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Use of overpressurized thin-layer chromatography (OPTLC) for the separation of amino-acids and polypeptides*

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Abstract: Overpressurized thin-layer chromatography (OPTLC) has been used for the separation of amino-acids and polypeptides. The effects of the ratio of pyridine to acetic acid as well as that of butanol to water on the selectivity and efficiency of separation have been investigated in detail. The influence of the nature of organic solvents in the eluent has also been studied. The optimal conditions have been elaborated and the practical applicability in pharmaceutical analysis of the separation system has been demonstrated.

Keywords: *Overpressurized thin-layer chromatography; OPTLC; optimization; amino-acids; polypeptides; insulin.*

Introduction

Several well established chromatographic methods have been published recently for the separation and determination of amino-acids and polypeptides. High-performance liquid chromatographic methods have been mainly used for this purpose. The interesting features of thin-layer chromatography (TLC) include simplicity, high efficiency and selectivity, independence of the detector in time and space, improvement of sensitivity of detection by chemical reaction on the plate, and the ability to detect every component. Thus many very difficult analytical problems can be solved even in small laboratories equipped with only basic TLC equipment. The theory and practice of the separation and determination by TLC of amino-acids and polypeptides have been discussed [1, 2].

The main disadvantage of TLC for the separation of amino-acids is associated with the use of high-viscosity solvent systems for development of the plates; this results in a long running time and thus a significant loss in separation efficiency. By the introduction of overpressurized thin-layer chromatography (OPTLC) by Tyihák *et al.* [3, 4] this unfavourable effect of low-mobility solvent systems can be avoided and the separation of amino-acids can be markedly improved [5, 6].

In the present work the applicability of the commonly used eluent comprising butanol, pyridine, acetic acid and water has been studied. In particular, the effects of the following parameters on the selectivity and efficiency of separation have been

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investigated: (a) the ratio of pyridine to acetic acid at constant butanol and water concentration; (b) butanol and water concentration, and (c) nature and concentration of organic solvents used in the eluent.

Experimental

Amino-acids and polypeptides were applied on the plate in 1-cm streaks in mixtures corresponding to about 2 μg of each component. The running distance for development of the chromatograms was 15 cm; when the chromatograms had been over-developed, a strip of filter paper was placed on the top of the plate.

HPTLC silica gel 60 F_{254} pre-coated chromatoplates (Merck Art. No. 5548) were used. A Chrompres 10 instrument (Labour MIM, Budapest, Hungary) was used for the experiments at 1 MPa membrane pressure and at 25°C. The flow rates were adjusted to 0.07 $cm^3\ min^{-1}$ (corresponding to about 40 min development time) for amino-acids and to 0.15 $cm^3\ min^{-1}$ (corresponding to about 25 min development time) for polypeptides, respectively. After development the plates were dried at 80°C under vacuum for 10 h to remove pyridine and acetic acid. The dried plates were evaluated by densitometry using OPTON KM-3 (Opton Feintechnik, G.F.R.) and Shimadzu CS-920 (AOL, Austria) spectrodensitometers in the reflectance mode at 200 nm, as well as after colour reaction with ninhidrin reagent (for amino-acids) at 500 nm and with toluidine in chlorine vapour (for polypeptides) at 500 nm, respectively. All solvents and reagents were of analytical grade and were obtained from Reanal (Hungary).

The compounds investigated are listed in Table 1. The polypeptides were synthesized at the Chemical Works of Gedeon Richter Ltd, and were considered to be of the highest available quality.

Table 1
Compounds investigated by OPTLC

Amino acids

(1) Glycine	(GLY)	(12) Tyrosine	(TYR)
(2) Alanine	(ALA)	(13) Tryptophan	(TRP)
(3) Valine	(VAL)	(14) Proline	(PRO)
(4) Leucine	(LEU)	(15) Hydroxyproline	(HYPRO)
(5) Isoleucine	(ILEU)	(16) Aspartic acid	(ASP)
(6) Serine	(SER)	(17) Glutamic acid	(GLU)
(7) Threonine	(THR)	(18) Asparagine	(ASN)
(8) Cysteine	(CYS)	(19) Glutamine	(GLN)
(9) Cystine	(CYS)2	(20) Lysine	(LYS)
(10) Methionine	(MET)	(21) Arginine	(ARG)
(11) Phenylalanine	(PHE)	(22) Histidine	(HIS)

