Hydrolyzed Vinyl Acetate–Divinylbenzene Copolymer Microspheres Containing L-Proline for Chiral Separation of Amino Acids

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Received: 8 December 2003 / Revised: 2 March 2004 / Accepted: 22 March 2004
Online publication: 30 June 2004

Abstract

A new packing material for ligand-exchange chromatography, L-proline-modified hydrolyzed vinyl acetate-divinylbenzene copolymer microspheres, has been prepared and evaluated. The microspheres, prepared by a one-step swelling and polymerization method, have a narrow bead size (as determined by scanning electron microscopy, SEM), a broad pore-size distribution (by nitrogen adsorption) and, especially, inhomogeneous structural composition (by differential scanning calorimetry). The support, which has an L-proline content of 0.35 mmol g⁻¹ (nitrogen analysis), provides good enantioselectivity and column efficiency. Twenty-three of the twenty-four common D,L-amino acids tested were resolved on a 250 mm × 4.6 mm i.d. column and mixtures of up to seven racemic amino acids were easily separated into their enantiomers. Two average pore diameters, volume-average (d_v) and surface-average (d_s) pore diameters, are defined. The ratio d_v/d_s is used to characterize the broadness of the pore-size distribution. It is shown that the widely used formula $d = 4V_p/S$ for cylindrical pores, where V_p is pore volume and S the specific surface area, gives the surface-average pore diameter.

Keywords

Column liquid chromatography
Chiral separations
Hydrolyzed vinyl acetate–divinylbenzene copolymer
Microspheres
Amino acids

Introduction

Among the modes of separation of liquid chromatography (LC) ligand-exchange chromatography (LEC) is an important technique for analytical and preparative separation of racemic amino acids into their constituent enantiomers. In this method complexes of transition metal

ions and the enantiomeric molecules are formed. Chiral separation is a result of differences between the free energies of the intermediate diastereomeric complexes formed.

In the early stage of the method so-called ‘macronet isoporous’ polystyrene (PS) particles containing cyclic amino acids, L-hydroxyproline, L-proline, etc.,

were developed as packing materials [1, 2]. The ‘macronet isoporous’ PS is a polymer in which pores are formed as a result of homogeneous cross-linking of linear macromolecules by rigid bridges. This polymer, with a special pore structure, has many advantages, for example enhanced swelling ability, pressure resistance, exchange capability, permeability, and favorable kinetic properties. More than twenty common amino acids have been resolved with this polymer. Rapid separation was also achieved with the fine particles. For example, a microbore column (100 mm × 1 mm i.d.) packed with angular 5–10-μm particles enabled complete resolution of a mixture of three racemic amino acids into six components under isocratic conditions in 70 min at room temperature [3]. This packing material did not find widespread application, because of difficulty of preparation and the irregular shape of the particles.

L-Amino acid-bonded silica supports are another material used for enantioselective LEC. It has been reported that 250 mm × 4.6 mm i.d. columns packed with 5–10-μm beads could separate mixtures of five to seven racemic amino acids in 60 min at 50 °C and flow rates from 2 to 5 mL min⁻¹ [4, 5].

The most impressive support is PS (degree of polymerization 14–90) chiral phase-bonded silica microspheres [6–8]. A recent result showed that a mixture of eleven racemic amino acids was resolved in 150 min on a 250 mm × 4.6 mm i.d. column with bead size 10 μm; the flow rate was 0.5 mL min⁻¹ and the tempera-

ture 75 °C [9]. Many other polymeric supports, for example polyacrylamide [10, 11], poly(hydroxyethyl methacrylate) [12], hydroxylated polyether [13], and urea–formaldehyde resin [14, 15], have been evaluated but none was as efficient as the bonded silica.

Formation of complexes of transition metal ions with enantiomeric molecules is a slow process, the efficiency of LEC is, therefore, often lower than that of the conventional HPLC. PS chiral phase bonded silica provides both high selectivity and efficiency; because the long flexible PS chains carrying the chiral selectors and bonded to the silica surface swing freely in the mobile phase, the analyte molecules can easily approach the selectors for complexing.

