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LIGAND-EXCHANGE CHROMATOGRAPHY OF ENANTIOMERIC AMINO ACIDS ON COPPER-LOADED CHIRAL BONDED SILICA GEL AND OF AMINO ACIDS ON COPPER(II)-MODIFIED SILICA GEL

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SUMMARY

A method has been developed for the synthesis of a chiral bonded phase that consists in attaching L-proline to triethoxypropylaminosilane and then bonding the final product to the silica gel microparticles. After treatment with copper(II) sulphate and elution with ammonia–water–acetonitrile mixtures, resolution of DL-tryptophan, phenylalanine and tyrosine is observed.

A novel method has been found for ligand-exchange chromatography. Pure silica gel is treated with an ammoniacal solution of copper(II) sulphate; formation of copper(II) silicate occurs, the copper(II) ions being held strongly.

A combined ligand-exchange and normal-phase partition mechanism was demonstrated and iso-capacity factor curves were determined for some amino acids. Examples of separations of 20 common amino acids and of fragments of Met-enkephalin are given, using isocratic elution and monitoring the column effluent at 210 nm with a UV detector.

INTRODUCTION

Ligand-exchange chromatography evolved in 1961[1] from the fundamental work of Walton and Stokes[2], published in 1954.

Ligand-exchange chromatographic techniques involve the formation of labile complexes between solutes and a complex-forming ion. This complex-forming ion is usually fixed in a stationary phase by ionic, covalent or coordination bonds. Extensive reviews of this subject have been published[3–5]. Classical ligand-exchange packings are ion-exchange or chelating resins (*e.g.*, iminodiacetate). Good results have been obtained by using pure silica gel with a metal deposited on it[6,7], ligands chemically bonded to silica and then loaded with copper(II)[8,9], or metal ions coordinated with a hydrophobic chelating agent in the mobile phase in conjunction

with a reversed-phase packing[10,11]. An application of ligand-exchange chromatography is the resolution of optical isomers[12] on a chiral bonded phase loaded with a transition metal[13–15] or with chiral chelate metal additives in the mobile phase[16–18].

The aim of this work was to develop ligand-exchange chromatography on chiral bonded silica and on pure silica gel, their surfaces being modified by ammoniacal solutions of a transition metal salt[19]. The experiments combined the high selectivity of ligand-exchange chromatography with the high efficiency of small particles. The silica-based material is more sensitive to hydroxyl ions than to ammonia molecules and has good stability in organic-rich media, even with high ammonia concentrations.

Iso-capacity factor curves (the set of points representing the percentage of water and the ammonia concentration in the mobile phase that give the same capacity factor, k', for a solute) have been determined for amino acids by use of a three-solvent system that permitted the percentage of water and the ammonia concentration to be varied. Combined normal-phase partition and ligand-exchange chromatography is demonstrated.

EXPERIMENTAL

Chromatographic apparatus

Two chromatographic systems were used for all of the experiments. The first consisted of three syringe pumps, Models 8510 and 8520 (two of them with mixing chambers) (Varian, Palo Alto, Calif., U.S.A.), called A, B and C. The second was a Spectra Physics Model 8000 with three solvent flasks, also called A, B and C (Spectra Physics, Santa Clara, Calif., U.S.A.).

Stop-flow injection was used with the three syringe pump system. The detector was a Varichrom (Varian) with a cell volume of 8 μl. The wavelength was usually 210 nm. The chromatographic columns were not thermostated.

Mobile phases

Solutions A, B and C were made up with doubly distilled water, acetonitrile (Spectrosol; SDS, Peypin, France) and ammonia (R. P. Normapur; Prolabo, Paris, France), respectively. The carefully measured volumes of acetonitrile and water or ammonia solution were mixed and degassed before the pumps or flasks A, B and C were filled. Because of a volume contraction on mixing water and acetonitrile, the calculated ammonia concentrations did not always correspond to the actual ammonia concentrations in the mobile phase. When necessary, the real value was checked by titrating the effluent with hydrochloric acid 0.1 N (Titrisol; Merck, Darmstadt, G.F.R.). In the ranges studied (20–70% water, 0.1–1.5 M ammonia), the relative error never exceeded 2%. Three series of experiments were carried out, as shown in Fig. 1, with the solutions specified in Table I. The shaded triangles in Fig. 1 are the accessible mobile phases in each experiment obtained by mixing A, B and C with known proportions using the three-pump system or the Spectra Physics chromatograph. The percentage of water and ammonia concentration at an experimental point were easily determined by using these shaded triangles. With a two-solvent system, points corresponding to an experiment would have fallen only on a straight line on a two-dimensional graph.

TABLE I

COMPOSITIONS OF SOLUTIONS A, B AND C

Experiment	*Solution*		
	A	*B*	*C*
First	CH_3CN	H_2O	50% H_2O 50% CH_3CN $[NH_3] = 0.5\ M$
Second	50% H_2O 50% CH_3CN	17% H_2O 83% CH_3CN $[NH_3] = 1.5\ M$	70% H_2O 30% CH_3CN $[NH_3] = 1.5\ M$
Third	25% H_2O 75% CH_3CN	17% H_2O 83% CH_3CN $[NH_3] = 1.5\ M$	70% H_2O 30% CH_3CN $[NH_3] = 1.5\ M$

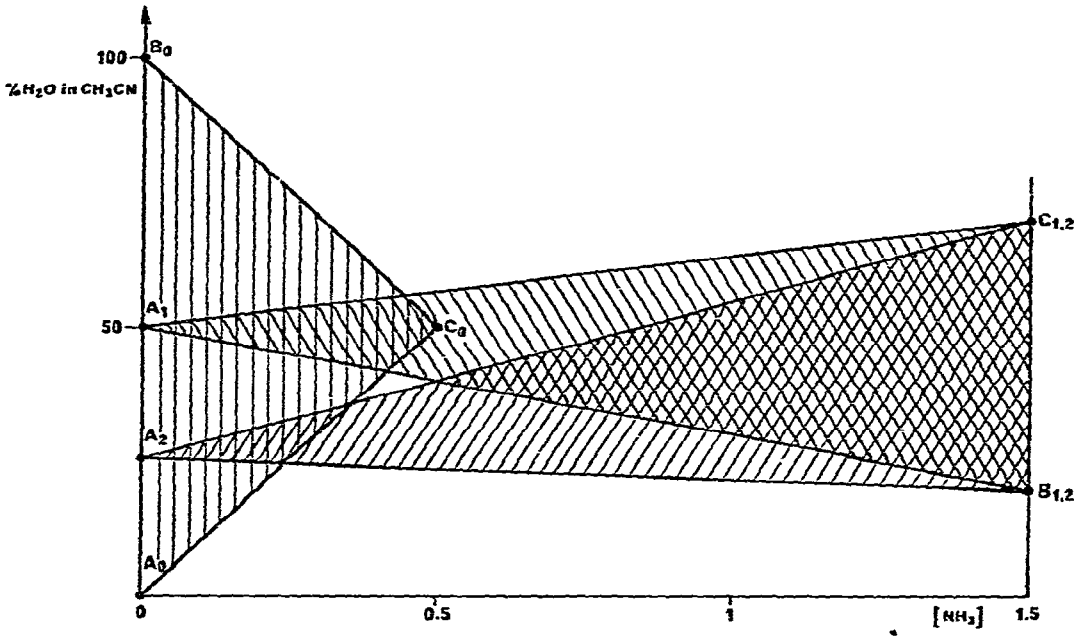


Fig. 1. Accessible mobile phases obtained by mixing solutions A, B and C.