Polypeptides

small		medium–large	large
(1) Arg–Lys–Asp	(TP-3)	(4) L-6-ketopiperidine-2-carbonyl-L-leucil-L-prolinamide (RGH-2202)	(7) Aprotinin
(2) Arg–Lys–Asp–Val	(TP-4)		(8) Insulin
(3) Arg–Lys–Asp–Val–Tyr	(TP-5)		
		(5) Glypressin	
		(6) Oxytocin	

Results and Discussion

An eluent system comprising butanol, acetic acid and water in different ratios has recently been used for the separation of amino-acids both in normal and ultra-micro chambers [5, 6]. For the normal chamber system, data are also available from work with pyridine as a fourth component in the eluent [7, 8]. To the authors' knowledge, no systematic study has been published on the role of the eluent components in the selectivity of separation; this four-component eluent mixture has not been utilized for OPTLC.

Optimization of the solvent systems for amino-acids

Figure 1 shows the dependence of *Rf*-values on the ratio of pyridine and acetic acid in the eluent when their total concentration and the concentration of butanol and water are constant.

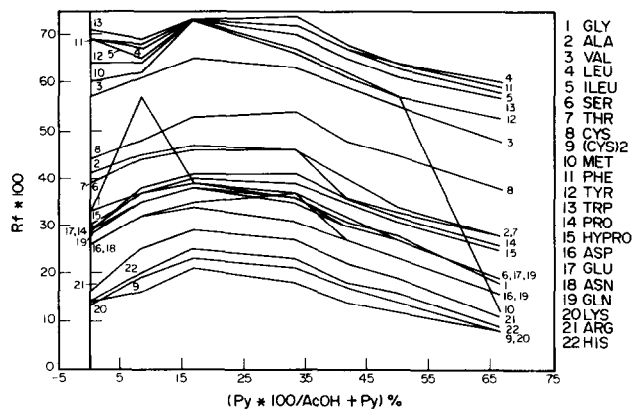

Figure 1
Dependence of *Rf*-values for amino-acids on the ratio of pyridine to acetic acid. Instrument: Chrompres 10, Flow rate: 0.07 cm^3 min^{-1}. Plate: HPTLC silica gel 60 F_{254}; eluent: butanol–(pyridine–acetic acid)–water (55:35:15, v/v/v); evaluation: Opton KM-3 spectrodensitometer, in reflectance mode at 500 mm after colour reaction with ninhidrin.

A 1:2 (v/v) ratio of pyridine to acetic acid provides the best conditions for the separation (Fig. 1). It can also be concluded that, by adding pyridine to the mixture of butanol, acetic acid and water, the selectivity of the separation cannot be significantly improved; however, better peak shape and enhanced efficiency of the separation of some selected amino-acids can be achieved. In Fig. 2 the densitograms obtained by using the pyridine in the mobile phase are shown for some selected amino acids.

The influence of butanol and water concentration on the *Rf*-values using a constant total concentration and ratio of pyridine and acetic acid is illustrated in Fig. 3. With an increase in the butanol concentration (water-lean eluent) only a small change in *Rf*-values can be observed. When a water-rich eluent is used, the spot shape deteriorates resulting in a significant loss in separation efficiency.

For further optimization of separation, the replacement of butanol by other organic solvents was investigated. In Fig. 4 the most interesting results were observed when the butanol was replaced by acetone (Fig. 4A), and methyl ethyl ketone (Fig. 4B).