The aim of the work described in this paper was to develop an efficient polymer support by simulating the pore structure of PS chiral phase bonded silica. Vinyl acetate (VAc)–divinylbenzene (DVB) copolymer microspheres were prepared in which the copolymerization reactivity ratios of the two monomers were very different. In copolymerization of styrene (S) and VAc the reactivity ratios are $r_S = 19$ –60 and $r_{VAc} = 0.01$ –0.16; this means that the growing PS and poly(vinyl acetate) (PVAc) chains both prefer to react with S than VAc and the molecules obtained have an inhomogeneous monomer distribution [16]. In our work the highly crosslinked DVB-rich domains formed first in the cores of the precipitated particles whereas the low crosslinked VAc-rich domains and even PVAc chains, when DVB was exhausted, were at surface of the particles. Agglomeration of the particles formed pores. After hydrolysis of acetate groups hydrophilic vinyl alcohol (VAl)-rich low-crosslinked networks with free poly(vinyl alcohol) (PVAI) chains were obtained; these were further modified with L-proline via epichlorohydrin. In this way a support with a pore structure similar to that of PS chiral phase bonded silica was prepared; this should give good ligand-exchange properties. Compared with bonded silica, polymeric packings have two important advantages—easy preparation and high chemical stability.

We have noticed that during characterization of pore size not enough attention has been paid to the statistical basis of pore size. Because pore sizes of a porous material obtained from different statistical analyses are often very different, the surface-average pore size d_s , the

volume-average pore size d_v , and their ratio d_v/d_s were defined and the possible consequence was discussed.

Experimental

Materials

DVB (DVB 49.3%, ethylstyrene 44.0%, diethylbenzene 6.7%) was obtained from Nankai University Chemical Plant. VAc (chemically pure), and PVAI (molecular weight = 77,000, degree of hydrolysis = 88%) were obtained from Beijing Organic Chemical Plant. Other chemicals—dodecyl sulfate (SDS), benzoyl peroxide (BPO), copper acetate ($Cu(OAc)_2$), and acetic acid (HOAc) (chemically pure), and solvents acetonitrile (MeCN), etc. (chromatographic or analytically pure) were obtained from Beijing Chemical Plant. Twenty-four D,L, twenty-two L, and eight D amino acids were used for LEC. Most of these (45 out of 54) were produced in China. D-serine, D,L- and L-norleucine, D,L-phenylalanine, and L-citrulline were purchased from Sigma, L-asparagine and D,L-proline from Aldrich, D-glutamic acid from Serva, and D,L-ethionine from Fluka. All the amino acids were of biochemical standard grade.

Preparation of L-Proline-Epoxy-Hydrolyzed VAc–DVB Copolymer Microspheres

VAc–DVB Copolymer Microspheres

The microspheres were prepared by a one-step swelling and polymerization method. A 2000 mL three-necked flask was charged with a homogeneous solution of 10 g PVAI, 3 g SDS, and 1000 mL distilled water and the solution was stirred at 120 rpm in a water bath at 25 °C. PS seed suspension (10 mL seed particles, 2.5 μ m in diameter, molecular weight 5800 in 50 mL distilled water) was added to the flask and an emulsion of monomer was then added dropwise over 4 h. This emulsion was prepared from a solution of 6 g PVAI, 3 g SDS, and 1000 mL distilled water and a solution of 200 mL monomer mixture and 2.4 g BPO, by use of an ultrasonic disrupter. The monomer mixture contained 50 mL DVB and 150 mL VAc. After complete absorption of the monomer mixture by the seed particles the temperature of the water bath was kept at 75 °C for 16 h and then at 95 °C

for 3 h. The copolymer microspheres were washed with water, methanol, and toluene and dried at 60 °C.

Hydrolyzed Microspheres

Aqueous NaOH solution (1%, 500 mL) was placed in a 1000-mL three-necked flask. A suspension of 70 mL VAc–DVB copolymer microspheres in 200 mL methanol was added in 30 min at 55 °C with stirring at 60 rpm. The temperature was kept at 55 °C for 5 h, then 65 °C for 4 h, and finally at 75 °C for 3 h. The hydrolyzed microspheres obtained were washed with water and dried at 60 °C.

