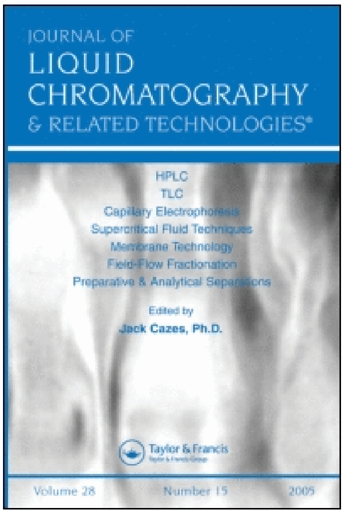

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NEW ORGANIC MONOSIZED MICROSPHERES FOR USE IN ENANTIOMER SEPARATIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Monosized nonporous resin particles were prepared in aqueous medium by polymerisation of urea with formaldehyde in the presence of

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soluble Fe(III) hydrolysis polymers. Measurements of the geometrical parameters showed that the material particle size distribution was in the range 2.5-3.5 μm (average particle diameter 2.99 μm with standard deviation 0.49), and had a sphericity index of 0.851. Particle pore-size was determined by gel permeation chromatography. The microspheres exhibited exceptional mechanical strength, chemical stability in the pH range between 1-13, and low tendency toward swelling in the solvents commonly used in liquid chromatography. The synthesis of a chiral urea-formaldehyde based resin for the enantiomer separation of underivatized phenylalanine by ligand exchange chromatography is reported.

INTRODUCTION

Silica-bonded packings are widely used in high performance liquid chromatography (HPLC), although these materials have certain disadvantages, which limit their use and shorten column lifetimes. These restrictions are mainly related to the Si-O-Si-C bonds, which are unstable in acidic and basic aqueous solvents, and to irreversible adsorption of basic solutes due to the residual surface silanol groups (1). Attempts to overcome such drawbacks were made by developing silica particles coated with a polymeric layer (2-4). These sorbents, which exploit a combination of mechanical rigidity and chemical stability, show low efficiency and loading capacity due to the difficulty of obtaining uniformly distributed layers inside the porous structure of the particles. Recent improvements in polymer technology have led to the production of highly cross-linked polymeric packings, that match rigidity and low swelling properties, and allow high eluent flow rates and possess excellent mass transfer properties (5-9). Procedures for the production of monodispersed polymer particles have been described in detail (10,11). Most of these stationary phases are formed from cross-linked polystyrenedivinylbenzene. They can easily be functionalized by introducing them into network reactive groups (12), or using them without functionalization for reversed-phase chromatography. Other polymer packings currently available are the poly(acrylate)-based ones. Because of the presence in their matrix of labile ester linkages, polyacrylic sorbents can be useful over a restricted pH range; however, these materials exhibit higher chemical stability than silica gel. Polymeric sorbents have been designed to solve specific problems in liquid chromatography, i.e. the separation of underivatised amino acids, amines, biopolymers, carbohydrates and inorganic ions, as well in steric exclusion chromatography. Another field of great interest is the development of chemically stable chiral stationary phases (CSPs). Owing to the high cost of most of the chiral selectors, the potential of virtually indestructible resin-type CSPs appears to be enormous, even in view of their use for preparative scale separations. Therefore, as an alternative to silica gel packings, a number of CSPs made of synthetic and natural polymers are suitable for low pressure chromatography (13-18). However, very few chiral polymeric sorbents for HPLC have so far been designed (19-21), even though a number of chiral selectors, such as cyclodextrins, proteins, ion-pairing agents, etc. may exhibit high enantiodiscrimination when used in the presence of alkaline aqueous eluents (22).

Recently we reported the synthesis and chromatographic evaluation of a new kind of CSP consisting of non porous (NP) and rigid polyamide particles, obtained by copolymerization of urea (U), formaldehyde (F) and a chirally selective monomer, L-leucinamide (23). This packing showed excellent chemical stability and specific enantioselectivity toward a series of carboxylic acid racemates, but low column efficiency, due to the poor size uniformity of the particles. In the present work we deals with an improved procedure for the production of U-F based CSPs. Firstly, NP microbeads with narrow particle size distribution were prepared by adding to the reaction mixture of water soluble Fe(III) hydrolysis polymers. The synthesis was then extended to the preparation of a chiral sorbent containing (+) L-2-amino-5-ureidopentanoic acid moieties bonded to the polyamide matrix. The resolution of underivatized phenylalanine by ligand-exchange chromatography is reported.

