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Thin-Layer (Planar) Chromatography

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Introduction

Thin-layer chromatography (TLC) has found extensive application in protein chemistry including recovery of peptides in microgram and nanogram quantities for further primary structural analysis, identification of peptides in partial hydrolysates, in correlating the chromatographic properties of the intact peptides with those of individual amino acids, peptide mapping to characterize or to identify a protein available in very small quantities, resolution of diastereomeric and enantiomeric peptides without any derivatization, fractionation of proteins on the ultramicro scale, testing the optical homogeneity of synthetic peptides, and determination of relative molecular masses.

Application of TLC to the following aspects of peptide studies have also been reported: experimental studies of solute retention and support matrix effects in reversed-phase TLC (RP-TLC) of peptides; a rapid thin-layer immunochromatography method using monoclonal antibodies of two distinct specificities for quantitation of protein antigens; non-stoichiometric models for theoretical treatment of the chromatographic process on ion exchange phases; determination of amino acid configuration of synthetic peptide analogues on Chiralplate® with MeCN/MeOH/H_2O (4 : 1 : 1), prepared from the racemic aromatic amino acids; dependence of the silanophyl effect on the chemical structure of peptides and on the type of mobile phase; study of the salting out behaviour of some peptides with aromatic groups by adsorption TLC on cellulose; separation of peptides on Empore TLC sheets and blotting onto polyvinylidene difluoride (PVDF) membranes with subsequent gas-phase sequencing; analysis of peptide and protein hydrolysates by 2D cellulose TLC and densitometry and its application to luteinizing hormone.

A knowledge of the behaviour of peptides and proteins with both the mobile and stationary phases, particularly with respect to information about kinetics of diffusion, adsorption and desorption, denaturation or conformation changes, is required. Optimization of chromatographic separations of peptides and proteins means a complete resolution of all components in a minimum time, on a preparative scale and with the retention of bioactivity. Various principles of liquid chromatography have successfully been applied to TLC resolution of peptides and proteins, e.g. reversed-phase, size exclusion, ion exchange, etc. The different thin-layer materials used for the purpose include silica gel, cellulose, mixtures of silica gel and cellulose, hydroxyapatite and cross-linked dextran gel filtration media like Sephadex® (various grades form Pharmacia, Uppsala, Sweden). The ordinary porous silica-based stationary phases containing chemically bonded alkyl chains of varying lengths have several disadvantages such as low stability at alkaline pH values (pH > 8), secondary equilibria caused by low diffusion kinetics within the pores,

and ion-exchange effects due to ionized underivatized silanol groups. Therefore, alternative stationary phases are being developed, e.g. coated silica phases, polymer-based phases, and nonporous materials. The separation and purification of peptides and proteins by ion exchange offers advantages because of mild separation conditions providing higher bioactivity recovery.

Sample Preparation

Depending on the nature and source, the proteins may be digested before applying them to thin-layer plates; some of the methods reported in the literature are described below.

1. Proteins are dissolved in ammonium bicarbonate (0.5%, pH 8.0) and digested with trypsin (1% w/w) for 4 h at 37°C. Chymotrypsin (1% w/w) may be added for trypsin–chymotrypsin digest and the digestion continued for a further 4 h. The peptides are recovered by freeze-drying.
2. Proteins may either be alkylated with iodoacetic acid or oxidized with performic acid to render them susceptible to enzymatic digestion. The treated proteins are then dissolved in ammonium bicarbonate buffer (0.05 mol L^{-1}, pH 8.4) to a concentration of 2 mg mL^{-1} and TPCK-treated trypsin (L-(1-tosylamide-2-phenylethyl chloromethyl ketone)) is added to give a final enzyme-to-substrate ratio of 1 : 75. The digest is incubated for 5 h at 30°C, freeze-dried, and redissolved in 10% isopropanol for application to the plates.

For TLC of smaller peptides, the samples have either been synthesized or obtained commercially. The stock solutions (0.025 mol L^{-1}) are prepared in aqueous 2-propanol (10%) and are kept refrigerated when not in use. For proteins, solutions can be prepared in dilute saline solutions or in an appropriate buffer.

Preparation of Thin-Layer Plates

Commercially available precoated silica or cellulose plates have generally been used. Sometimes these plates have uneven coatings that can be checked by holding the plates against a light box and looking for dark streaks or patches that indicate uneven thickness. Such plates should be rejected or used for initial trial runs only. The following method has been widely used for making plates with cellulose powder. Preparation of thin-layer plates from Sephadex is also described.

Thin-Layer Plates from Cellulose

Cellulose powder is slurried with methanol/water (4 : 1, 200 mL). The slurry is poured into a Büchner funnel and is washed successively with 2-propanol/water/acetic acid (3 : 1 : 1, 300 mL); methanol/water (1 : 3, 200 mL); methanol/1 mol L^{-1} HCl (3 : 2, 200 mL); water (200 mL), and finally with methanol (200 mL). The powder is dried overnight *in vacuo* before use. The purified cellulose powder (15 g) is spread as a slurry over five plates (20 × 20 cm) at an initial thickness of 400 μm. The coated plates are allowed to dry overnight in a horizontal position before use.