Epoxy Microspheres

A suspension of 70 mL hydrolyzed microspheres in 100 mL dioxane containing 1 mL concentrated sulfuric acid was placed in a 500-mL three-necked flask. Epichlorohydrin (100 mL) was added dropwise to the suspension at 50 °C with stirring at 60 rpm. The temperature was then kept at 90 °C for 24 h. The epoxy microspheres obtained were washed with hot water and dioxane then dried at 60 °C.

L-Proline Microspheres

NaOH solution (2.5%, 20 mL) and L-proline solution (50%, 10 mL) were added consecutively to a suspension of 30 mL epoxy microspheres in 60 mL dioxane, with stirring. The reaction was then kept at 55 °C for 24 h. The L-proline microspheres obtained were washed with water and dioxane and dried at 60 °C.

Characterization of the Microspheres

Scanning electron microscopy (SEM; Philips XL30), nitrogen adsorption (Micromeritics ASAP2000), and differential scanning calorimetry (DSC; Perkin-Elmer DSC7) were used to characterize the VAc–DVB copolymer microspheres. Particle size and size dispersion, pore volume, specific surface area and pore-size distributions, and glass transitions of the microspheres were determined.

Elemental analysis was performed to determine the nitrogen content of the L-proline microspheres. The average L-proline content of the microspheres was thus obtained.

Fig. 1. SEM micrograph of VAc-DVB copolymer microspheres

Fig. 2. Nitrogen adsorption of VAc-DVB copolymer microspheres

Ligand Exchange Chromatography

The clean L-proline microspheres were placed in 100 mL of 2% copper acetate for one day to form the bonded L-proline-Cu(II) complex. On the second day the green microspheres were placed in a 250 mm × 4.6 mm i.d. column at 25 MPa with water as the mobile phase. A Shimadzu 3A LC apparatus with a UV254 detector was used for LEC. D,L-Amino acids were used to examine the separating power of the new packing material. D- and L-amino acids were used to determine the order of elution of the separated enantiomers. Samples (20 μ L of 0.02–0.1% solutions) were injected on to the column by means of a Rheodyne 7725 injector. The mobile phases were 70:30 (0.1 M NH_4OH + 0.03 mM $\text{Cu}(\text{OAc})_2$ –MeCN, pH 6.22, 70:30 (0.1 M NH_4OH + 0.1 M HOAc + 0.03 mM $\text{Cu}(\text{OAc})_2$ –MeCN, pH 4.66, and 70:30 (10 mM NH_4OH + 20 mM HOAc + 0.03 mM $\text{Cu}(\text{OAc})_2$ –MeCN, pH 4.69. pH was measured with a pH meter. Flow rates were 0.2–1.2 mL min^{-1} . Column temperatures were 30 and 75 $^{\circ}\text{C}$.

Results and Discussion

Microspheres

Size and Size Dispersion

Figure 1 shows the SEM micrograph obtained from VAc-DVB copolymer

Fig. 3. DSC thermogram of VAc-DVB copolymer microspheres. The bars indicate glass transitions

microspheres; from this the average diameter d and dispersion coefficient ϵ were obtained. ϵ is defined as $\epsilon = d/\sigma$, where σ is the standard deviation of the diameter. It is apparent that the prepared microspheres have the appropriate particle size ($d = 7.7 \mu\text{m}$) and narrow particle-size distribution ($\epsilon = 0.14$) for a packing material. The one-step swelling and polymerization method can be used to prepare monodisperse crosslinked PS microspheres ($\epsilon = 0.026$) [17]. If the monomer is very soluble in water the size distribution of the microspheres is broader ($\epsilon = 0.172$)

[18]. In our work, because the solubility of VAc in water is 2.5% (w/w) at 28 $^{\circ}\text{C}$ and the solubility of S is only $3.6 \times 10^{-2}\%$ (w/w) at 45 $^{\circ}\text{C}$ [19], the size distribution obtained is reasonable.