EXPERIMENTAL

Chemicals

$Fe(NO_3)_3 \cdot 9H_2O$, sodium bicarbonate, propylene glycol, urea, formaldehyde (40 % m/v) and copper (II) acetate were purchased from Carlo Erba (Milan, Italy). Other chemicals and solvents of reagent grade were obtained from Merck (Darmstadt, Germany). DL-, D- and L-phenylalanine, and (+) L-2-amino-5-ureidopentanoic acid were from Sigma (St. Louis, MO, USA). n-Alkanes, and tetrahydrofuran (THF) and methanol of HPLC grade were purchased from Fluka (Buchs, Switzerland). Water was filtered through Millipore (Bedford, MA, USA) type GS (0.22 µm) filter-disks.

Synthesis of monosized particles

Two spherical microparticulate materials were synthesised according to the following procedures.

Packing I. To 10.7 g of Fe $(NO_3)_3 \cdot 9H_2O$ (26.5 mmol), dissolved in 50 ml of bidistilled water, were added under stirring small aliquots (300 mg) of sodium bicarbonate for a total of 5 g, the final pH of the red solution was 2.20. Then to the solution were added propylene glycol (150 ml), urea (13.5 g, 0.225 mol) and 40% m/v formaldehyde (40 ml, 1.06 mol). Viscosity measurements of the mixture at 25 °C gave $\delta = 1.072$ g/cm^3 and $\eta = 8.22$ cP (dynamic viscosity). The solution was transferred to a round bottomed glass flask connected to a rotary flash evaporator, without applying vacuum, and allowed to react at room temperature overnight. The material was collected on a filter, washed with water, and then treated under stirring with 50 ml of 3 N HCl for 20 min, filtered and washed successively with water, water-methanol (1:1), methanol and acetone. After drying under vacuum at 40 °C, 5.30 g of resin were obtained. Elemental analysis gave : N = 30.83; C = 29.34; H = 5.48.

Packing II. Spherical particles containing a chiral monomer were prepared according to the procedure described above using 14.8 g (0.08 moles) of (+) L-2-amino-5-ureidopentanoic acid, 5.1 g (0.084 moles) of urea and 30 ml (0.8 moles) of 40% (v/m) formaldehyde. Elemental analysis gave : N = 16.17; C = 52.28; H = 6.88. Acid-base titration (24) showed that the resin contained 1.7 mequiv. of COOH groups per gram.

Column packing

Column packing was obtained by the high performance slurry technique, commonly used for silica-based sorbents. A suspension of resin (3.5 g in 25 ml methanol) was forced at 6000 p.s.i. through stainless-steel tubes (150 x 0.46 cm I.D.) using methanol as pressurising solvent.

Particle and pore size measurements of packing I

Particle geometric parameters were measured on a Model VIDS IV (Analytical Measuring Systems, Cambridge, UK) image analyser, equipped with a 286 Unibit IBM computer and a VIDS IV software, and with a Model TK-870E camera. The analyser was connected to a Model MP 3502M (Swift & Son, London, UK) trinocular polarising microscope, with a 500 x magnification lens. Data obtained on 51 randomly selected beads were elaborated using STAT graphic software. A sphericity index value of 0.851 and an average diameter of 2.99 μm (with standard deviation of 0.49) were obtained.

Scanning electron micrographs (SEM) were performed on a Cambridge 100 scanning secondary electron microscope using the gold-sputtered procedure.

Pore-size properties were investigated by gel permeation chromatography (GPC) (25). The following procedure was used: the column was connected to the solvent delivery pump and to a refractive index (RI) detector (Water Associates, Model R401) and then conditioned with anhydrous tetrahydrofuran. Solute standards included n-alkanes (n-pentane, n-hexane, n-decane, n-dodecane and n-pentadecane), and a poly(styrene) standard with narrow molecular weight range of 20.000 (Aldrich). Duplicate injections of each aliphatic hydrocarbon gave retention volumes similar to V_0 obtained for poly(styrene) standard.