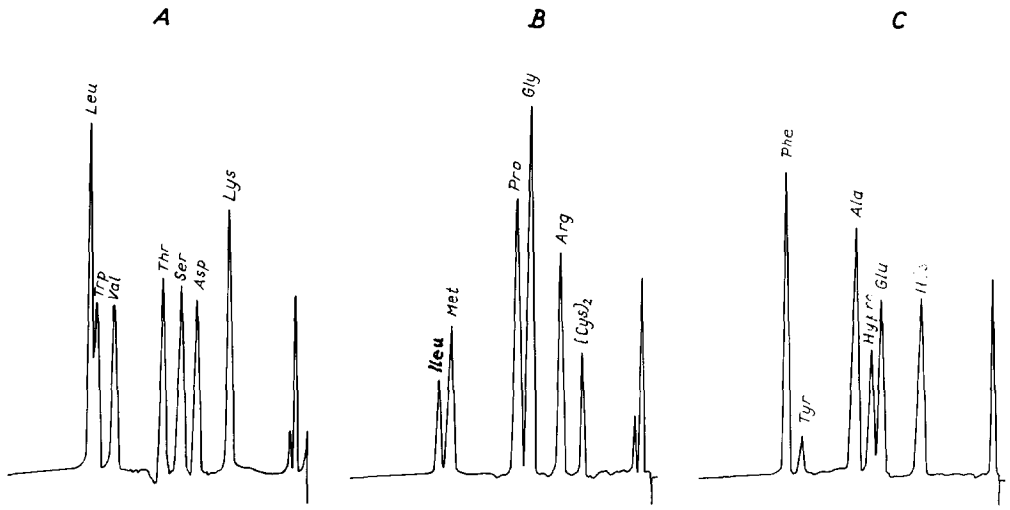

Figure 2
Separation of amino-acids using the optimal ratio of pyridine and acetic acid. Eluent: Butanol–pyridine–acetic acid–water (55:10:25:15, v/v/v/v). Other conditions: as in Fig. 1.

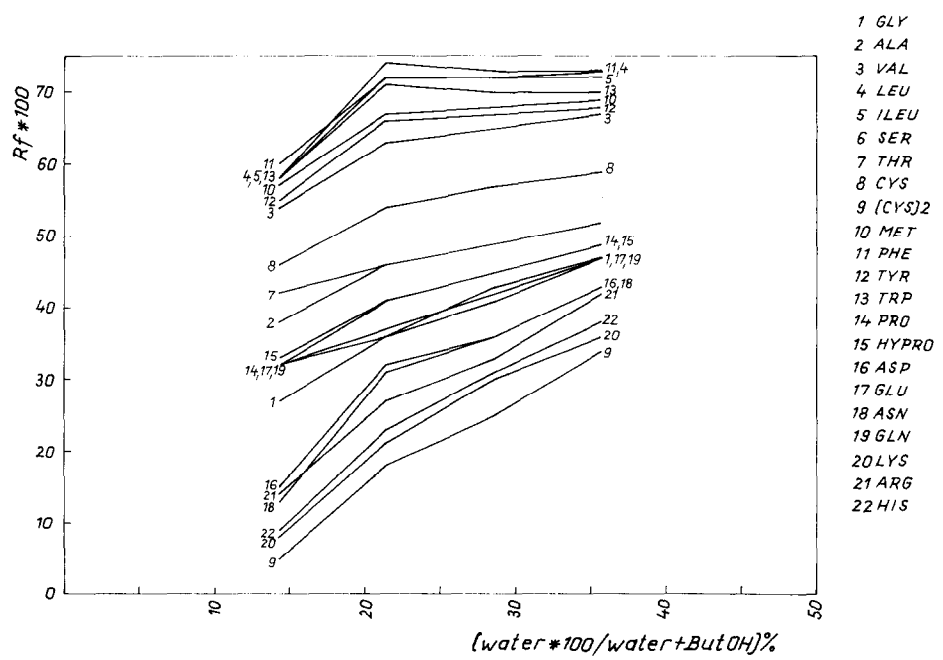

Figure 3
Influence of butanol and water concentration on the separation of amino-acids. Eluent: (Butanol–water)–pyridine–acetic acid (70:10:20, v/v/v). Other conditions: as in Fig. 1.