Volume-Average and Surface-Average Pore Diameters

From nitrogen adsorption measurements two sets of data, incremental pore volume (ΔV_i) and incremental pore surface area (ΔS_i) versus pore-diameter range (Δd_i) and pore diameter (d_i) are obtained with the assumption of cylindrical pores

Table 1. Chiral separations of amino acids

Amino acid	Mobile phase 1, 30 °C			Mobile phase 2, 30 °C			Mobile phase 2, 75 °C			Mobile phase 3, 30 °C			Mobile phase 4, 30 °C		
	t_1	t_2	α	t_1	t_2	α	t_1	t_2	α	t_1	t_2	α	t_1	t_2	α
Asp	3.5			14			9			5			16	18	1.17
Glu	3.5			14			10			5			18		
Asn	10	11	1.14	18	22	1.30	13	16	1.35	8	9	1.29	13	17	1.47
Gln	10			18	20	1.15	12	13	1.13	5.5			12	12.5	1.07
Ser	10	12	1.29	18	26	1.59	13	17	1.47	6	7	1.67	13	19	1.71
Thr	11	12	1.13	22	30	1.46	15	22	1.67	8.5	10	1.38	17	22	1.40
Tyr	11	16	1.63	40	94	2.52	27	51	1.16	12	23	2.47	33	73	2.40
Met	21			32	36	1.15	30	34	1.27	10			37	41	1.12
Eth	27			50	56	1.13	42	48	1.16	12			56	61	1.10
Cys-Cys	68	74	1.09												
Ala	17	17.5	1.04	12			14			5			11		
Val	22	26	1.21	20	30	1.65	19	29	1.69	6.5	8.5	2.00	17	25	1.64
Nle	38	40	1.06	36	44	1.25	28	35	1.36	8	8.5	1.14	32	38	1.18
Ile	32	39	1.24	34	48	1.47	28	38	1.43	8	9	1.29	26	36	1.47
Leu	37			40			34	36	1.07	9			34		
Phe	40	52	1.32	84	138	1.68	54	82	1.57	21	31	1.61	65	94	1.50
β -Phe	40	52	1.32	82	144	1.80	53	84	1.64	20	32	1.77	64	96	1.54
Trp	83	130	1.59	147	310	2.14	74	140	1.95	34	67	2.12	89	220	2.55
Pro	31	44	1.46	13	20	1.82	12	16	1.53	5	6	3.00	11	16	1.77
Orn	30			10	12	1.36	10	12	1.36	5			8	10	1.57
Lys	48			10	12	1.36	9	11	1.44	5			7	8	1.40
Cit	13	14	1.10	13	15	1.24	11	13	1.31	5	5.5	2.00	12	14	1.27
His	29	63	2.31	16	24	1.70	14	19	1.53	11	15	1.62	16	23	1.61
Arg	84	93	1.11	14	18	1.42	11	13	1.31	5	5.5	2.00	9	10.5	1.33
Formic acid, t_0	3.0			4.5			4.5			4.5			4.5		

Mobile phase 1. 70:30 (0.1 M NH_4OH + 0.03 mM $\text{Cu}(\text{OAc})_2$)–MeCN; mobile phase 2. 70:30 (0.1 M NH_4OH + 0.1 M HOAc + 0.03 mM $\text{Cu}(\text{OAc})_2$)–MeCN, pH 6.22; mobile phase 3. 70:30 (0.1 M NH_4OH + 0.2 M HOAc + 0.03 mM $\text{Cu}(\text{OAc})_2$)–MeCN, pH 4.66; mobile phase 4. 70:30 (10 mM NH_4OH + 20 mM HOAc + 0.03 mM $\text{Cu}(\text{OAc})_2$)–MeCN, pH 4.69, t_1 , t_2 , and t_0 are retention times (min).