Thin-Layer Plates from Gel Filtration Media

Sephadex G-100 (6 g) or Sephadex G-200 is suspended in 100 mL of the solvent (e.g. 0.5 mol L^{-1} NaCl solution). Care should be taken to ensure that no aggregates are present in the final gel suspension. The dextran gels usually take 48 h to proceed to complete swelling. Thoroughly cleaned and dry glass plates (10 cm × 20 cm) are coated with a 0.9 mm-thick layer of a suitable thin-layer spreader. The plates are kept in a closed vessel containing a dish of the solvent and stored in the horizontal position for at least 18 h before use. The layers may be stored for fairly long periods in a wet chamber; if they dry out or show cracking, a very mild spray with buffer solution is applied to regenerate the layers.

Development of Chromatograms

The silica gel or cellulose-based chromatograms are developed in the usual manner, while the development of gel plates is carried out as shown in **Figure 1**.

Various solvent systems, support materials, and detection procedures for the TLC of a variety of proteins and peptides have been summarized in

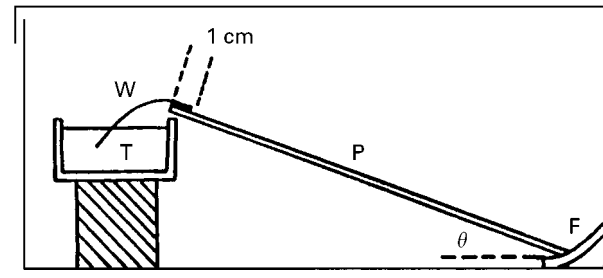

Figure 1 Apparatus for thin-layer chromatography of proteins. Solvent (0.5 M NaCl) is led to plate P by means of Whatman No. 3 filter paper wick W. The wick should overlap about 1 cm onto the gel layer. T is the solvent trough.

Table 1. The hR_F values for some of these systems have been recorded in **Tables 2–6**.

For two-dimensional peptide mapping, conventional chromatography follows electrophoresis. The plate is dampened with electrophoresis buffer, taking care not to smudge the applied sample, and run at 1000 V fir 40–90 min, for 20 cm × 20 cm plates. The electrophoresis plate is removed from the apparatus and dried overnight in a fume hood (there should be no smell of acetic acid on the plates). The plate is then developed using a suitable chromatographic solvent (Table 1). The composition of the solvent is critical for the mobility of peptides. More organic solvent in the mixture tends to increase the relative mobility of the hydrophobic peptides, since the stationary phase is hydrophilic. Some of the solvent systems used for two-dimensional work are mentioned in **Table 7**.

Detection of Peptides on Thin-Layer Chromatograms

Various peptides and proteins are located on the thin layer chromatograms by using ninhydrin, fluorescamine, o-phthalaldehyde, iodine vapours or UV. Quantitation is performed by densitometric scanning or by spectrophotometry after eluting the peptides; immunochromatography using monoclonal antibodies has also been used for quantification.

Detection on Cellulose or Silica Gel Plates

The plates can be viewed using a long-wavelength (366 nm) UV source or stained with a suitable reagent as described in **Table 1**. Certain other detection methods are described below.

1. *Morin reaction*. The dried chromatograms are sprayed with a 0.05% solution of morin (3,5,7,2′,4-pentahydroxyflavone) in methanol, and heated for 2 min at 100°C. The *N*-protected amino acids and peptide derivatives give yellowish-green fluorescence on a green fluorescent background, or dark absorption spots under UV. The detection limit is about 2 μg per spot.
2. *Iodine starch reaction*. The chromatogram is placed in an iodine vapour atmosphere for 5 min. The excess iodine is removed by leaving the plate in the open air, and then the layer is sprayed with 1% aqueous starch solution. The peptides (and amino acids) give blue spots.

Detection on Gel Layers

The gel layer is covered with dry filter paper (Whatman 3 mm), avoiding bubble formation and is carefully smoothed down over it. The layer and the paper are dried together at 120°C or the paper is carefully peeled off the layer and dried at 110°C.

The proteins on the paper are detected by the usual paper chromatographic methods. The dried paper is immersed for 15 min in Amido black 10 B (0.6 g) dissolved in a mixture of methanol or ethanol (750 mL), water (450 mL), and glacial acetic acid (100 mL) and washed three times with 1% acetic acid for 30 min each time. Alternatively, the paper is stained in 1% solution of Bromophenol blue saturated with mercuric chloride for 5 min and then washed five times with 0.5% acetic acid for 30 min each time.

Recovery of Peptides

After detection, the peptide spots on the cellulose or silica gel thin layers are carefully scraped and are transferred to 150 mm long Pasteur pipettes that have been tightly plugged with one-quarter of a glass fibre membrane filter (20 mm diameter, Sartorious SM 13400) and prewashed with 2 mL of 6 mol L^{-1} HCl; 200 μL of 6 mol L^{-1} HCl containing 0.02%-β-mercaptoethanol is added to each of the Pasteur pipettes and the peptide is extracted at room temperature for 15 min. The HCl is then forced through the filter with nitrogen (1 atm).

TLC of Diastereomeric and Enantiomeric Dipeptides

Successful separation of dipeptide diastereomers, either as the free peptides or as the *N*-protected methyl esters, has been reported. Starting from pure L-methionine and DL-alanine, Np-S-L-Met-DL-Ala-O-Np and Np-S-L-Met-L-Met-DL-Ala-O-Np were synthesized and the separation of diastereomeric *p*-nitrophenyl (Np) esters of *N*-protected di- and tripeptides was achieved on silica gel F_{254} precoated (Merck) plates. TLC separation of diastereomeric dipeptides has been well documented.

