.2 | 4878 |
| 60 | 33.2 | 1807 | 22.5 | 2667 | 15.2 | 3947 | 32.6 | 1840 | 16.7 | 3593 | 8.5 | 7059 |
| 80 | 34.8 | 2299 | 28.2 | 2837 | 32.8 | 2439 | 33.1 | 2417 | 17.1 | 4678 | 8.7 | 9195 |
| 100 | 37.4 | 2673 | 35.8 | 2793 | 45.1 | 2217 | 33.7 | 2967 | 17.0 | 5582 | 9.0 | 11111 |
| 120 | 40.6 | 2956 | 45.6 | 2631 | 62.7 | 1914 | 34.1 | 3519 | 17.5 | 6857 | 9.1 | 13186 |
| 140 | 42.8 | 3271 | 55.7 | 2513 | 98.2 | 1426 | 34.2 | 4094 | 17.7 | 7865 | 9.2 | 15217 |
| 160 | 48.2 | 3319 | 65.4 | 2446 | 120.4 | 1329 | 34.8 | 4598 | 18.0 | 8889 | 9.4 | 17021 |
| 200 | – | – | – | – | – | – | 36.3 | 5510 | 18.2 | 10989 | 9.3 | 21505 |
| 240 | – | – | – | – | – | – | 36.9 | 5962 | 18.5 | 11892 | 9.5 | 23158 |
| 280 | – | – | – | – | – | – | 37.3 | 7507 | 19.2 | 14583 | 9.7 | 28866 |
| 320 | – | – | – | – | – | – | 38.8 | 8247 | 19.6 | 16327 | 10.2 | 31373 |

* 1, Silpres N-1 (10–11 μm), $N_s$ (normal saturated) chamber; 2, Silpres N-2 (5–6 μm), $N_s$; 3, Silpres N-3 (2–3 μm), $N_s$; 4, Silpres N-1, PUM chamber of linear type; 5, Silpres N-2, PUM; 6, Silpres N-3, PUM.

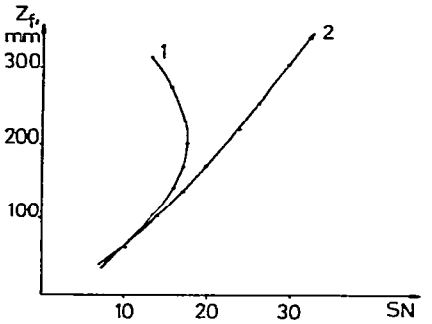


Fig. 2. Variation of separation number ($SN$) with the solvent migration distance ($Z_f$) on silica gel 60 precoated TLC plate (Merck). Solvent, methylene chloride; flow-rate of solvent, 20 $cm^3/h$. 1, Normal saturated TLC chamber; 2, linear PUM chamber. Calculation of separation numbers according to Kaiser[7].

In conventional TLC *n*-butanol–acetic acid–water (4:1:1) is one of the best eluents for the separation of amino acids, but the long analysis time and poor resolution and sensitivity make this procedure inadequate for efficient chromatography. Re-chromatography with this viscous solvent system gives better results[9]. Of course, the use of a fine-particle sorbent layer results in an even separation time with considerable spot or band diffusion which limits the resolution of amino acids.

Comparisons of conventional and overpressured TLC techniques for the separation of amino acids were carried out with normal and linear pressurized chambers using HPTLC plates and *n*-butanol–acetic acid–water (4:1:1) as the eluent.

Fig. 3 shows that the one-dimensional separation of 21 protein amino acids is inadequate with the longer distance. The movement of the viscous eluent was very


Fig. 3. One-dimensional separation of a mixture of 21 amino acids on a silica gel 60 $F_{254}$ HPTLC plate in a normal unsaturated ($N_{us}$) chamber. Solvent, *n*-butanol–acetic acid–water (4:1:1); development distance, 9.3 cm; running time, 250 min; reagent, ninhydrin; $s_0$ (start distance) increase diagonally; marker substances at layer's edges.


Fig. 4. One-dimensional separation of a mixture of 21 amino acids on a silica gel 60 $F_{254}$ HPTLC with impregnated edges in a PUM chamber of the linear type. Solvent, *n*-butanol–acetic acid–water (4:1:1); running distance, 16 cm; running time, 47 min; external pressure on the membrane, 1.2 MPa; flow-rate of solvent, 10 $cm^3/h$; other conditions as for Fig. 3; $s_0$ (start distance) increases diagonally.

slow (250 min for 9.3 cm) and the bands became diffused. In the PUM chamber development for 16 cm gave better results (Fig. 4) but the efficiency is unsatisfactory.