Abbreviations: Asp, asparagic acid; Glu, glutamic acid; Asn, asparagine; Gln, glutamine; Ser, serine; Thr, threonine; Tyr, tyrosine; Met, methionine; Eth, ethionine; Cys-Cys, cystine; Ala, alanine; Val, valine; Nle, norleucine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; β -Phe, β -phenylalanine; Trp, tryptophan; Pro, proline; Orn, ornithine; Lys, lysine; Cit, citrulline; His, histidine; Arg, arginine.

(Fig. 2). The pore volume ($D_v(d)$) and pore surface area ($D_s(d)$) differential distributions are therefore obtained.

$$D_v(d) = \Delta V_i / \Delta d_i$$

$$D_s(d) = \Delta S_i / \Delta d_i$$

Consequently, two average pore diameters, the volume-average (d_v) and surface-average (d_s) pore diameters, can be defined.

$$d_v = \sum d_i D_v(d) \Delta d_i / \sum D_v(d) \Delta d_i \\ = \sum d_i \Delta V_i / \sum \Delta V_i$$

$$d_s = \sum d_i D_s(d) \Delta d_i / \sum D_s(d) \Delta d_i \\ = \sum d_i \Delta S_i / \sum \Delta S_i$$

The ratio d_v/d_s characterizes the pore-size distribution. For monosized pores $d_v/d_s = 1$ whereas for broad pore-size distributions the ratios are much greater than unity.

The experiment also gives pore volume V_p and specific surface area S . With the assumption of cylindrical pores an average pore diameter is obtained as $d = 4V_p/S$; this is widely used in characterization of porous solids [20, 21]. Now it can be shown to be the surface-average pore diameter:

$$d_s = 4V_p/S = 4\sum(d_i \Delta S_i / 4) / \sum \Delta S_i \\ = \sum d_i \Delta S_i / \sum \Delta S_i$$

Figure 2 gives $d_v = 34.3$ nm, $d_s = 16.9$ nm, and $d_v/d_s = 2.03$, so the microspheres have a broad pore-size distribution. Calculating with V_p (0.478 mL g^{-1}) and S (147 $\text{m}^2 \text{g}^{-1}$), $d_s = 4V_p/S = 13.0$ nm, which is close to the value obtained from the pore surface distribution.

d_v and d_s are obtained from different statistical bases. d_v reflects the contribution of pore volume whereas d_s reflects that of pore surface area. Thus, d_v should be more appropriate for LC modes related to pore volume and d_s should be more appropriate for LC modes related to pore surface. Definitions of d_v and d_s are necessary not only for differentiating between pore-diameter values obtained by different means but also for deepening our understanding of separation mechanisms. As far as we are aware, however, such definitions have not been proposed [20–23], although similar definitions for agglomerated particles of porous solids appeared more than 30 years ago [24].

In swelling and polymerization pore structure is controlled by monomer, porogen, and reaction temperature. The percentage of pore volume (porosity) correlates well with the percentage of porogen used [25, 26]. Assuming the polymer density $\rho = 1.2 \text{ g cm}^{-3}$, the porosity of the prepared microspheres = $V_p/(V_p + \rho^{-1}) = 0.37$. This means the volume fraction of porogen in the reaction mixture is almost 0.37. In our recipe we added no extra porogen except the diethylbenzene contained in the DVB and the seed polymer, of which the volume fractions were only 0.02 and 0.05, respectively. Neither diethylbenzene nor the seed polymer are major contributors to the porosity obtained, which is possibly mainly because of the presence of water in the reaction mixture. Water is very soluble in VAc and is stabilized by the emulsifier in the oil droplets, which can be absorbed with VAc by the seed polymer at the swelling stage.

L-Proline Content

The final product is Polymer-O- $\text{CH}_2\text{-CH(OH)-CH}_2\text{-L-proline}$. Elemental anal-

ysis gives the nitrogen content as 0.48% (w/w), from which the average L-proline content of the microspheres is 0.35 mmol g⁻¹. This value is in the same range as for LEC supports with agglomerated pores. For example, for L-amino acid bonded silica, PS chiral phase bonded silica, poly(hydroxyethyl methacrylate), and urea-formaldehyde resin the values are 0.6–0.7 [5], 0.56–0.63 [6], 0.3 [12], and 0.27 mmol g⁻¹ [15], respectively. LEC supports with network pores have fixed ligand contents as high as 2.8–3.4 mmol g⁻¹ for the ‘macronet isoporous’ PS [1, 2] and 3.0 mmol g⁻¹ for polyacrylamide [10].