Typical examples of the separation of enantiomeric dipeptides on Chiralplates® are given in Table 5. It was observed that the antipodes with *C*-terminal L-configuration always gave a smaller R_F value than the corresponding enantiomeric dipeptide with *C*-terminal D-configuration. The method also resolves diastereomeric dipeptides. A comparison of resolution of four isomeric Try-Try, Ala-Ala, Phe-Phe, Tyr-Tyr, Lys-Ala, and Asp-Ala mixtures on Chiralplates® and on microcrystalline cellulose plates showed that the separation of L,L and D,D pairs of all tested dipeptides was better on microcrystalline cellulose plates while L,D, and D,L, pairs were better separated on Chiralplates® (**Figure 2**A, B).

Table 1 Solvent systems, support materials, and detection procedures for TLC of proteins and peptides

No.	*Protein/peptide*	*Developing system*	*Detection*
1	Carbazo peptides	(a) *n*-Butanol/acetone/acetic acid/5% NH_4OH/water (9 : 3 : 2 : 4) (b) *n*-Butanol/acetic acid 5% NH_4OH (11 : 6 : 3) (c) *n*-Butanol/acetic acid/pyridine/water (15 : 3 : 10 : 12) (d) *n*-Butanol/acetic acid/5% NH_4OH/H_2O two-phase mixture (6 : 1 : 1 : 2)	0.2% Ninhydrin spray (*n*-Butanol/2 N acetic acid, 95 : 5) followed by a spray of a saturated solution of $K_2Cr_2O_7$ in conc. H_2SO_4
2	Leu-Try-Leu	$CHCl_3$/(34%)NH_4OH/ethanol (2 : 1 : 2)	Dried for 2 h at 60°C, sprayed with 1% (v/v) triethylamine in acetone followed by 0.1% fluorescamine in acetone
3	Np-*S*-L-Met-DL-Ala-O-Np, Np-*S*-L-Met-L-DL-Ala-O-Np, Np-*S*-Met-Ala-Met-OMe	Solvents and hR_F given in Table 3	Iodine
4	*N*-*t*-butoxy peptides	Ethanol/pyridine/water/acetic acid (5 : 5 : 3 : 1)	Exposure to chlorine followed by spraying with *o*-toluidine reagent or dicarboxidine reagent
5	Lysino alanine	(a) Butanol/anhydrous acetic acid/water (4 : 1 : 1) (b) Phenol/water (3 : 1)	Ninhydrin
6	Peptides from proteases	(a) Chloroform/methanol (3 : 1) (b) Chloroform/methanol/acetic acid (45 : 4 : 1)	(a) Aqueous 2% sodium nitroprusside (b) 4% Ninhydrin in acetone/pyridine/acetic acid (97 : 3 : 2) (c) 1-Naphthol/NaBrO (Sakaguchi reagent)
7	Insulin	(a) 0.1 N HCl/96% ethanol (1 : 1) (b) 0.5 N HCl/ethanol/acetone (5 : 3 : 0.5)	0.5% Ninhydrin in acetone
8	Pepsin, trypsin and α-chymotrypsin	(a) 0.1 N HCl/ethanol (1 : 1) (b) 0.5 N HCl/ethanol/acetone (5 : 3 : 0.5)	0.5% Ninhydrin in acetone, heated at 100°C
9	Angiotensin	(a) *n*-Butanol/acetic acid/water (4 : 1 : 1) (b) *n*-Butanol/pyridine/water (15 : 10 : 3)	Ninhydrin
10	Bradykinin	*iso*-Propanol/methyl acetate/conc. ammonia (9 : 7 : 4)	UV
11	Dansyl peptides from ATP-creative phosphotransferase	Methyl acetate/iso-propanol/conc. ammonia (9 : 7 : 4)	UV after exposure to dioxane vapours
12	Glycopeptides from *Escherichia coli* and *Bacillus megaterium*	*n*-Butanol/pyridine/acetic acid/water (130 : 20 : 6 : 24)	Chlorine/toluidine
13	Lipopeptides from *Nocardiea asteroids*	Chloroform/methanol (1 : 1)	P_2O_5, 130°C
14	Cyclotripeptides from *Aspergillus ochraceus*	Five different solvent systems	Chlorine/toluidine, $KMnO_4$
15	Basic polypeptides	(a) *n*-Butanol/25% ammonia/water (34 : 3 : 3) (b) Chloroform/methanol (9 : 1)	Ninhydrin/chlorine/toluidine
16	Lipase, diastase, papain, emulsin, invertase	96% Ethanol/0.1 N HCl (1 : 1)	0.5% Ninhydrin in acetone, heated at 100°C for 10 min
17	Lipopeptides from *E. coli*	Chloroform/methanol/water (68 : 25 : 4)	Radioactivity