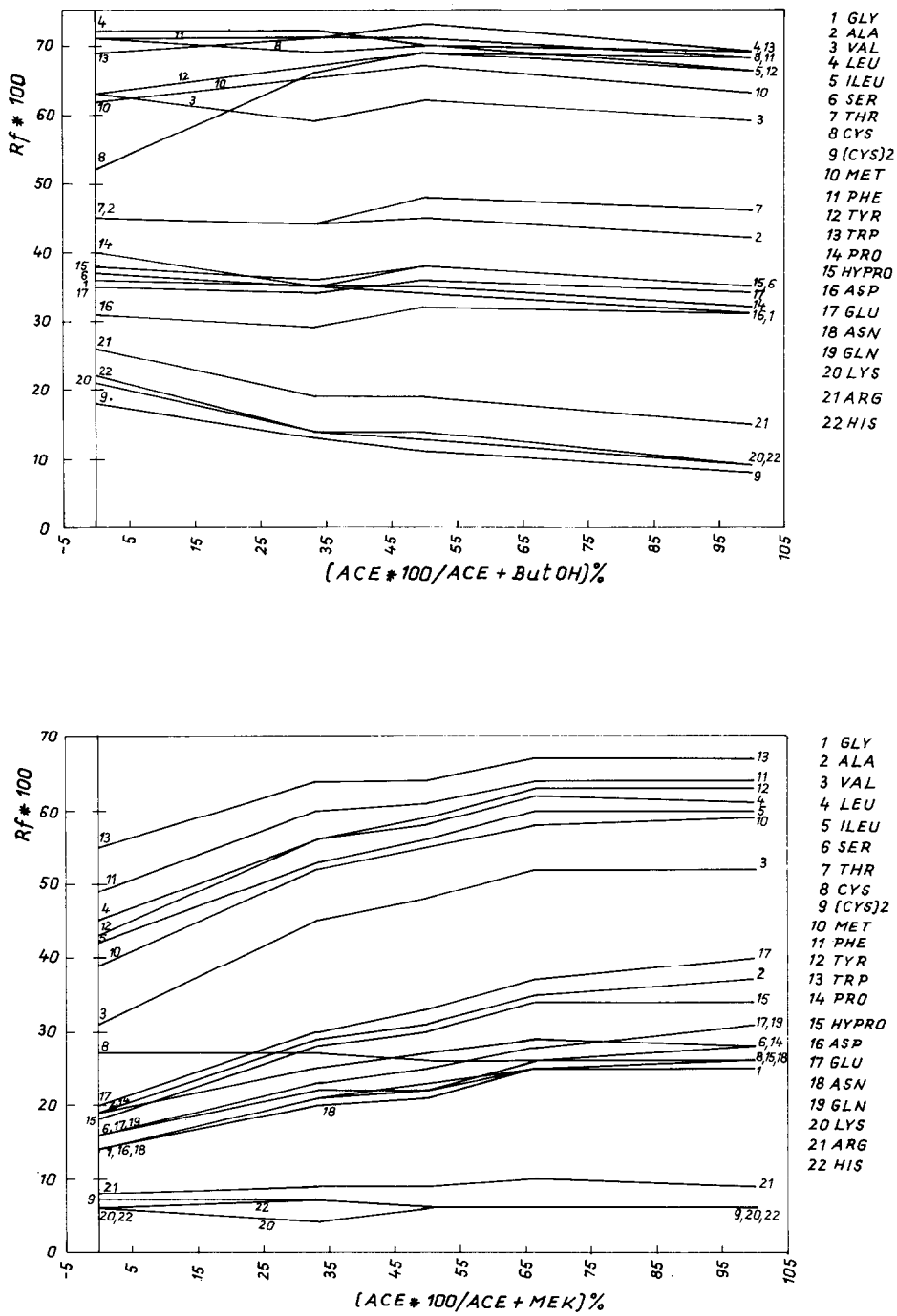

Figure 4
Dependence of *Rf*-values for amino-acids on the nature and concentration of organic solvents in the eluent. Eluent "A": (acetone–butanol)–pyridine–acetic acid–water (55:10:15:20, v/v/v/v). Eluent "B": (acetone–methylethyl ketone–pyridine–acetic acid–water (55:10:20:15, v/v/v/v).

It can be seen that the replacement of butanol by aliphatic ketones can change the elution order. From these experiments it can be concluded that for amino-acids the selectivity of separation can be improved by changing the nature of organic solvents; the resolution can be enhanced through the efficiency of separation by finding the optimal ratio of pyridine to acetic acid and the retention of the components can be controlled by the water concentration.

Optimization of the solvent system for polypeptides

The model compounds can be classified into three groups: small polypeptides, medium–large polypeptides and large polypeptides. Figure 5 shows the dependence of the *Rf*-values of three groups of polypeptides on the ratio of pyridine to acetic acid under conditions similar to those in Fig. 1.

It can be seen that with the exception of oxytocin and RGH-2202, where retention is practically independent of the ratio of pyridine to acetic acid, the *Rf*-values of polypeptides decrease with an increase in pyridine concentration. A 1:1 (v/v) ratio of pyridine to acetic acid seems to be the most useful in practice.