Chromatography

The liquid chromatographic system consisted of a Perkin Elmer (Norwalk, CT, USA) Model Series 400 solvent delivery pump equipped with a Rheodyne 7125 injection valve, and connected to a Varian (Walnut Creek, CA, USA) Model 2550 variable wavelength detector. Chromatographic data were acquired using a PE Nelson Model 1020 Plus PC-based integrator (Perkin Elmer) or recorded on a Shimadzu (Kyoto, Japan) Chromatopac CR3A integrator.

The mobile phase used for the enantiomer separations consisted of 0.05 M acetate buffer prepared from acetic acid solutions, the pH was adjusted to 8.5 with potassium hydroxide, and to the eluent a small amount of copper (II) acetate was added (0.1 mM). Before use the eluent was filtered through a Millipore MF filter (0.45 μm) and degassed with helium. After washing with water, the column was loaded with copper (II) ions by flushing with a 0.05 M copper (II) acetate aqueous solution.

Samples were prepared by dissolving suitable amounts of D-, L- and DL-phenylalanine in the eluent in order to obtain a concentration of approximately 5 mg/ml. Sample volumes of about 2-4 μl were injected for the experiments.

RESULTS AND DISCUSSION

Two main steps are known to occur in the polymerisation between formaldehyde and urea to form resins (26). The first is an addition reaction of formaldehyde to introduce the hydroxymethyl group and is illustrated in the equation below:

$$\text{R-NH}_2 + \text{HCHO} \longrightarrow \text{R-NHCH}_2\text{OH}$$

The second step involves the linking together of monomer units with the formation of linear polymer chains. This condensation reaction is catalysed by acids.

$$\begin{gathered} n\text{NH-CH}_2\text{OH} + n\text{H}_2\text{NR} \longrightarrow \\ \longrightarrow (\text{-HNCONHCH}_2\text{NHCONHCH}_2\text{-})_{n/2} \end{gathered} \tag{1}$$

Acidic groups contained on the surface of inorganic polymer materials (for example silanols in silica gel) are known to favour reaction *(1)* with the formation of spherical shaped agglomerates coarceving the solid particles. According to this reaction, Iler (27) developed a method for the production of porous silica gel particles for HPLC, and, using a similar procedure, Stout *et al.* (28) reported the preparation of macro porous microspheres for SEC, the porogenic sol silica being subsequently removed from the agglomerates with hydrofluoric acid. In the present work we used water soluble Fe(III) hydrolysis polymers as the catalysing hydroxides.

Several metal ions are known to yield soluble polymers as intermediates in the precipitation of insoluble hydroxide (29). Hydrolysis polymers have been extensively studied by gel filtration chromatography and identified as polydispersed products with high molecular weight (30-33). Owing to the acidic character of such inorganic compounds, urea-formaldehyde polymers were expected to grow around the Fe(III) hydrolysis species, giving rise to monosized particles with high mass compactness. Data related to the geometric parameters of 51 randomly chosen particles (Packing I) were statistically processed and are reported as a frequency histogram in Figure 1. Scanning electron micrography (SEM) of the particles are shown in Figure 2. Figure 2b shows the microporous surface structure of beads at 24 K magnification.

Microporosity was confirmed by gel chromatography of a series of different molecular weight hydrocarbons and poly(styrene) standards. As expected, the compactness of linear polymer agglomerates gives the sorbent excellent mechanical strength. Figure 3 shows the back