Table 1 *Continued*

No.	*Protein/peptide*	*Developing system*	*Detection*
18	Angiotensin antagonists	(a) Chloroform/acetone (5 : 1) (b) *n*-Butanol/acetic acid/water (4 : 1 : 1)	Ninhydrin
19	Vasopressin and xytocin, tripeptide amides	Butanol/acetic acid/water (4 : 1 : 1)	UV
20	*N*-methyl oligopeptides	(a) Chloroform/methanol (9 : 1) (b) Chloroform/acetone (8 : 2 or 9 : 1)	Chlorine/toluidine, UV, iodine UV, iodine
21	Dansyl peptides of lipoproteins	Three different solvent systems	Ninhydrin
22	Glycoprotein-derived oligosaccharides	Propanol/acetic acid/water (3 : 3 : 2)	Orcinol
23	Various peptides	Chloroform/methanol/aq.17% ammonia (2 : 2 : 1)	Sprayed with 0.1% solution of 4-chloro-7-nitrobenzofuran in ethanol, followed by 50% methanol (adjusted to pH 11 with (NaOH)
24	Enantiomeric and diastereomeric dipeptides (on Chiralplate)	Solvents and hR_F given in Table 5	0.1%
25	*Boc*-βAla-Try-Met-Asp-Phe	Chloroform/methanol/acetic acid (18 : 2 : 1); sec-butanol/3% ammonia (25 : 11 and 3 : 1); ethanol/acetic acid/water (20 : 6 : 11); *n*-butanol/acetic acid/water (4 : 1 : 5, upper phase)	Ninhydrin, Ehrlich reagent, fluorescamine reagent
26	Oligopeptides (8 standard samples)	(a) *iso*-Amyl alcohol/ethanol/anhyd. acetic acid/pyridine/water (175 : 50 : 13 : 175 : 150) (b) *iso*-Propyl alcohol/ethanol/aq.ammonia (4 : 4 : 3)	Ninhydrin Ninhydrin
27	Lysino alanine	(a) *iso*-Propyl alcohol/1 M HCl (3 : 1) (b) *tert*-Butyl alcohol/acetone/methanol/water/conc. ammonia (4 : 4 : 1 : 14 : 5); electrophoresis in glacial acetic acid/98% formic acid/water (17 : 5 : 28, pH 2)	
28	Ribonuclease B, globin, R17-bacteriophage coat protein, carboxymethylated protein, subunits from BI component of TYMV; tryptic digest of glyceraldehyde 3-phosphate dehydrogenase of yeast	(a) *n*-butanol/pyridine/acetic acid/water (15 : 12 : 3 : 12) (b) *iso*-Butanol/pyridine/water (7 : 7 : 6) (c) *iso*-Amyl alcohol/pyridine/water (7 : 7 : 6) (d) Butanol/acetic acid/water (5 : 1 : 4), upper phase	Cd-Ninhydrin spray followed by heating at 60°C
29	Dipeptides with Leu/Ile as *N*-terminal residues	Solvent and hR_F given in Table 4	Cd-Ninhydrin spray followed by densitometry
30	Several di- and tripeptides (see Table 2)	Solvents and hR_F given in Table 2	Cd-Ninhydrin spray followed by heating at 60°C for 15 min
31	*S*-Carboxymethyl insulin chain-A, Ala-Leu-Gly, Leu-Leu-Val-Tyr, Glu-Gly-Phe	(a) Pyridine/acetic acid/water/acetone (2 : 4 : 79 : 15) at pH 4.4, electrophoresis (b) *n*-Butanol/pyridine/acetic acid/water (915 : 10 : 3 : 12)	(a) 0.3% Ninhydrin in collidine/acetic acid/ethanol (3 + 100 + 87) (b) 1% Fluorescamine (w/v) in acetone (c) 0.05% *o*-Phthaladialdehyde in methanol (w/v) containing 0.2% β-mercaptoethanol and 0.09% Brij-35; sheets pre- and postsprayed with 10% triethylamine in methylene chloride

Table 1 *Continued*

No.	*Protein/peptide*	*Developing system*	*Detection*
32	Tryptic peptides from polyoma virus	(a) Acetic acid/0.5% pyridine, pH 3.5 (25 V cm^{-1}) for electrophoresis (b) *n*-Butanol/acetic acid/water/pyridine (5 : 1 : 4 : 1)	Ninhydrin spray
33	Ribonuclease S protein	(a) 1.25 mol L^{-1} Pyridine acetate buffer, pH 6.45, 400 V for electrophoresis (b) Butanol/acetic acid/pyridine/water (30 : 6 : 20 : 24), TLC	Cd-Ninhydrin
34	Actinomycinhydrolysate	(a) Formic acid/acetic acid, pH 6.0 10 mA, electrophoresis (b) Butanol/water/acetic acid (5 : 4 : 1) TLC	Cu-Ninhydrin, fluorescamine
35	Acid hydrolysates of collagen	(a) *iso*-Propanol/butanone/1 mol L^{-1} HCl (12 : 3 : 5) (b) 2-Methyl-2 butanol/butanone/acetone/methanol/conc. ammonia (5 : 4 : 7 : 1 : 4)	Cd-Ninhydrin, *o*-phthaldialdehyde
36	Cystine peptides	Butanol/acetic acid/water (71 : 7 : 22)	Ninhydrin
37	Streptococcal peptides	Aq. 10% trichloroacetic acid	Ninhydrin
38	Enantiomeric dipeptides, Try-Try, Ala-Ala, Phe-Phe, Tyr-Tyr, Lys-Ala, Asp-Ala (synthesized)	Pyridine/water (2 : 1 or 4 : 1) at $-10^{\circ}C$	Ninhydrin
39	Bovine serum-albumin, bovine γ-globulin, α-chymotrypsin, cytochrome *c*, haemoglobin, lysozyme, myoglobin, ovalbumin, ovomucoid, pepsin, ribonuclease, thyroglobulin, trypsin (Sephadex G-100, G-200)	Solvent and R_{Hb} given in Table 6	Staining in 0.2% Ponceau S in 10% aq. acetic acid for 30 min followed by washing with water
40	Ornithine carbamoylphosphate transferase (Sephadex G-200, G-200 superfine)	0.5 mol L^{-1} NaCl	Amido black 10B in methanol/water/glacial acetic acid (5 : 4 : 1)
41	α-Chymotrypsin, aldose, catalase, bovine serum albumin, ovalbumin, lysozyme, phosphorylase b, β-galactosidase, alkaline phosphatase, lactate dehydrogenase, glyceraldehyde phosphate dehydrogenase (Sephadex G-75 and G-100)	6 mol L^{-1} guanidine hydrochloride (descending chromatography	On paper, by spraying with diazotized sulfanlic acid
42	Small peptides on aluminium-backed thick silica gel	–	Fluorescence
43	Cystine and its peptides on polyamide or silica gel	–	Spraying with dilute 2-nitro-5-sulfothiobenzoate
44	Peptides from soya bean meals on $KHSO_4$-coated silica gel plates	Butanol/dioxane/water (4 : 5 : 1)	Treated with saturated thiobarbituric acid solution 85% H_3PO_4 (1 : 1), then with ethanolic naphthoresorcinol/20% H_2SO_4 (10 : 1), heated at $105^{\circ}C$
45	Phenyl alanine peptides on ammonium tungstophosphate-coated silica gel	0.1 mol L^{-1} Nitric acid/1 mol L^{-1} ammonium nitrate	