Chemicals and packings

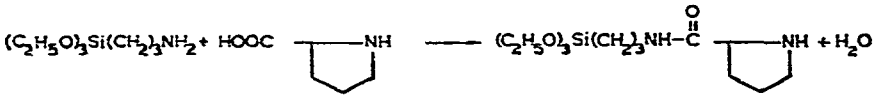
Experiments were performed using Partisil 5 silica gel, mean particle size 7 μm (Whatman, Clifton, N.J., U.S.A.). One experiment was performed using Spherosil Normatom XOA 600, mean particle size 5 μm (Prolabo).

All chemicals were obtained from Merck and Prolabo.

Chiral bonded phase columns

Our approach consisted in synthesizing a chiral silane in homogeneous liquid media before bonding it to the silica gel matrix.

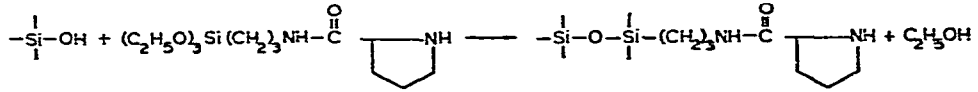
Synthesis of the chiral silane. The reaction is as follows:



A 7.5-g (0.065 mole) amount of L-proline was added to 17.5 ml (0.075 mole) of 3-triethoxysilyl propylamine in 175 ml of pyridine. The mixture was stirred for 15 h at room temperature, refluxed under anhydrous conditions for 5 h, then kept at 0° for 15 h. The unreacted L-proline (about 2.5 g) was separated by filtration and the solution was evaporated (70°, 17 mmHg). Additional L-proline was removed by recrystallization from dry benzene. Subsequently the solution was vacuum dried at 60° and 15 g of N-[3-(triethoxysilylpropyl)]-L-prolinamide were obtained. Elemental analysis gave C = 49.8% (calc. 52.8%), H = 8.9% (calc. 9.4%), N = 8.8% (calc. 8.8%).

The optical rotation of the chiral silane was $[\alpha]_{579}^{23} = -27°$ to $-29°$ (for $C = 1.5\%$, pyridine) and the structure was confirmed by ^{13}C Fourier-transform nuclear magnetic resonance spectroscopy. The yield of this reaction was about 72%.

Preparation of the L-proline bonded phase. The schematic reaction is as follows:



A 5-g amount of Partsil 5 was suspended in 70 ml of dry toluene, and adsorbed water removed by azeotropic distillation with toluene. When the distillation temperature reached 109° a solution of 2.5 g of N-[3-(triethoxysilylpropyl)]-L-prolinamide in 15 ml of toluene was added. The mixture was then refluxed under anhydrous conditions for 8 h. The bonded silica was washed with dry toluene, ethanol, water, ethanol, toluene and finally diethyl ether. Bonded silica was vaccum dried at 60° for 8 h.

Elemental analysis for the various products gave C = 9.5–11%, H = 2.1–2.7% N = 2.8–3.2%.

After filtration, about 1 g of N-[3-(triethoxysilylpropyl)]-L-prolinamide was recovered by evaporation, no racemization was observed.

Columns. The columns (25 cm × 4.8 mm I.D.; stainless steel) were packed with L-proline bonded phase in the conventional manner by preparing a slurry in carbon tetrachloride and forcing it into the column at 400 atm using ethanol and a Haskel pump (DST 150). After packing, a $2 \cdot 10^{-2}$ *M* solution of copper(II) sulphate was allowed to percolate through the column. Equilibrium was reached when about 0.7 mole of copper(II) ions per mole of L-proline were fixed, indicating that both Cu^{2+}(Pro) and $Cu^{2+}(Pro)_2$ were formed in the stationary phase (checked by elemental analysis). The column was then connected to the chromatographic apparatus and equilibrated with a water–acetonitrile (47:53)–0.17 *M* ammonia mobile phase. During this equilibration, proline and copper(II) sulphate were partly eluted, showing that not all the chiral moieties were equally strongly linked, perhaps because of polymerization during the bonding procedure. When equilibrium was

reached, 1 mole of copper(II) ions was fixed per mole of L-proline and the total absence of sulphate anions was revealed by elemental analysis. The structure of the chiral site shown in Fig. 2 is therefore probable.

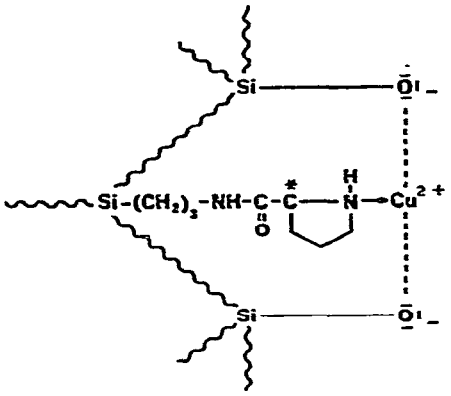


Fig. 2. Possible configuration of the chiral site after treatment with ammonia–water–acetonitrile mobile phase.

Copper(II)-modified silica gel columns

Stainless-steel columns of variable length and 4.8 mm I.D. were packed by the slurry technique with Partisil 5. A $1 \cdot 10^{-2}$ or $2 \cdot 10^{-2}$ *M* copper(II) sulphate–1 *M* ammonia solution was then allowed to percolate through the columns. Equilibrium was reached when about 0.75 mmole of copper(II) per gram of silica gel was fixed (cadmium and nickel columns were prepared in the same way). After washing, the analysis of the modified silica gel revealed no sulphate anions, which indicates that the formation of copper(II) silicate did occur. Copper(II) ions were strongly fixed in the silica matrix as the copper(II) concentration in the eluates never exceeded 0.4 ppm even with strong eluents (checked by atomic-absorption spectrometry.)