The dependence of the retention of polypeptides on the water concentration of the eluent at constant butanol concentration (Fig. 6) and on the ratio of butanol to water at a constant ratio and total concentration of pyridine and acetic acid (Fig. 7) are shown.

The retention of large polypeptides (insulin and aprotinin) is highly dependent on the ratio of butanol to water. However, a sharp change of the *Rf*-values of these large polypeptides can occur within a definite water concentration (within 20–30% for insulin and 40–50% for aprotinine) (Fig. 7). A similar but smaller change in the retention can be

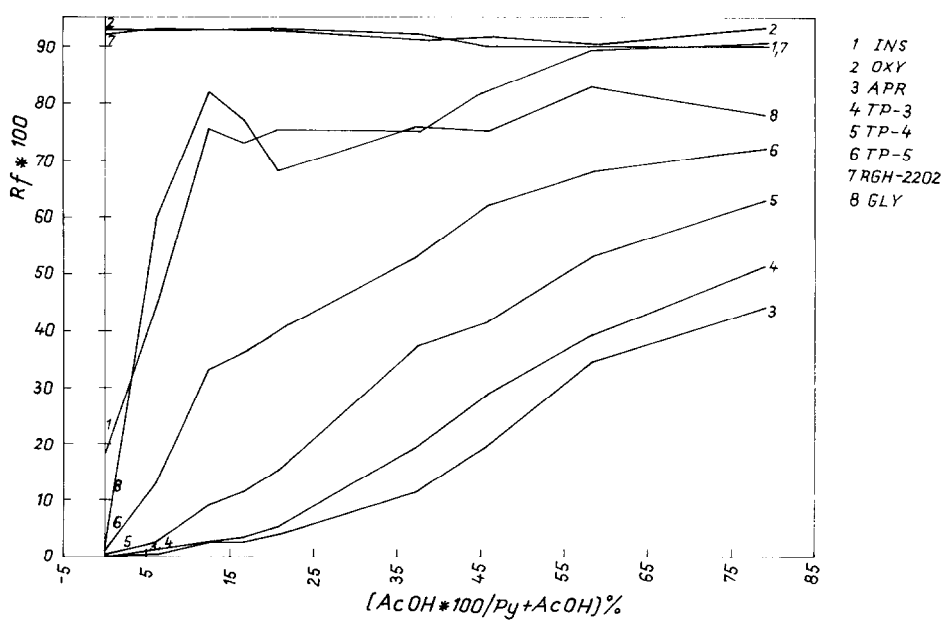

Figure 5
Dependence of *Rf*-values for amino-acids on the ratio of pyridine to acetic acid. Eluent: butanol–(pyridine–acetic acid)–water (30:48:22, v/v/v). Other conditions: as in Fig. 1, except the flow rate (0.15 cm^3 min^{-1}) and colour reaction (toluidine reaction).

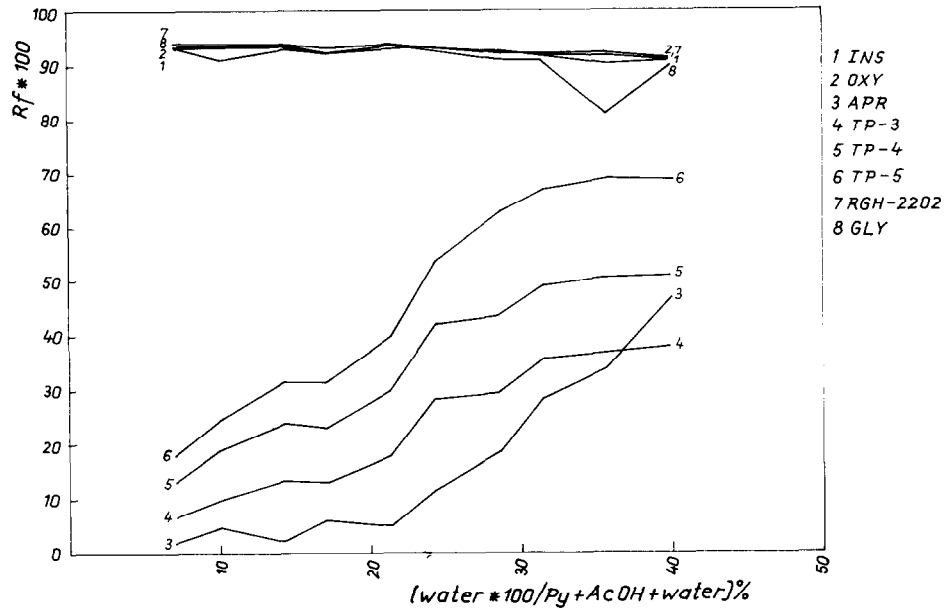

Figure 6
Influence of water concentration on the retention of polypeptides. Eluent: butanol–pyridine–acetic acid (30:35:35, v/v/v) with water. Other conditions: as in Fig. 5.