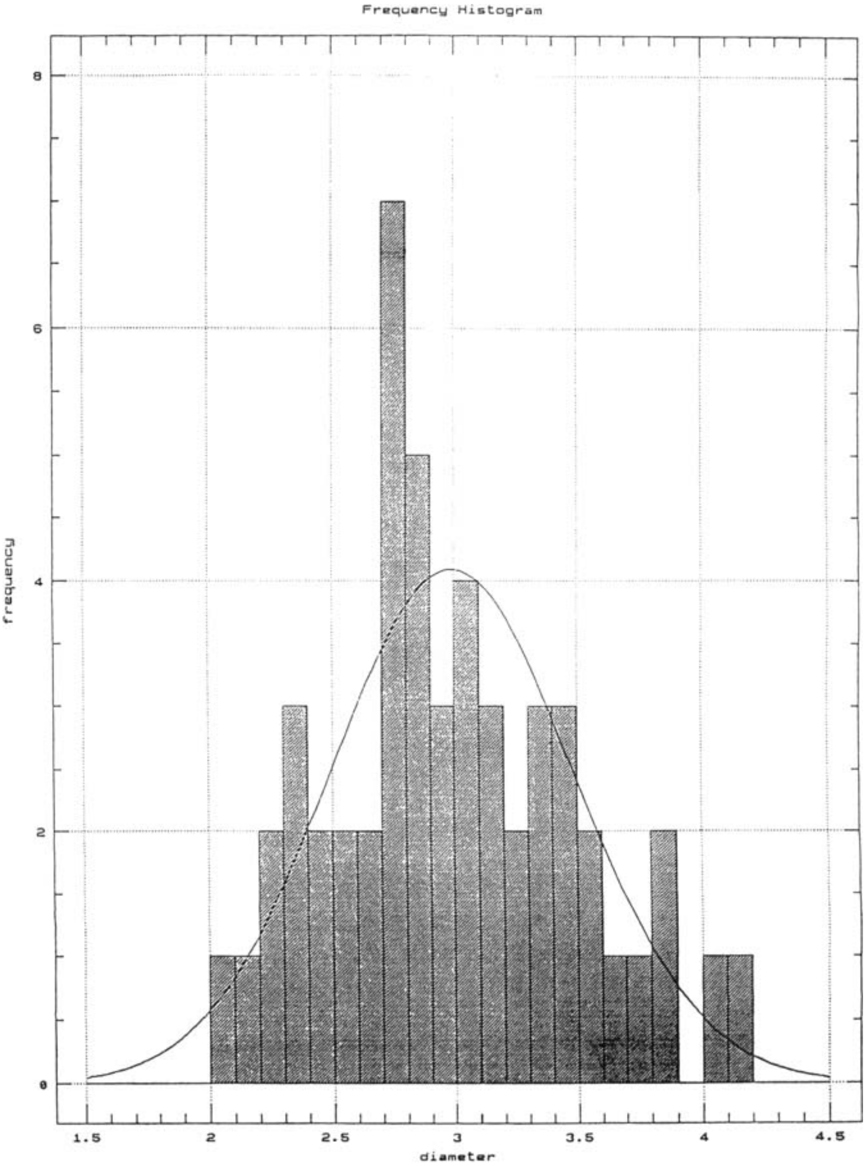


FIGURE 1: Particle size diameter distribution of the urea-formaldehyde resin represented as frequency histogram.