All columns modified by the same procedure gave reproducible results. When they were eluted with a water-rich mobile phase, they were not stable for long periods and needed to be replaced; however, this is not a major drawback considering the low cost of the packing. When they were eluted with water-poor (<50%) mobile phases and the ammonia concentration was greater than 1 *M*, the silica gel seemed to be stable, but copper(II) ions were very slowly washed out (*ca.* 0.4 ppm in the eluates). In the other instances, the columns were stable from several weeks to several months.

RESULTS AND DISCUSSION

Resolution of amino acids on the chiral bonded phase (L-proline)

Of the eight amino acids tested only three were resolved with the L-proline bonded phase: tryptophan, phenylalanine and tyrosine. Typical chromatograms are shown in Figs. 3 and 4; the D-isomers were always eluted first. The L-proline bonded phase shows a decreasing selectivity in the order DL-tryptophan > DL-phenylalanine > DL-tyrosine. Attempts to resolve DL-β-(3,4-dihydroxyphenyl)alanine (DOPA)

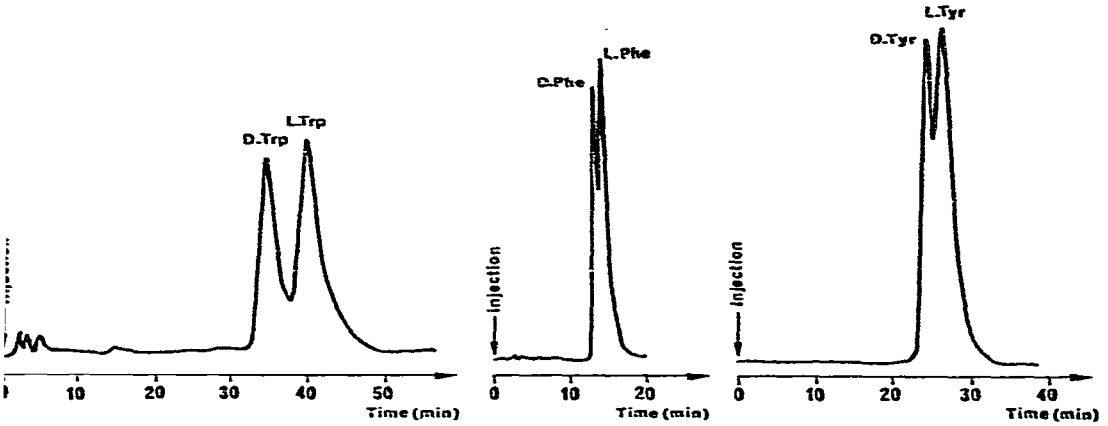


Fig. 3. Resolution of DL-tryptophan, -phenylalanine and -tyrosine. Column: 25 × 0.48 cm I.D. Packing: Cu(II)-modified L-prolylpropionamide bonded Partisil 5. Mobile phase: water–acetonitrile (47:53)–0.15 *M* ammonia. Flow-rate: 1 ml/min. ΔP: 50 atm. Detection: UV at 210 nm.

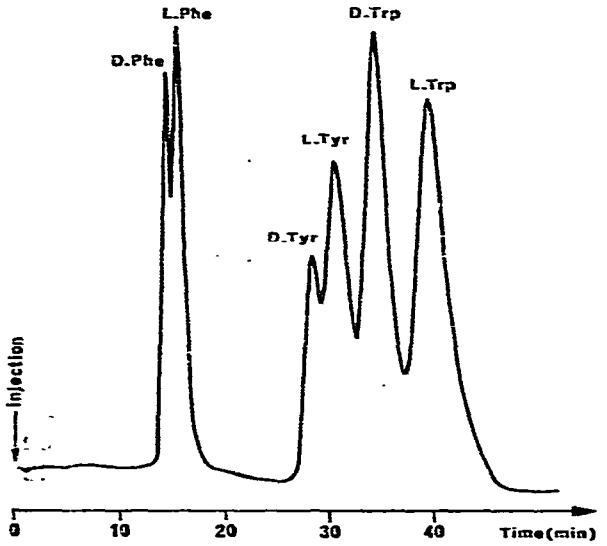


Fig. 4. Simultaneous resolution of DL-phenylalanine, –tyrosine and –tryptophan. Conditions as in Fig. 3, except mobile phase is water–acetonitrile (48:52)–0.125 *M* ammonia.

failed. Both the aromatic and hydrophobic character of the solutes seem to facilitate chiral recognition by the support.

An experiment was performed with DL-tryptophan to demonstrate the effect of the mobile phase composition on the selectivity (Fig. 5). Six mobile phases were tested, chosen such as to keep constant the capacity factor of the D-isomer by simultaneous variation of the percentage of water and the ammonia concentration. Variations of 30% in the water content and 0.3 *M* in the ammonia concentration, produced no improvement in resolution, despite the increase in efficiency with the