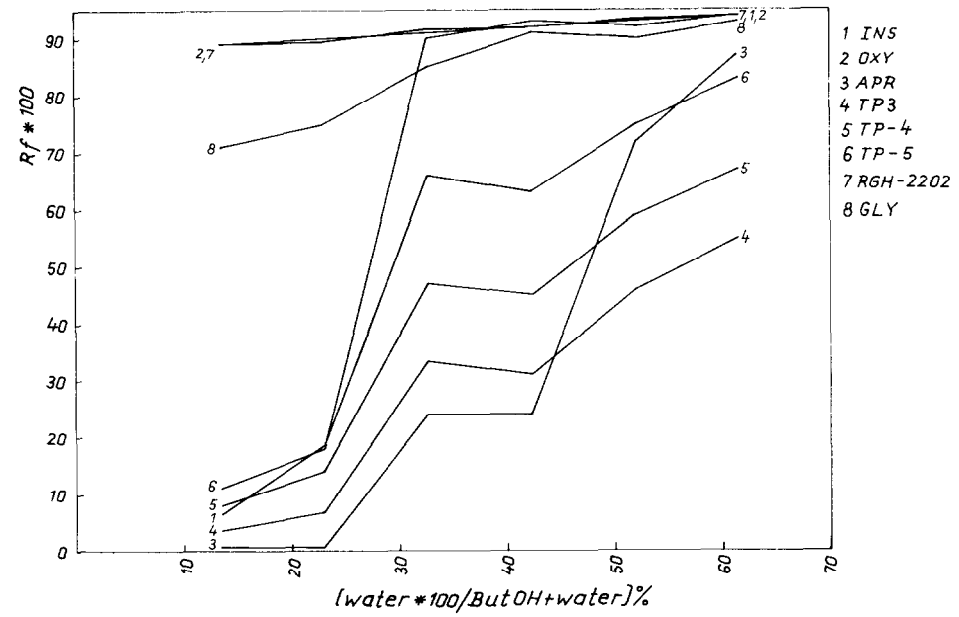

Figure 7
Dependence of *Rf*-values for polypeptides on the ratio of butanol to water. Eluent: (butanol–water)–pyridine–acetic acid (52:24:24, v/v/v). Other conditions: as in Fig. 5.

observed in the case of small polypeptides. The dependence of *Rf*-values on the butanol and water concentration for medium–large polypeptides is not so marked.

The replacement of butanol by other organic solvents in the eluent is demonstrated in Fig. 8 which shows that the use of butanol resulted in better selectivity than did other organic solvents.

From the experiments with polypeptides it can be stated that the selectivity of separation is mainly influenced by the concentrations of butanol and water in the eluent. The retention of polypeptides should be controlled by the ratio of pyridine to acetic acid. Organic solvents other than butanol do not offer better separation conditions. These observations differ considerably from those obtained for amino-acids.

Application of the separation system in pharmaceutical analysis

A few examples serve to demonstrate the applicability of the method in the industrial analysis of amino-acids and peptides. Figure 9 shows the separation of the small polypeptides TP-3, TP-4 and TP-5 using the optimal eluent system.

The second example shows the applicability of the method in the purity control of insulin. Figure 10 shows the densitogram of an insulin sample. The detection limit for insulin-like compounds is about 0.2%.

The third example relates to the determination of essential amino-acids in liver extract. In Fig. 11 the densitograms of dried liver extract (Fig. 11A), Sirepar^R (Fig. 11B) and Perhepar^R injections (Fig. 11C) are shown. The detection limit is less than 0.1 μg; this limit provides the possibility of determining amino-acids in concentrations below ppm.