In continuous developments the efficiency of the separation increased considerably (Fig. 5) but in this instance the input pressure of solvent reached the ex-


Fig. 5. One-dimensional separation of a mixture of 21 amino acids on a silica gel 60 $F_{254}$ HPTLC plate. Solvent, *n*-butanol–acetic acid–water (4:1:1); continuous development; running time, 70 min; flow-rate of solvent, 10 $cm^3/h$; external pressure on membrane, 1.2 MPa; marker substances at layer's edges; $s_0$ (start distance) increases diagonally.

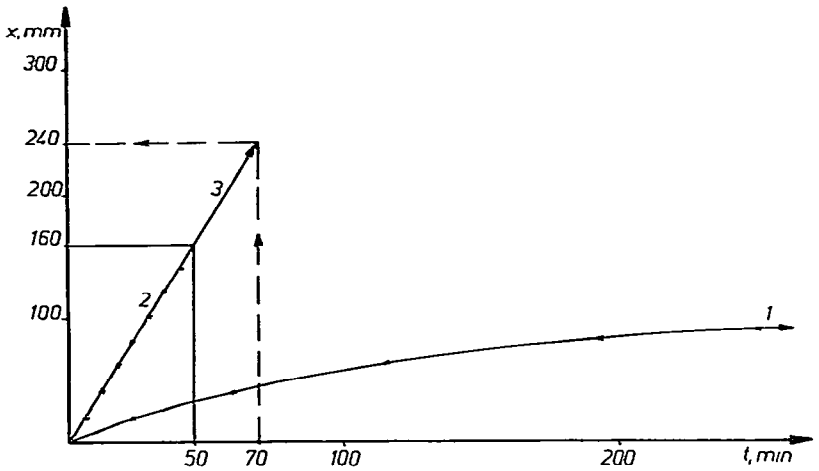


Fig. 6. Distance travelled by *n*-butanol–acetic acid–water (4:1:1) solvent on a silica gel 60 $F_{254}$ HPTLC plate in normal unsaturated and PUM chambers *versus* time. Conditions and data as for Fig. 3–5. 1, Normal development in $N_{us}$ chamber; 2, normal development in PUM chamber; 3, continuous development in PUM chamber.

ternal pressure on the membrane, so the separation could not be continued further (the external pressure maximum in the PUM chamber used is 1.2 MPa).

These preliminary results show that for the efficient separation of amino acids linear OPTLC will be the most effective (with continuous development, quantitative evaluation, etc.) among the liquid chromatographic techniques. The results will be reported in detail in a later paper series.

Fig. 6 shows the characteristics of solvent movement (distance *versus* time) in the various development modes; in the PUM chamber the relationship is linear.

For similar reasons to those for protein amino acids, separations on a fine-particle sorbent layer over a long distance may be useful for other types of substances, e.g., essential oils, sugars, lipids and nucleotides.

## REFERENCES

1 E. Tyihák, H. Kalász, E. Mincsovics and J. Nagy, *Proc. 17th Hung. Annu. Meet. Biochem., Kecskemét, 1977*; *C.A.*, 88 (1978) 15386.
2 E. Tyihák, E. Mincsovics and H. Kalász, *J. Chromatogr.*, 174 (1979) 75.
3 E. Mincsovics, E. Tyihák and H. Kalász, *J. Chromatogr.*, 191 (1980) 293.
4 F. Geiss, *Die Parameter der Dünnschicht-Chromatographie*, Vieweg, Braunschweig, 1972.
5 E. Tyihák, E. Mincsovics, P. Tétényi, I. Zámbó and H. Kalász, *Acta Horticult.*, 96 (1980) 113.
6 G. Guiochon and A. Siouffi, *J. Chromatogr. Sci.*, 16 (1978) 598.
7 R. E. Kaiser (Editor), *Einführung in die Hochleistungs-Dünnschicht-Chromatographie*, Institut für Chromatographie, Bad Dürkheim, 1976.
8 W. K. Paik and S. Kim, *Protein Methylation*, Wiley, New York, 1980.
9 J. G. Kirchner, *Thin-Layer Chromatography*, Wiley, New York, 2nd ed., 1978.