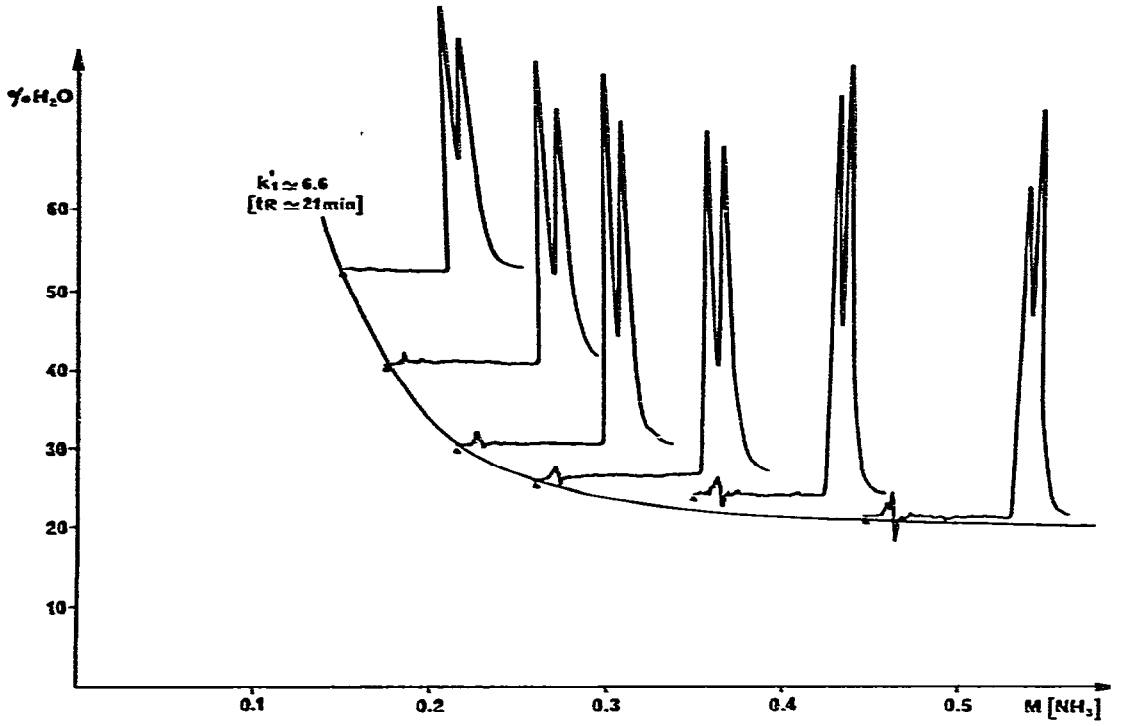


Fig. 5. Influence of mobile phase composition on the resolution of DL-tryptophan. Conditions as in Fig. 3, except for mobile phase composition: the injection points of each run are located at the mobile phase composition. The iso-capacity factor curve $k_1 \approx 6.6$ is shown.

ammonia concentration (*i.e.*, the selectivity decreases concomitantly). It seems that the resolution of the optical isomers does not depend significantly on the roles of normal-phase partition and the ligand-exchange mechanism.

These preliminary experiments prove that the silica-based material can be used successfully for the resolution of optical isomers by ligand-exchange chromatography. As the carboxylic group in L-proline is not available for the attachment of a copper(II) ion, ionic bonding takes place between silicate sites in the neighbourhood of the L-proline site and the copper(II) ion.

Further experiments on the synthesis of chemically bonded phases, particularly with the use of other silanes permitting the L-proline carboxylic group to be kept free*, are in progress.

Amino acid resolution on copper(II)-modified silica gel

Preliminary experiments were performed to determine the roles of the ammonia concentration and percentage of water on the capacity factors as illustrated by eight amino acids; iso-capacity factor curves were plotted on a two-dimensional graph of percentage of water *versus* ammonia concentration (Fig. 6). Amino acids

* A note on this subject appeared[20] during the preparation of this manuscript.

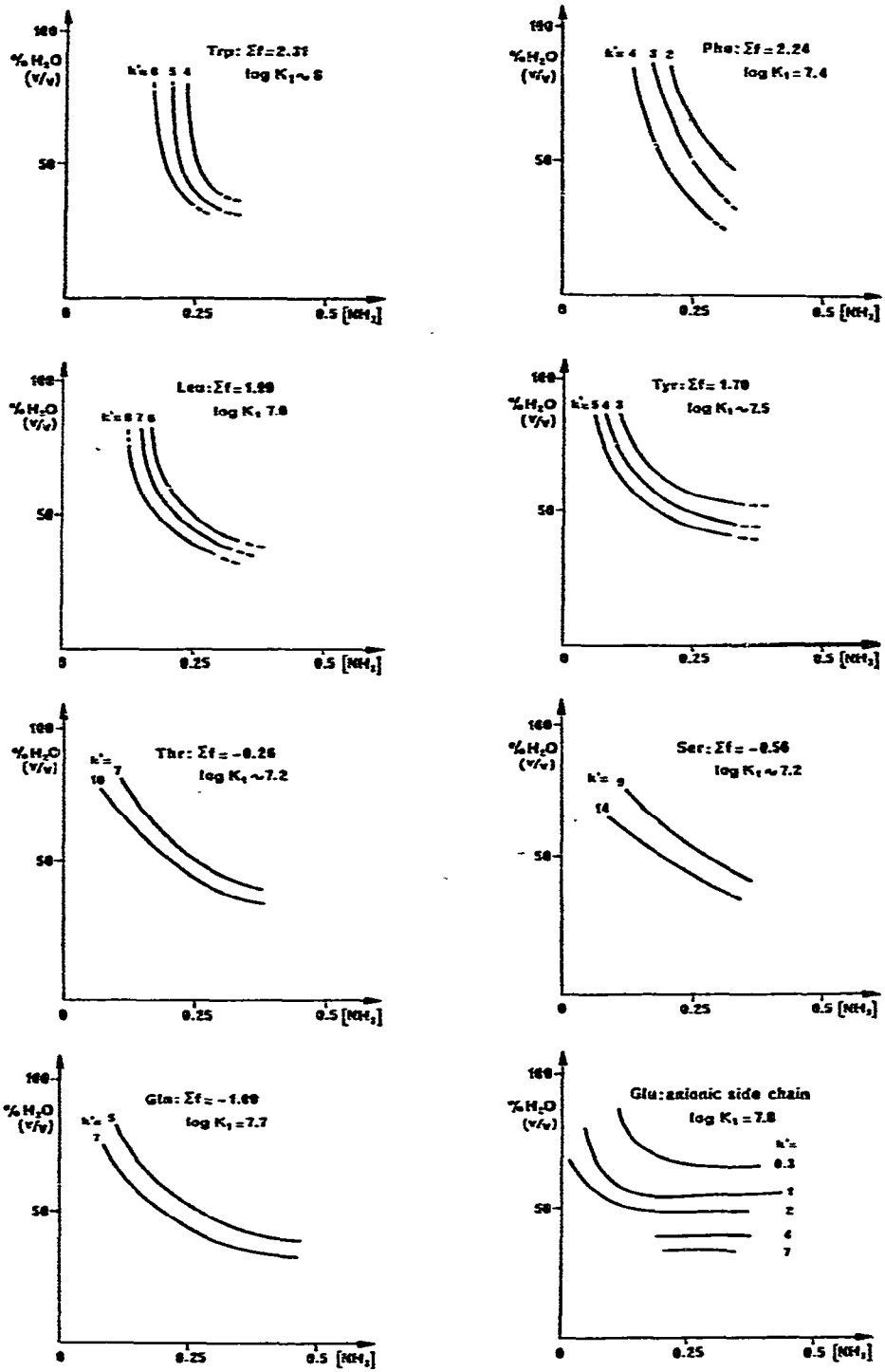
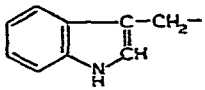
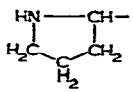
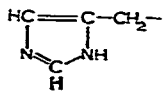


Fig. 6. Iso-capacity factor curves for eight amino-acids. Column: 15 × 0.48 cm I.D. Packing: Cu(II)-modified Partisil 5. Mobile phase: ammonia–water–acetonitrile mixtures.

have been classified according to the relative lipophilicities of their side-chains according to Rekker[21] (Table II). An exception is glutamic acid, because its side-chain is anionic in the mobile phases studied and is then the most hydrophilic (with aspartic acid side-chain) of all the amino acid side-chains.