Table 1 *Continued*

No.	*Protein/peptide*	*Developing system*	*Detection*
46	Small peptides	Suspended over aq. 2,4,6-trimethyl pyrylium tetrafluoroborate and alkaline with 0.5 mL of triethyl amine then adjusted to pH 3 by acetic acid (chamber temp 80°C)	SIMS
47	Peptides on silica gel 60H	Water or aq. methanol	Ninhydrin
48	Oleoyl-L-alanyl-L-alanine	Water/MeCN (7 : 3), pH 5.0	Ninhydrin
49	Peptides on cellulose, impregnated cellulose or alumina layers (reversed-phase)	Propan-1-ol as organic component	–
50	Enantiomeric peptides on Chir® plate	–	–
51	Peptide from *Schumanniophyton magnificum* with anti-cobra venom activity on silica, or silica impregnated with 5% liq. paraffin in petrol ether	Acetone/methanol/10% ammonia (3 : 1 : 1); butanol/methanol/10% ammonia (3 : 1 : 1); chloroform/methanol (1 : 1)	Spraying with Dragendorf reagent, 5% ferric chloride, or 2% ninhydrin in acetone
52	HBsAG (hepatitis B virus) segments in soln on silica	Ethyl acetate + stock soln of pyridine/acetic acid/water (20 : 6 : 11) in various proportions	Spraying with toluidine/KI after chlorination
53	Protein antigens	A sandwich assay format using monoclonal antibodies of two distinct specificities, on covalently immobilized to a defined detection zone on a porous membrane while other serves as a label. Sample is mixed with the coating, and the mixture is then passed along a porous membrane in detection zone.	Monoclonal antibodies immobilized to a defined detection zone on a porous membrane; blue colour
54	Thyrotropin hormone, on silica	Chloroform/methanol (9 : 1), or ethyl acetate + stock soln of pyridine/acetone/water (20 : 5 : 11) in ratio 6 : 4.	–

Layer material for S. nos 1–25, 42–45, 51–52, 54, silica gel, for S. nos 26–38, cellulose; for S. nos 39–41, Sephadex; for S. no. 49 cellulose or alumina; and for S. no. 50, Chir® plate.

Table 2 [a]hR_F values of peptides from L-amino acids after one-dimensional TLC on cellulose

Peptides	*Solvent systems*				
	A	*B*	*C*	*D*	*E*
Ala-Ala	65	26	55	68	58
Ala-Asp	56	1	45	44	19
Ala-Glu	64	5	58	56	29
Ala-Gly	50	17	36	46	46
Ala-Phe	94	52	86	84	85
Ala-Ser	52	17	36	41	49
Gly-Ala	52	18	37	43	46
Gly-Asp	43	0	30	29	13
Gly-Gly	34	13	22	29	34
Gly-His	7	16	5	16	32
Gly-Ile	81	49	65	80	75
Gly-Leu	82	51	67	87	80
Gly-Lys	14	11	2	21	27
Gly-Phe	75	50	65	76	67
Gly-Pro	47	17	39	45	44
Gly-Ser	32	13	22	28	26
Gly-Tyr	68	28	45	64	51
Gly-Val	72	36	56	73	62
Leu-Ala	97	56	88	90	89
Leu-Gly	82	52	67	84	83
Leu-Val	100	77	98	96	95
Val-Gly	67	38	56	70	69
Val-Leu	100	80	98	100	95
Ala-Gly-Gly	47	13	34	39	43
Glu-Cys-Gly	16	0	7	7	0
Gly-Gly-Gly	32	8	20	31	30
Val-Gly-Gly	65	28	52	65	61

[a]hR_F is the R_F value multiplied by 100.
Solvent systems: A, 2-propanol/butanone/1 M HCl (60 : 15 : 25); B, 2-methyl butan-2-ol/butanone/propanone/methanol/water/ammonia (10 : 4 : 2 : 1 : 3 : 1); C, 2-propanol/water (3 : 1); D, 2-propanol/water/acetic acid (15 : 4 : 1); E, 2-propanol/water/ammonia (15 : 4 : 1). All proportions are v/v.