The inhomogeneous structure of the VAc–DVB copolymer microspheres is revealed by two glass transitions in the DSC thermogram (Fig. 3). One transition (50 °C) is indicative of VAc-rich domains, the other (105 °C) of DVB-rich domains [27]. As discussed in the Introduction, the large difference between the copolymerization reactivity ratios of the two monomers VAc and DVB is responsible for the observed inhomogeneity. Because of this inhomogeneity, the hydrophilic low-crosslinked VAl-rich networks with free PVAL chains are at the surface of the pores and the L-proline concentration at the pore surface should be higher. This is a favorable situation for LEC separation.

Ligand-Exchange Chromatography

The retention times of the amino acids at a flow rate 0.6 mL min⁻¹ are listed in Table 1. The enantioselectivity, α , is defined as $\alpha = (t_2 - t_0)/(t_1 - t_0)$, where t_1 and t_2 are the retention times of the two enantiomers ($t_2 > t_1$) and t_0 is the retention time of formic acid on the column. Representative chromatograms are shown in Figs. 4–7. Twenty-three of the twenty-four common D,L-amino acids tested were resolved; of these more than half were completely resolved. The enantioselectivity of the support is better than that of the corresponding bonded silica and polystyrene chiral phase bonded silica. With L-proline as selector the bonded silica cannot resolve alanine, leucine, cystine, and glutamine [5] and the polystyrene chiral phase bonded silica cannot resolve alanine, serine, threonine, methionine, and asparagic acid [6]. The column efficiency is slightly less than that

Fig. 4. Separation of the amino acids, in order of elution, D-Tyr, L-Tyr, D-Val, L-Val, L-Pro, D-Pro, D-Trp, and L-Trp on a 250 mm × 4.6 mm i.d. column with 70:30 (0.1 M NH₄OH + 0.03 mM Cu(OAc)₂)–MeCN as mobile phase at flow rates of 0.6 mL min⁻¹ (left) and 1.2 mL min⁻¹ (right); the column temperature was 30 °C and the UV254 detector was used

Fig. 5. Separation of the amino acids, in order of elution, D-Lys, L-Lys, D-Arg, L-Arg, D-Thr, L-Thr, D-Ile, D-Tyr, L-Ile, D-β-Phe, L-Tyr, D-Trp, L-Phe, and L-β-Trp on a 250 mm × 4.6 mm i.d. column with 70:30 (0.1 M NH₄OH + 0.1 M HOAc + 0.03 mM Cu(OAc)₂)–MeCN, pH 6.22, flow rate 0.6 mL min⁻¹, as mobile phase. Left: column temperature 30 °C, D- and L-Trp are not included. Right: column temperature 75 °C, the pairs L-Lys and D-Arg, and D-Ile and D-Tyr, co-elute as single peaks. The UV254 detector was used

of the bonded silica, as shown by the HETP of the last peaks in the chromatograms (0.16 mm compared with 0.1 mm) [5, 9]. However, for the first time the polymer column can resolve mixtures of up to seven racemic amino acids into the corresponding enantiomers.

Acidity of Mobile Phases

In this study basic and acidic mobile phases were used. Chiral separation of racemic cystine has not been reported. This amino acid only dissolves in the basic mobile phase and is partly resolved with our support. It is apparent that the analytes have the longest retention times if the neutral mobile phase is used. Figure 5 shows the chromatogram, obtained by use of a neutral mobile phase, in which a mixture of seven racemic amino acids is