TABLE II

RELATIVE LIPOPHILICITIES OF AMINO ACID SIDE-CHAINS

Amino acid	*Abbreviation*	*Side-chain*	*Σf*
Tryptophan	Trp		2.31
Phenylalanine	Phe	C_6H_5–CH_2–	2.24
Leucine	Leu	$(CH_3)_2CH$–CH_2–	1.99
Isoleucine	Ile	CH_3–CH_2–$CH(CH_3)$–	1.99
Tyrosine	Tyr	4-OH–C_6H_4–CH_2–	1.70
Valine	Val	$(CH_3)_2CH$–	1.46
Cystine	Cyst	1/2(–CH_2–S–S–CH_2–)	1.11
Methionine	Met	CH_3–S–$(CH_2)_2$–	1.08
Proline	Pro		1.01
Cysteine	Cys	HS–CH_2–	0.93
Alanine	Ala	CH_3–	0.53
Lysine	Lys	NH_2–$(CH_2)_4$–	0.52
Glycine	Gly	H–	(0.00)
Aspartic acid	Asp	HOOC–CH_2–	−0.02
Glutamic acid	Glu	HOOC–$(CH_2)_2$–	−0.07
Histidine	His		−0.23
Threonine	Thr	CH_3–$CH(OH)$–	−0.26
Serine	Ser	HO–CH_2–	−0.56
Asparagine	Asn	NH_2–CO–CH_2–	−1.05
Glutamine	Gln	NH_2–CO–$(CH_2)_2$–	−1.09
Arginine	Arg	NH_2–$(CHNH)$–NH–$(CH_2)_3$–	?

The iso-capacity factor curves are more vertical for lipophilic than for hydrophilic amino acids. It is clear that variations in the percentage of water lead to greater variations in the capacity factors of hydrophilic than of lipophilic amino acids, and variations in ammonia concentration to greater variations in the capacity factors of lipophilic than of hydrophilic amino acids. Experiments carried out with proline, hydroxyproline and basic amino acids gave similar results and led to the same conclusions.

Examples of the variation of capacity factors with mobile phase composition are given in Fig. 7. If the ammonia concentration is very low the ligand-exchange mechanism governs the retention time and its variations; at very high ammonia concentrations the normal-phase partition mechanism partly governs the retention time

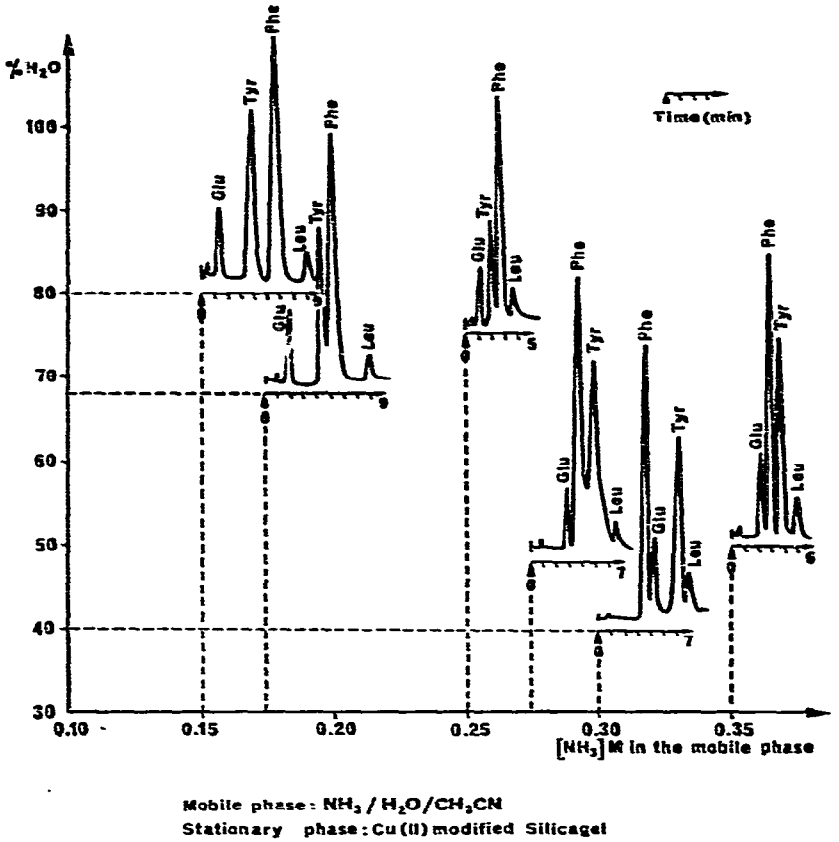


Fig. 7. Example of variation of k' with mobile phase composition. Column and packing as in Fig. 6. Mobile phase: the injection points of each run are located at the mobile phase composition. Flow-rate: 1.7 ml/min. $30 < \Delta P < 80$ atm (as a function of the viscosity of the mixture).

and governs its variations. In the range studied (ammonia concentration 0.15–1.5 M and 25–70% of water in acetonitrile), both normal-phase partition and ligand-exchange mechanisms govern the variations of the capacity factors of the amino acids. A first, but simple approximation shows that iso-capacity factor curves are hyperbolic[22]; the capacity factor (k') curve has the value of its horizontal asymptote proportional to $10^{-\Sigma f}/k'$ and of its vertical asymptote proportional to K/k', Σf being the relative lipophilicity value according to Rekker[21] (except for glutamic and aspartic acids) and K being the constant of the equilibrium

$$\overline{\text{Cu}(\text{NH}_3)_i^{2+}} + \text{A}^- \rightleftharpoons \overline{\text{Cu}(\text{NH}_3)_{i-1}\text{A}^+} + \text{NH}_3$$

where A^- is the anionic form of an amino acid and the bar indicates the stationary phase.

An important conclusion is that when two solutes are eluted with the same retention time for a given mobile phase, they can be easily separated not only by

decreasing the eluent strength, but also by increasing it, with appropriate selection of the percentage of water and ammonia concentration. Both a better resolution and shorter analysis time are thus obtained (see Fig. 7). Also, different variations of the percentage of water and ammonia concentration permit different elution orders to be achieved.