Separation of Peptides on Reversed-Phase Plates Impregnated with Ion Pair Reagents

Successful separations of a large number of di- and tripeptides, and some tetra- and pentapeptides, on home-made silanized silica gel plates and reversed-phase (RP-2, RP-8, RP-18) plates impregnated with dodecylbenzenesulfonic acid, anionic and cationic detergents such as triethanolaminedodecylbenzene sulfonate (DBS), sodium dioctylsulfosuccinate (Na-DSS), and *N*-dodecylpyridinium chloride (*N*-DPC), and on ammonium tungstophosphate layers have been reported in the literature. The separation conditions and hR_F values of some polypeptides of moderate size and of similar structure on home-made layers of silanized silica gel and RP-2 plates are recorded in **Table 8**. The separation of such peptides is, however, a difficult problem of analytical importance.

Table 3 hR_F values for dipeptides and tripeptides

Peptides	hR_F	*Solvent*
Np-*S*-Met-Ala-O-Np		
L-L	77	
L-D	67	A
Np-*S*-Met-Met-Ala-O-Np		
L-L-L	58	
L-L-D	66	B
Np-*S*-Met-Ala-Met-OMe		
L-L-L	56	
L-D-L	52	C

Solvents: A, acetic acid/diethyl ether (1.5 : 20, v/v); B, acetic acid/diethyl ether (0.2 : 20, v/v); C, diethyl ether/iso-propanol (20 : 0.25, v/v). Np = p-nitro phenyl-.

The different solvent systems and chromatographic conditions used for these separations are summarized in **Table 9**. The description of the peptides and their structures is omitted. These systems may thus provide helpful guidance for choosing or developing a solvent system according to the actual requirement of the experiment.

Table 4 Chromatographic behaviour of dipeptides from L-amino acids on thin layers of cellulose

Peptides	hR_F			*Colour yield* ($mm^2\ \mu mol^{-1}$) $\times 10^{-4}$	
	A	*B*	*C*	*405 nm*	*490 nm*
Ile-Ala	90	68	56	3.5	7.8
Ile-Gly	84	53	46	3.7	5.2
Ile-Glu	94	13	13	10.1	23.0
Ile-Leu	100	88	86	1.6	14.6
Ile-Lys	58	50	41	86.6	22.5
Ile-Met	94	79	80	3.7	8.4
Ile-Phe	100	81	89	4.5	13.6
Ile-Pro	89	60	63	2.9	3.5
Ile-Ser	87	53	38	5.8	13.7
Ile-Try	100	83	83	2.6	10.8
Ile-Val	100	80	83	5.8	14.5
Leu-Ala	97	58	–	2.9	5.1
Leu-Gly	82	52	0	2.9	5.6
Leu-Leu	99	66	83	3.8	8.5
Leu-Met	100	71	75	9.6	15.1
Leu-Phe	99	73	79	6.9	10.5
Leu-Ser	88	61	41	4.5	9.8
Leu-Try	100	73	77	7.7	13.0
Leu-Tyr	97	67	66	8.9	10.8
Leu-Val	100	77	–	3.1	5.0

Solvents: A, 2-propanol/butanone/1 mol L^{-1} HCl (12 : 3 : 5); B, 2-methyl-2-butanol/butanone/propanone/methanol/water/0.88 NH_3 solution (10 : 4 : 2 : 1 : 3 : 1); C, *n*-butanol/butanone/water/0.88 NH_3 (80 : 5 : 17 : 3).

Table 5 Resolution of enantiomeric and diastereomeric dipeptides

Enantiomeric dipeptide		hR_F *values*			
		A		*B*	
L-*isomer*	D-*isomer*	L	D	L	D
Gly-L-Phe,	Gly-D-Phe			57	63
Gly-L-Leu,	Gly-D-Leu			53	60
Gly-L-Ile,	Gly-D-Ile			54	61
Gly-L-Val,	Gly-D-Val			58	62
Gly-L-Try	Gly-D-Try			48	55
D-Leu-L-Leu,	L-Leu-D-Leu	19	26	48	57
D-Ala-L-Phe,	L-Ala-D-Phe	21	26	59	65
D-Met-L-Met,	L-Met-D-Met	29	33	64	71

Diastereomeric dipeptides	hR_F *in eluent B*
L-Leu-L-Leu	45
L-Leu-D-Leu	53
L-Ala-L-Ala	64
L-Ala-D-Ala	70
L-Ala-L-Phe	59
L-Ala-D-Phe	65
L-Met-L-Met	62
L-Met-D-Met	72

Solvent system: A, methanol/water/acetonitrile (1 : 1 : 4, v/v); B, methanol/water/acetonitrile (5 : 5 : 3, v/v). Length of run 13 cm. Detection: 0.1% ninhydrin reagent.