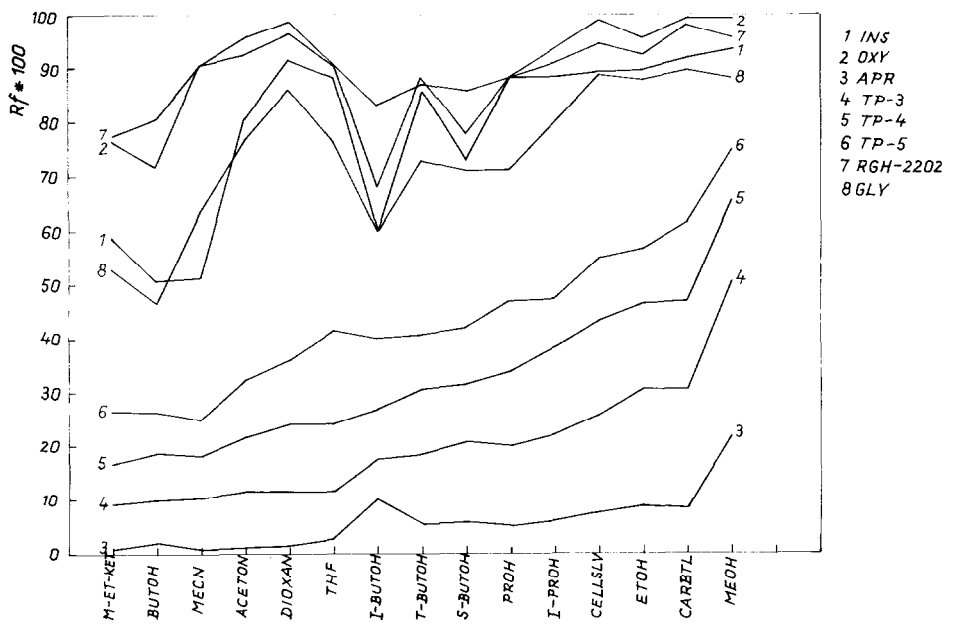

Figure 8
Dependence of *Rf*-values for polypeptides on the nature of the organic solvent in the eluent. Eluent X–pyridine–acetic acid–water (45:15:15:5, v/v/v/v); X = organic solvents. Other conditions: as in Fig. 5.

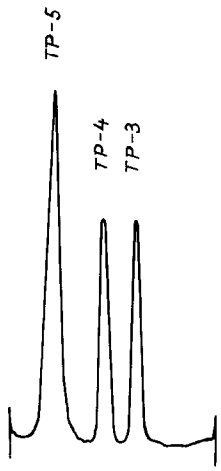

Figure 9
Separation of small polypeptides. Eluent: butanol–pyridine–acetic acid–water (45:15:15:25, v/v/v/v). Other conditions: as in Fig. 5.

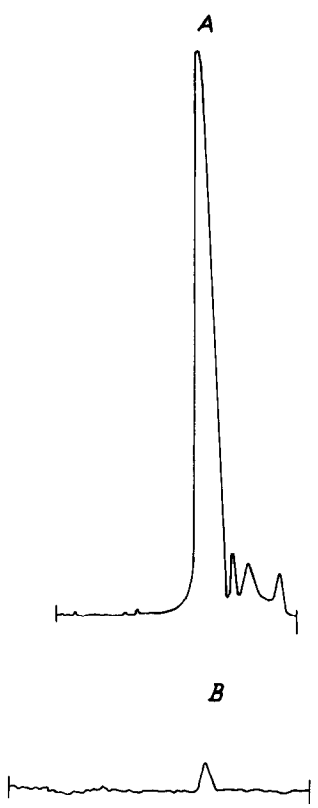

Figure 10
Densitogram of insulin sample. Eluent: butanol–pyridine–acetic acid–water (45:20:15:20, v/v/v/v). Other conditions: as in Fig. 11.

Figure 10A: 200 μg bovine insulin; **Figure 10B:** 1 μg bovine insulin.