Kinetics

Diffusion of mobile ligands and their exchange in the coordination sphere of the sorbed metal govern the kinetics of ligand-exchange chromatography[5]. The rate-determining step for the commonly used granular resin material is the particle diffusion of ions and molecules in the polymeric phase, often giving broad and asymmetric peaks. This drawback is overcome by the use of small silica particles. Fig. 8 shows the influence of ammonia concentration on the capacity factor of phenylalanine and on the plate number evaluated for this peak. In the range studied, the plate number is proportional to the ammonia concentration. The rate-determining step is then the rate of desorption of the solute from the coordination site (*i.e.*, the rate of replacement of amino acids by ammonia molecules in the coordi-

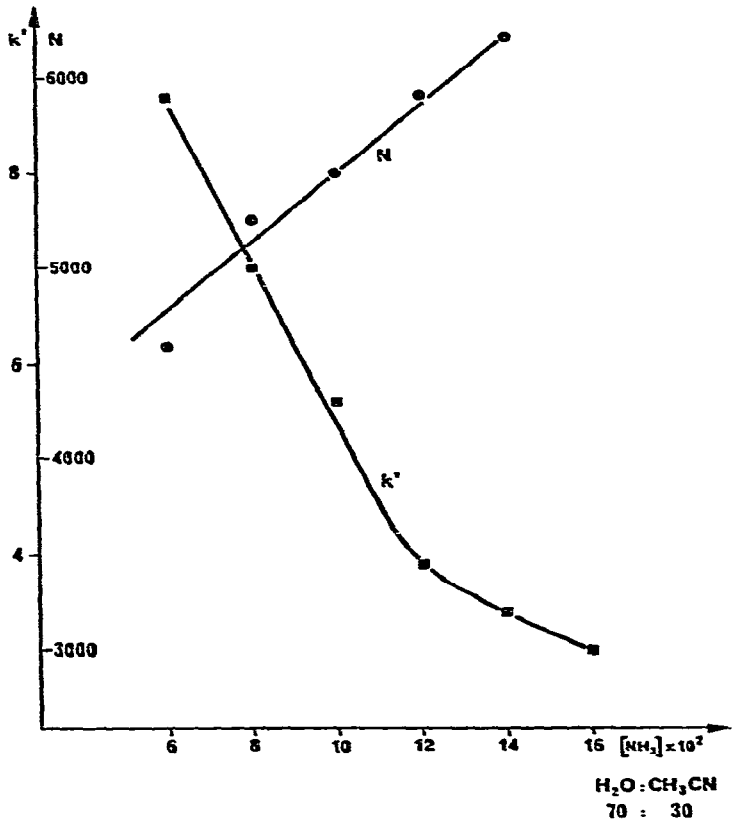


Fig. 8. Influence of ammonia concentration on the plate number (N) and the capacity factor (k'). Mobile phase: water–acetonitrile (70:30)–ammonia. In the range studied, N is roughly proportional to the ammonia concentration.

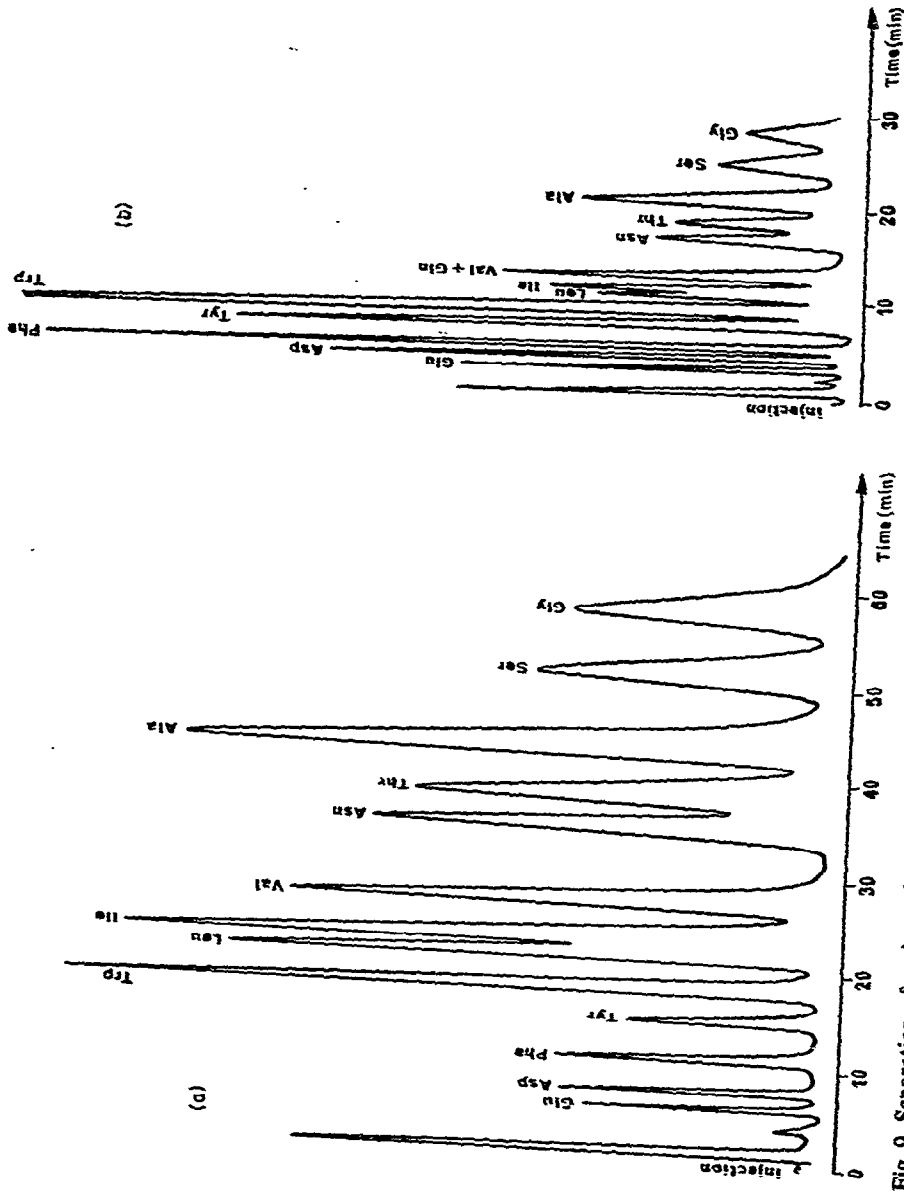


Fig. 9. Separation of amino-acids on Cu(II)-modified silica gel. Column: 15 × 0.48 cm I.D. Packing: (a) Cu(II)-modified Partisil 5; (b) Cu(II)-modified Spherosil XOA 600. Mobile phase: (a) water–acetonitrile (52:48)–0.15 *M* ammonia; (b) water–acetonitrile (50:50)–0.17 *M* ammonia. Flow-rate (a) 1 ml/min; (b) 1.7 ml/min. ΔP: 30 atm. Detection: UV at 210 nm.

nation sphere of the metal ion). It is obvious that ligand-exchange chromatography is an efficient method if the ammonia concentration is not too low (> 0.12 *M*), the retention times of solutes then being adjusted if necessary by the percentage of water in the mobile phase. The reduced plate height for an ammonia concentration of 0.15 *M* was about 10 for a reduced mobile phase velocity of 10. Therefore, about 2200 theoretical plates were obtained with Partisil 5 (mean particle diameter 7 μm) for a 15-cm column length.