Table 6 $^aR_{Hb}$ values of proteins on Sephadex plates

Protein	*Molecular weight* $\times 10^{-2}$	$^aR_{Hb}$	
		A	*B*
Cytochrome-C	13.0	0.68	0.74
Ribonuclease	13.6	0.68	0.74
Lysozyme	14.5	0.65	0.70
Myoglobin	16.9	0.79	0.80
α-Chymotrypsin	22.5	0.87	0.87
Trypsin	23.8	0.83	0.86
Ovomucoid	27.0	0.94	1.03
Pepsin	35.0	0.99	1.04
Ovalbumin	45.0	1.03	1.04
Haemoglobin	68.0	1.00	1.00
Bovine serum albumin	65.0	1.28	1.54
Bovine γ-globulin	180.0	1.28	1.54
Thyroglobulin	650.0	1.33	1.83
Macroglobulin	1000		1.86

$^aR_{Hb} = d_P/d_{Hb}$, where d_P and d_{Hb} are the distances traversed by the test protein and by hemoglobin, respectively.
A, plates of Sephadex G-100, developed in 0.5 mol L^{-1} NaCl solution; B, plates of Sephadex G-200, developed in 0.5 mol L^{-1} NaCl solution.

Table 7 Conditions for fingerprinting tryptic peptides on silica gel G thin-layer plates (20 cm × 20 cm × 0.1–0.25 mm)

First dimension: electrophoresis
pH 3.5, pyridine/acetic acid/water (2 : 20 : 978, v/v) 1000 V, 45 min
pH 6.5, pyridine/acetic acid/water (100 : 3 : 897, v/v) 1000 V, 40 min
pH 4.7, *n*-butanol/pyridine/acetic acid/water (2 : 1 : 1 : 18, v/v)

Second dimension: chromatography at 25°C
Chloroform/methanol/ammonium hydroxide (2 : 2 : 1, v/v)
n-Propanol/ammonium hydroxide (7 : 3, v/v)
n-Butanol/pyridine/acetic acid/water (97 : 75 : 15 : 60, v/v), pH 5.3

Determination of Relative Molecular Mass of Proteins

Since there is a close correlation between the logarithm of the relative molecular mass of a protein and its chromatographic behaviour (the distance covered in a constant time on a given layer), TLC of proteins and polypeptide chains on cross-linked dextran gel provides a method that allows rapid estimation of relative molecular masses of polypeptides. Relative molecular masses of several polypeptides estimated via TLC on Sephadex G-75 and G-100 plates developed with 6 mol L^{-1} guanidine hydrochloride are shown in **Table 10.** Sephadex G-75 is used for the chromatography of polypeptide chains with relative

Table 8 hR_F values of polypeptides on home made layers of silanized silica gel and on RP-2 plates

Compound	*Silanized silica gel*		*RP-2*	
	A	*B*	*B*	*C*
Angiotensin III inhibitor	47	76	75	81
Angiotensin III	16	55	53	71
Angiotensin II	37	75	73	79
Angiotensin I	22	63	59	75
Melittin	00	00	00	52
Glucagon	00	33	26	72
Insulin B chain	00	36	31	72
Actinomycin C_1	00	03	02	25
Actinomycin V	00	04	03	22
Actinomycin I	00	08	05	32

A, 1 mol L^{-1} acetic acid in 30% methanol; B, 1 mol L^{-1} acetic acid + 3% potassium chloride in 50% methanol; C, 3% potassium chloride in water/methanol/tetrahydrofuran (4 : 3 : 3). Migration distance was 11 cm for home-made layers and 6 cm for RP-2 plates. Actinomycins were yellow; other compounds were located by 1% ninhydrin in pyridine/acetic acid (5 : 1). Actinomycins I and V differ from C_1 by having one of the two prolines replaced by 4-hydroxy- and 4-ketoproline, respectively.

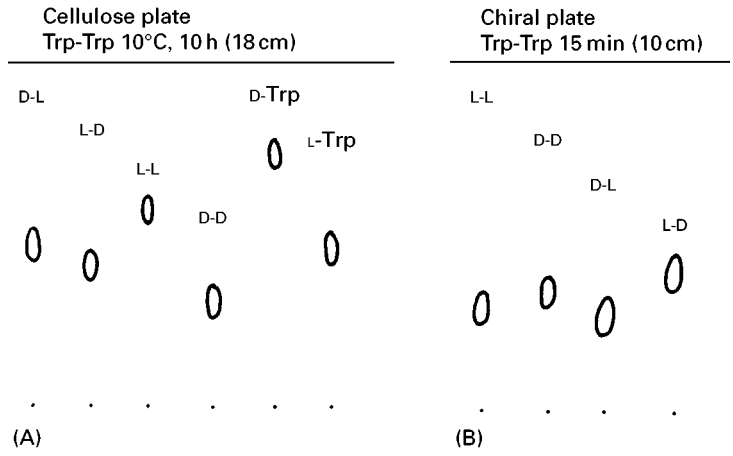

Figure 2 Chromatograms of Trp-Trp isomers. (A) Microcrystalline cellulose plate; eluent, pyridine/water (2 : 1). (B) Chiral plate; eluent, methanol/water/acetonitrile (50 : 50 : 200).