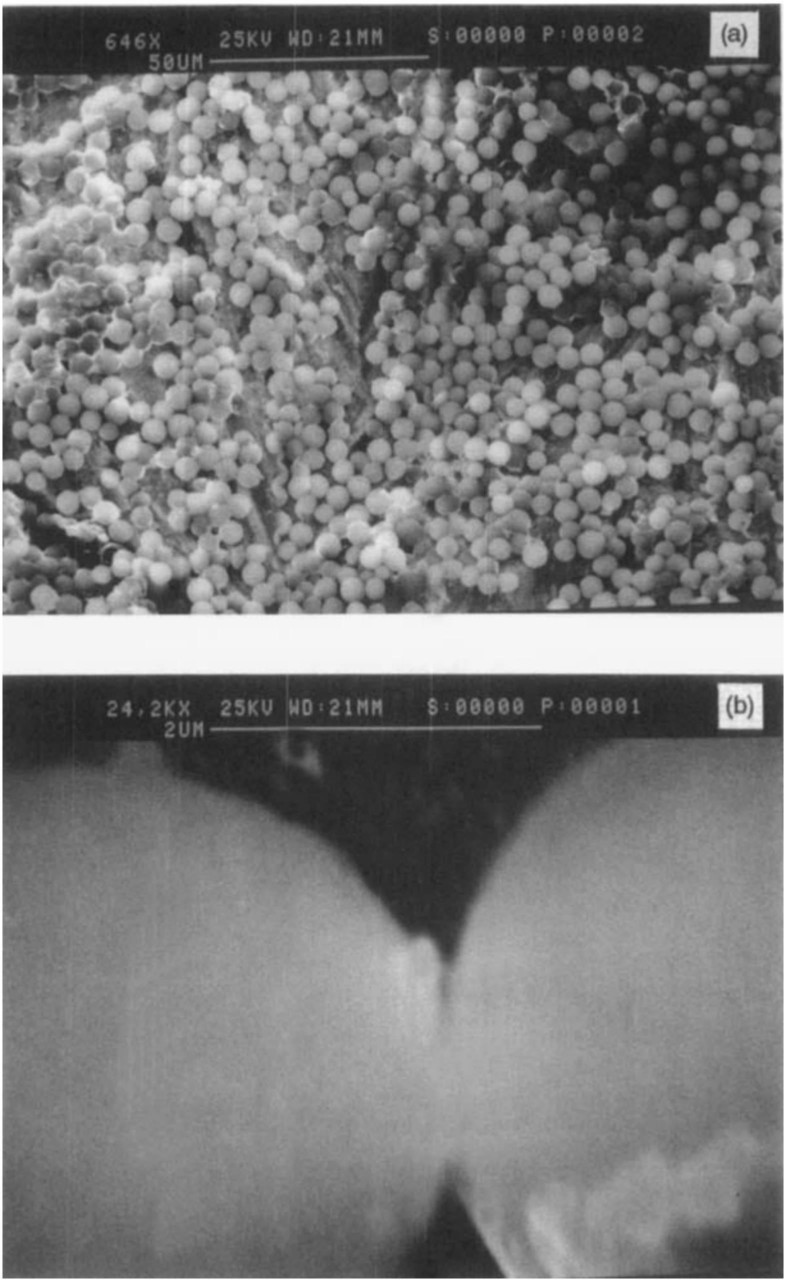


FIGURE 2: Scanning electron micrography (SEM) of the (a) spherical polyamide gel particles; (b) surface structure, performed at 24 K magnification.

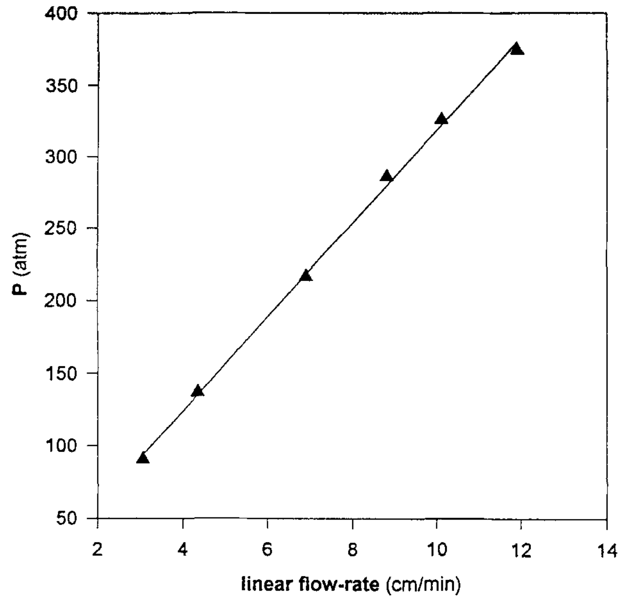
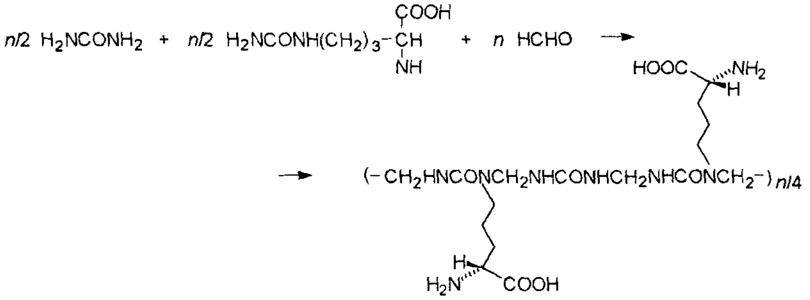


FIGURE 3: Pressure plot for a column packed with urea-formaldehyde resin *vs.* linear flow-rate. Eluent, water-methanol (1:1 v/v).

pressures of a packed column as a function of the linear velocity using a water-methanol mixture (1:1 v/v) as eluent. This plot is comparable to that found when using silica based packings.

Two procedures can be used for the functionalisation of the sorbent : a) grafting the superficial matrix by acylation and conversion of polyamide moieties to the corresponding iminium salts (34) or b) direct copolymerization of urea and formaldehyde in the presence of an urea-derivative selector. The second method appears of greater applicability and reproducibility of yield because it avoids a multi-step modification of the original structure and insures an uniform distribution of the bonded species. In both cases modified sorbents may exhibit selectivity only by the superficial bonded ligands, the inner ones being masked from the gel's non porous morphology.