Applications

Figs. 9–12 illustrate some separations of amino acids and peptides. Acidic and neutral amino acids are easily resolved (except for methionine and glutamine) with the mobile phases chosen for these examples. The capacity factors are very sensitive to both the percentage of water and ammonia concentration, so that the composition of the mobile phase must be carefully adjusted. The use of isocratic runs allows the mobile phase to be recycled.

The separation of proline, hydroxyproline and basic amino acids is shown in Fig. 10. Three examples were chosen, two on a 5-cm column packed with copper-

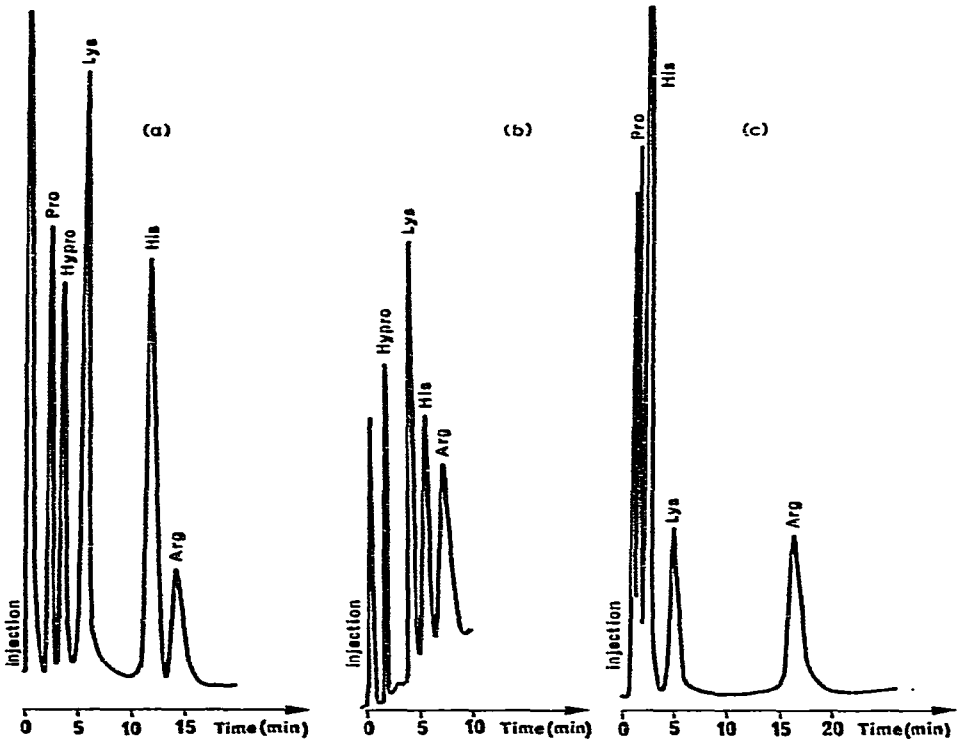


Fig. 10. Separation of proline, hydroxyproline and basic amino acids. Column: (a) and (b) 5 × 0.48 cm I.D.; (c) 15 × 0.48 cm I.D. Packing: (a) and (b) Cu(II)-modified Partisil 5; (c) Cd(II)-modified Partisil 5. Mobile phase: (a) water–acetonitrile (45:55)–1.27 *M* ammonia; (b) water–acetonitrile (70:30)–1 *M* ammonia; (c) water–acetonitrile (45:55)–1 *M* ammonia. Flow-rate: 1.7 ml/min. ΔP: (a) 20 atm; (b) 30 atm; (c) 55 atm. Detection: UV at 210 nm.

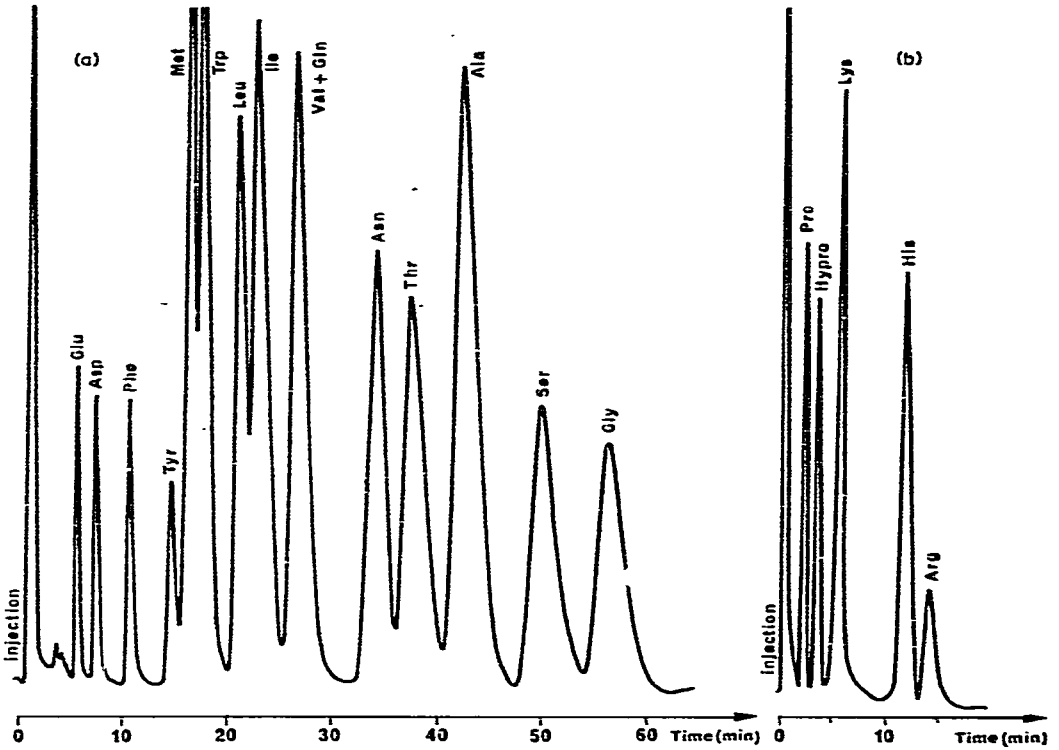


Fig. 11. Separation of 20 amino acids by the use of two isocratic runs. Column: (a) 15 × 0.48 cm I.D.; (b) 5 × 0.48 cm I.D. Packing: Cu(II)-modified Partisil 5. Mobile phase: (a) water–acetonitrile (52:48)–0.15 *M* ammonia; (b) water–acetonitrile (45:55)–1.27 *M* ammonia. Flow-rate: (a) 1 ml/min; (b) 1.7 ml/min. ΔP: (a) 30 atm; (b) 20 atm. Detection: UV at 210 nm, 0.1 a.u.f.s. Sample: Glu, Asp, Phe, Tyr, Trp (5 nmole of each); Met, Gln (0.15 μmole of each); Leu, Ile, Val, Asn, Thr, Ser, Gly (0.2 μmole of each); Ala (0.4 μmole); Pro, Hyp, Lys, His, Arg (undefined).