Table 9 TLC conditions for the separation of peptides on impregnated and RP silica gel plates

1. RP-2, RP-8, RP-18, plates impregnated with 4% HDBS
   (a) 1 mol L^{-1} acetic acid in methanol/water (1 : 1)
   (b) 1 mol L^{-1} acetic + 0.2 mol L^{-1} HCl in methanol/water (1 : 1); at high methanol percentage, i.e. methanol/water (4 : 1), the R_F values increased and more polar peptides gave elongated spots. The RP plates cannot be used with aqueous organic eluents containing more than 30% water.
2. Untreated thin layers of silanized silica gel or impregnated with different detergents
   (a) Water/methanol/acetic acid (64.3 + 30 + 5.7), for untreated plates
   (b) 0.1 mol L^{-1} or 0.05 mol L^{-1} HCl + 1 mol L^{-1} acetic acid in 30% methanol (pH 1.25 or 1.55); 0.1 mol L^{-1} NaCl + 1 mol L^{-1} acetic acid in 30% methanol (pH 2.75 or 3.30); 0.1 mol L^{-1} sodium acetate + 0.1 mol L^{-1} acetic acid in 30% methanol (pH 5.10); 1 mol L^{-1} sodium acetate in 30% methanol (pH 8.15), for plates impregnated with 4% HDBS.
   (c) Water/acetic acid (7 : 3 or 1 : 1), for plates impregnated with 4% HDBS.
   (d) 0.1 mol L^{-1} acetic acid + 0.1 mol L^{-1} sodium acetate in 30% methanol, 1 mol L^{-1} acetic acid in 30% methanol; 1 mol L^{-1} sodium acetate in 30% methanol, for plates impregnated with 4% N-DPC. Alkaline elements, which cannot be used in RP column chromatography, can be used here. Separation on layers impregnated with N-DPC is better with an eluent of pH 5.10 than with an eluent of pH 2.75.
3. Layers of ammonium tungstophosphate + $CaSO_4$. (1/2)H_2O in the ratio 4 : 2
   (a) Aqueous solutions of ammonium nitrate (1 mol L^{-1}, 2 mol L^{-1})
   These plates provided compact spots and good resolutions. Mainly di-, tri-, tetra- and homopeptides were resolved by the above methods. The peptides were detected by spraying the wet layers with a solution of 1% ninhydrin in pyridine/glacial acetic acid (5 : 1) and then heating the layers at 100° for 5 min.

HDBS, Dodecylbenzenesulfonic acid; *N*-DPC, *N*-dodecylpyridinium chloride.

Table 10 Relative molecular masses of proteins on Sephadex plates

Protein	M_r	*No. of PC*	M_r *of PC*	M_r *results (SD)*
Lysozyme	14 500	1	14 500	17 500(520)
Lactate dehydrogenase	12 600	4	31 500	31 000(500)
Glyceraldehyde phosphate dehydrogenase	140 000	4	35 500	35 500(1200)
Alkaline phosphatase	41 000	1	41 000	41 500(800)
Phosphorylase b	188 000	2	94 000	98 000(500)
β-Galactosidase	135 000	1	135 000	132 000(400)

SD, standard deviation; PC, polypeptide chain.
Results are mean of 15 runs.

molecular masses less than 100 000, while Sephadex G-100 is used for higher molecular mass proteins. The solutions of proteins are prepared in guanidine hydrochloride ($10\ mg\ mL^{-1}$), and cytochrome-*c* ($10\ mg\ mL^{-1}$) is used as an internal standard. Descending chromatography is carried out at room temperature under an inclination angle of 25° to the horizontal. After 3 h of development the quotient R_s protein/R_s cytochrome *c* is calculated for each protein–cytochrome *c* combination. A standard calibration line is obtained by plotting the log relative molecular masses of standards against protein–cytochrome quotient; the relative molecular masses of the unknown proteins are calculated from this plot.

See also **II/Affinity Separation:** Immunoaffinity Chromatography. **Chromatography:** Size Exclusion Chromatography of Polymers. **Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Ion Pair Thin-Layer (Planar) Chromatography; Layers; Spray Reagents. **III/Amino Acids and Derivatives:** Chiral Separations. **Impregnation Techniques: Thin-Layer (Planar) Chromatography. Appendix: 1/Essential Guides for Isolation/Purification of Enzymes and Proteins.**

Further Reading

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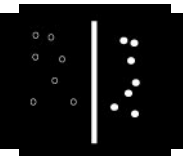
PERVAPORATION: MEMBRANE SEPARATIONS



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Introduction

The future of membranes in liquid–liquid separation lies in their potential to replace conventional unit operations such as distillation and cryogenic separation. Pervaporation, which has elements in common with reverse osmosis and membrane gas separation, is a liquid–liquid membrane separation process that can be employed for aqueous–organic or organic–organic separations. The most developed area of pervaporation is the separation of aqueous–organic mixtures but a vast potential lies in the area of organic–organic separations, specifically in the separation of azeotropic organic mixtures, where conventional separation processes tend to be complex and uneconomical.

The first pervaporation studies were carried out in late 1950 by Binning and coworkers at American Oil. However the process was not commercialized owing to lack of technology to prepare a membrane that would withstand the commercial application. By the 1980s, membrane technology was advanced to the extent that a commercially viable pervaporation technology could be developed. However, the only commercialized applications today are the alcohol dehydration and separation of volatile organics from aqueous solutions. A few pilot-plant studies have been carried out on the industrially more significant organic–organic separations.

The applications of pervaporation can be categorized as follows: (1) dehydration of organic solvents; (2) removal of volatile organic compounds from aqueous streams; and (3) separation of organic–organic mixtures. There is a tremendous amount of literature on the first two applications. Pervaporation has been successful in these applications because the properties of organic components are very different from water and exhibit distinct membrane permeation properties. The feed solutions are also relatively non-aggressive and do not chemically degrade the membrane. However, in the case of separation of organic–organic mixtures, it is much more difficult to select membranes that would exhibit selectivities for one component over the other. This article