Fig. 6. Separation of the aromatic and cyclic amino acids, in order of elution, L-Pro, D-Pro, D-Tyr (shoulder), D-His, L-His, D-Phe (shoulder), L-Tyr, L-Phe, D-Trp, and L-Trp on a 250 mm × 4.6 mm i.d. column with 70:30 (0.1 M NH₄OH + 0.2 M HOAc + 0.03 mM Cu(OAc)₂)–MeCN, pH 4.66, flow rate 0.6 mL min⁻¹, as mobile phase; the column temperature was 30 °C and the UV254 detector was used

Fig. 7. Separation of the basic amino acids D-Lys, L-Lys and D-Orn, D-Arg (shoulder), L-Orn and L-Arg, D-Cit, L-Cit, D-His, and L-His on a 250 mm $\times$ 4.6 mm i.d. column with 70:30 (10 mM NH_4OH + 20 mM HOAc + 0.03 mM $\text{Cu}(\text{OAc})_2$ –MeCN, pH 4.69, as mobile phase at flow rates of 0.6 mL min^{-1} (left) and 0.2 mL min^{-1} (right); the column temperature was 30 $^\circ\text{C}$ and the UV254 detector was used

resolved. If the acidic mobile phase is used repulsive interactions occur between the protonated analytes and the central Cu(II) complexed with the chiral selectors; this results in reduced retention times. Long retention times are usually required for separation of aromatic and cyclic amino acids in LEC. Figure 6 shows these amino acids can be separated much more quickly, without loss of resolution, by use of an acidic mobile phase.

Ionic Strength of Mobile Phases

The effect of mobile phase ionic strength was studied by comparing the mobile phases 70:30 (0.1 M NH_4OH + 0.2 M HOAc + 0.03 mM $\text{Cu}(\text{OAc})_2$ –MeCN, pH 4.66, and 70:30 (10 mM NH_4OH + 20 mM HOAc + 0.03 mM $\text{Cu}(\text{OAc})_2$ –MeCN, pH 4.69. In the same way as for buffer solutions the ionic strength decreases when the solution is diluted, but the pH remains almost constant. We can see that, in general, the low ionic strength acidic mobile phase is more suitable for LEC separations, twenty D,L-amino acids, including five basic amino acids (Fig. 7), are resolved with this mobile phase.

In LEC the decisive interaction is the complexing interaction of the Cu(II)–fixed ligand–mobile ligand, which is responsible for the enantioselectivity, and the retention of the solutes is determined by a combination of contributions from several different types of interaction with the stationary phase [6]. Our data are in agreement with this general statement. Secondary interactions can, however,

interfere with the decisive interaction. Our study shows that the complexes are better formed if the low ionic strength mobile phase is used, therefore more racemic amino acids can be resolved.

Column Temperature

The column temperature has a substantial effect on the chiral separation. As column temperature increases retention times decrease and column efficiency increases [8, 15]. This is because the Cu(II) complexes with the amino acids are less stable at high temperature and the ligand exchange rate increases. Comparison of the two chromatograms in Fig. 5 clearly reveals the temperature effect. The *HETP* of the last peaks were estimated as 0.30 mm at 30 $^\circ\text{C}$ and 0.16 mm at 75 $^\circ\text{C}$.

Flow Rate

Figure 4 shows the separations achieved at two flow rates. Although the retention time is reduced when flow rate is increased from 0.6 to 1.2 mL min^{-1} , resolution remains almost unchanged. Figure 7 shows that a low flow rate can sometimes be used to achieve better resolution, if the increased retention time is still acceptable.

Conclusions

1. Because of the large difference between the copolymerization reactivity ratios of the monomers, VAc–DVB co-

polymer microspheres have an inhomogeneous structure. Because the low crosslinked VAl-rich networks with free PVAL chains are at the surfaces of the pores, the chiral selector L-proline is also concentrated on the pore surface. This provides a support with good characteristics for use in LEC.

2. Two average pore diameters, the volume-average (d_v) and surface-average (d_s) pore diameters are defined. The ratio d_v/d_s is used to characterize the pore-size distribution. It is pointed out that the widely used formula $d = 4V_p/S$ gives the surface-average pore diameter.

Acknowledgement

The authors are grateful to NSFC for the grant 29635010.

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