(II)-modified silica gel and the third on a 15-cm column packed with cadmium(II)-modified silica gel. Fig. 11 illustrates a good approach to the resolution of protein amino acids with two isocratic runs, one with a 15-cm column and the other with a 5-cm column, using copper(II)-modified Partisil for both. The mobile phases chosen are not the only ones that solve the problem. Several other orders of elution of amino acids can be obtained with variation of the percentage of water and ammonia concentration in the mobile phase.

Fig. 12 illustrates a specific biological problem: the enzymatic degradation of the Met-enkephalin, a neuroendocrine pentapeptide (Tyr–Gly–Gly–Phe–Met). Two isocratic systems were used in conjunction. The first was for the separation of the Met-enkephalin, tetra- and tripeptides and Phe-Met, the second for that of dipeptides and amino acids. These systems permitted the separation of all possible fragments of Met-enkephalin with an analysis time of about 15 min. It was then easy to observe the appearance evolution of peptidic fragments consecutive to the enzymatic degradation. Direct injection of a 5-μl sample from the enzymatic media was performed without storage of frozen samples[23,24].

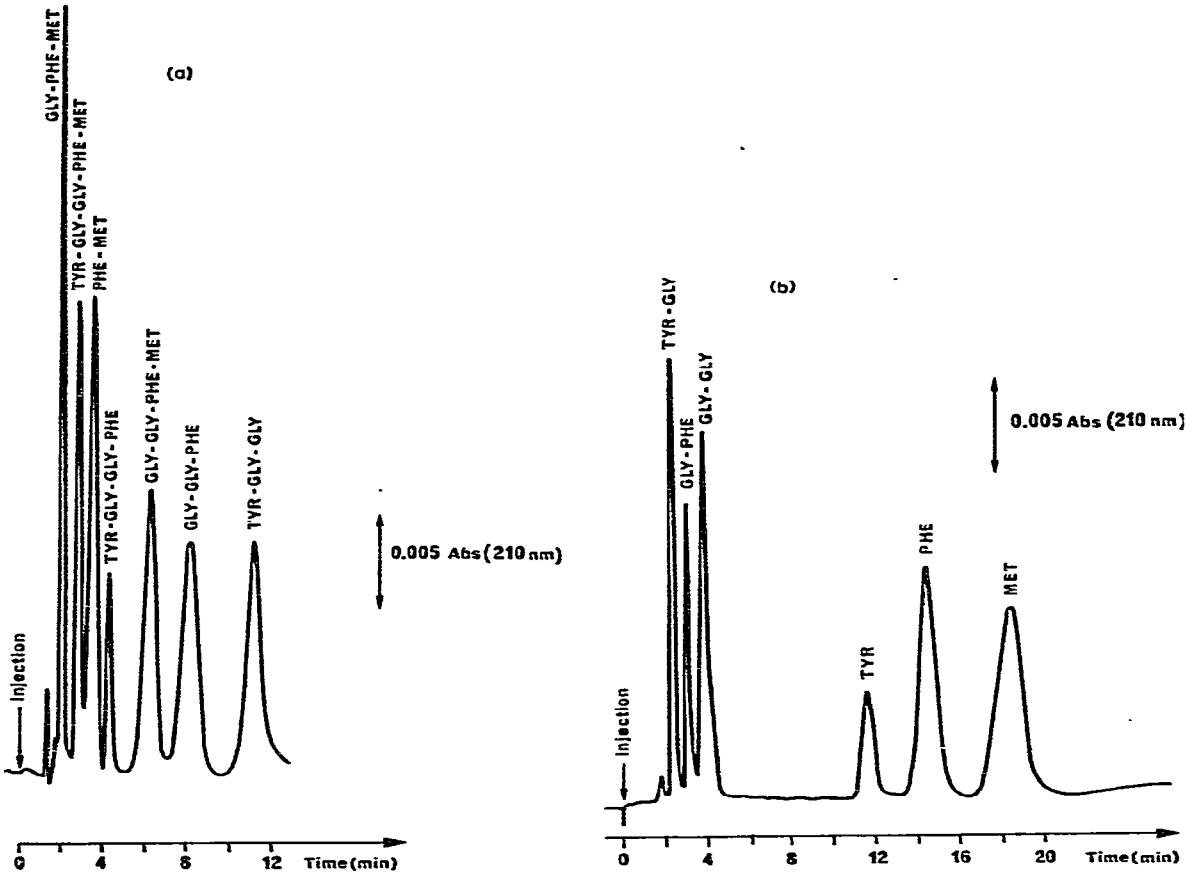


Fig. 12. Separation of the possible degradation fragments of Met-enkephalin by two isocratic runs. Column: 20 × 0.48 cm I.D. Packing: Cu(II)-modified Partisil 5. Mobile phase: (a) water–acetonitrile (13:87)–0.125 *M* ammonia; (b) water–acetonitrile (70:30)–0.2 *M* ammonia. Flow-rate: 1.5 ml/min. ΔP: (a) 50 atm; (b) 100 atm. Detection: UV at 210 nm.

CONCLUSIONS

The high selectivity of the ligand-exchange procedure combined with the high efficiency obtained with the microparticulate silica (7 μm) make ligand-exchange chromatography a powerful tool. The simplicity of the preparation of the metal-modified silica gel columns and the variety of possible applications (separations of amines, aziridines, sulphur-containing compounds, hydroxy and keto compounds, unsaturated and aromatic compounds, biogenic amines and alkaloids, carbohydrates and amino sugars and more generally all ligands) should make the method increasingly popular. The choice of the mobile phase composition (percentage of water in acetonitrile and ammonia concentration) permits the simple regulation of solute capacity factors, thus providing a rapid and effective solution for specific problems.

Metal-modified silica gel having simultaneously chiral chemically bonded sites may provide a means of resolving enantiomeric pairs. In this particular field

of ligand-exchange chromatography, the optimization of both efficiency and selectivity are fundamental for the achievement of a separation. The present results and work in progress on combined normal-phase partition and ligand-exchange chromatography on metal-modified silica gel should provide further useful information.

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