The copolymerization procedure was used to prepare a packing containing an amino acid derivative, (+) L 2-amino-5-ureidopentanoic acid as the chirally selective monomer (packing **II**). According to reaction (*1*) monomers are assumed to copolymerise as in the reaction below :



(2)

Elemental analysis and acid-basic titration measurements showed that only a part of the chiral selector reacts in accordance with equation (2), and is available for the enantiodiscriminative process. However, ligand coverage was far higher then that commonly obtained by grafting reactions on silica gel (35), and appears comparable to the high yield of surface modification of poly(styrene) beads (12).

Figure 4 shows the resolution of free DL-phenylalanine on packing **II**, and the enantiomer elution sequence, L > D. Owing to the lack of hydrophobic interaction on the polyamide matrix (data not reported), the complexation interaction between the enantiomer analytes and the bonded Cu(II)-ligand complexes seems to be the dominant one, and the chiral recognition similar to that proposed by Davankov to explain the

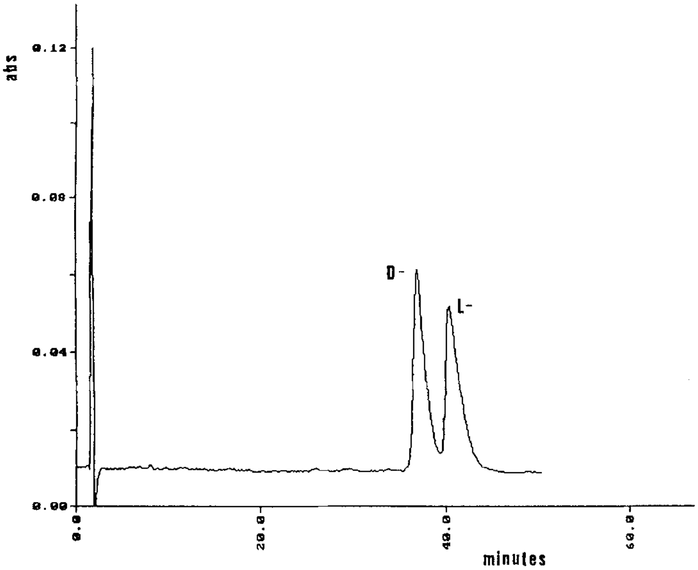


FIGURE 4: Enantioseparation of underivatized phenylalanine. Column (150 x 4,6 mm I.D.) packed with (+) L-2-amino-5-ureidopentanoic acid-urea-formaldehyde resin. Eluent, 0.05 M potassium and 0.1 mM copper (II) acetate buffer at pH 8.5; flow-rate, 0.8 ml/min; detection, UV at 254 nm; room temperature.

resolution of underivatized amino acids on grafted polyacrylamide-type resins (36). According to this model, the =C=O carbonyl group present in the selector moiety probably occupies the lower axial site of the Cu(II) coordination sphere, hindering the rotation of the D-amino acid and side chain, and, thus decreasing its retention in comparison with the L-enantiomer. Chromatographic data are summarised in Table I. A resolution (R_S) value of 1.32 appears reasonably high as in the separation of free amino acid enantiomers.

Concluding, urea and formaldehyde were found to copolymerize in monosized microspheres that exhibited excellent properties as packing

TABLE 1

Chromatographic data for the separation of underivatized phenylalanine enantiomers. Column, 150 x 0.46 cm I.D.; eluent, 0.05 M potassium and 0.1 mM copper (II) acetate buffer at pH 8.5; flow-rate, 0.8 ml/min; UV detector, 254 nm; room temperature.

k'_D	k'_L	α^a	N^b	Rs^c
20.1	22.1	1.1	3691	1.32

[a] $\alpha = k'_L / k'_D$; [b] $N = 5.545\ (t_R / W_h)^2$, refereed for the second eluted peak; [c] R_S, calculated from the fundamental resolution equation.

for high-performance liquid chromatography. Because a large number of mono- and polyfunctional amides are commercially available or can easily be synthesised, several new stationary phases with a wide range of selectivity could be produced. Owing to the low cost of the starting compounds, the procedure described in the present work appears of considerable interest in the development of new CSPs to be used for preparative scale separations.

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