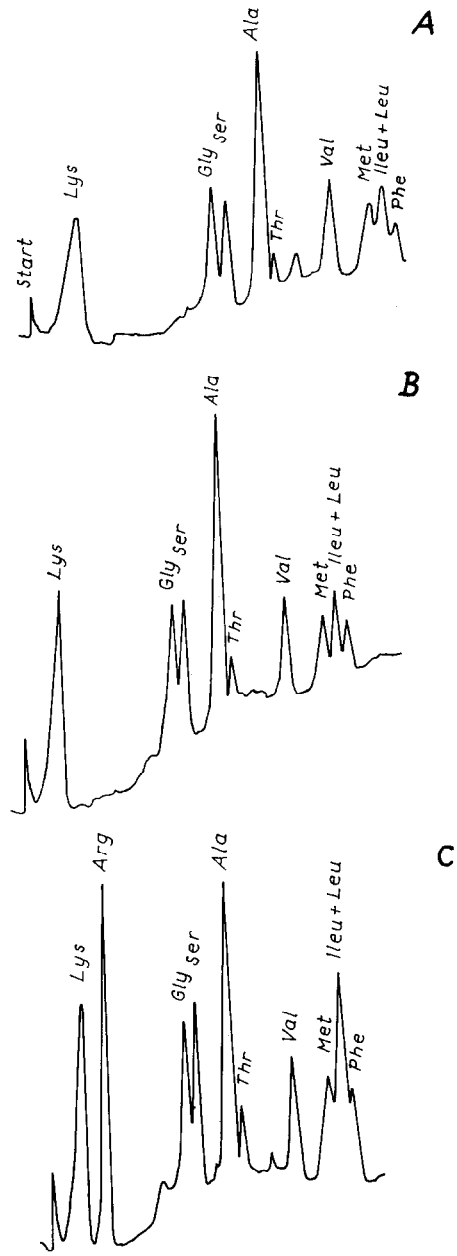

Figure 11
Identification of free amino-acids in liver extract. Eluent: acetone–methyl ethyl ketone–pyridine–acetic acid–water (40:20:5:15:20, v/v/v/v/v). Over-running up to 25 cm distance. Evaluation after ninhidrin reaction at 250 nm in the reflectance mode. Other conditions as in Fig. 5. Densitograms of liver extract powder (A), Sirepar^R injection (B) and Perhepar^R injection (C).

Conclusions

From the experiments the following conclusions can be drawn:

(a) Amino-acids and polypeptides can be separated within a reasonable time and with satisfactory resolution by the aid of an OPTLC technique. The constant linear flow can eliminate the unfavourable effect of highly viscous solvents on the separation efficiency and can provide an excellent opportunity for optimization of the solvent systems;

(b) For amino-acids the selectivity of separation is mainly influenced by the nature of the organic solvent in the eluent. The efficiency of separation (spot shape) can be improved by finding the optimal ratio of pyridine to acetic acid; retention of the compounds can be controlled by the water concentration;

(c) For polypeptides the selectivity of separation can be affected by the ratio of butanol and water; the retention of polypeptides can be controlled by the ratio of pyridine to acetic acid. Butanol was found to be the most useful organic solvent in the eluent.

The separation of amino-acids and polypeptides in a butanol–pyridine–acetic acid–water system can also be influenced by the use of inorganic salts and ion-pair reagents in the eluent. This work is in progress.

References

[1] M. Brenner, A. Niederwieser and G. Pataki, in *Thin-Layer Chromatography* (E. Stahl, Ed.), 2nd edition, pp. 730–756. Springer, Berlin (1969).
[2] S. Blackburn, in *CRC Handbook of Chromatography, Amino acids* (G. Zweig and J. Sherma, Eds), Vol. I. CRC Press Inc., Boca Raton, USA (1983).
[3] E. Tyihák, E. Mincsovics and H. Kalász, *J. Chromatogr.* **174**, 75–81 (1979).
[4] E. Mincsovics, E. Tyihák and H. Kalász, *J. Chromatogr.* **191**, 293–300 (1980).
[5] E. Tyihák, E. Mincsovics, H. Kalász and J. Nagy, *J. Chromatogr.* **211**, 45–51 (1981).
[6] B. Polyák, M. Ábrahám and L. Boros, in *Chromatography the State of the Art* (H. Kalász and L. S. Ettre, Eds.), pp. 591–599. Akadémiai Kiadó, Budapest (1985).
[7] E. Escher, M. Bernier and P. Parent, *Helv. Chim. Acta* **66**, 1355–1365 (1983).
[8] B. Oray, M. Jahani and R. Gracy, *Anal. Biochem.* **125**, 131–138 (1982).

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