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Retention and selectivity of flavanones on homopolypeptide-bonded stationary phases in both normal- and reversed-phase liquid chromatography

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Abstract

Three linear polymers of repeating amino acid units, or homopolypeptides, have been individually covalently bonded to microparticulate silica and evaluated for liquid chromatographic separations. The retention and selectivity of seven flavanones were investigated on these stationary phases and a structurally similar, commercially available reference stationary phase, Chiraspher. All three of the homopolypeptide stationary phases retain solutes in the normal-phase mode. The aromatic-containing homopolypeptide stationary phases also retain solutes in the reversed-phase mode. Selectivity values for the flavanones were higher in the normal-phase mode; chiral selectivity was observed for the amphiphilic homopolypeptide stationary phase in the reversed-phase mode. The retention mechanism of each stationary phase is suggested based on the chemical nature and conformation of the corresponding homopolypeptide ligand.

1. Introduction

Conformational transitions of stationary-phase ligands as a function of mobile-phase composition and column temperature have been investigated in relation to fundamental retention and selectivity in liquid chromatography (LC) [1]. In both theoretical treatments and practical applications of reversed-phase LC, the conformational ordering of alkyl chains has been thoroughly investigated [2–4]. Conformational properties of chiral stationary-phase ligands in liquid chromatography have been directly related to, and in some instances simply inferred, in the mechanism of enantiomeric recognition. For instance, (+)-poly(triphenylmethacrylate) forms a helical coil structure that has been directly related to its enantioselectivity as a stationary-phase ligand [5–7]. The process of chiral recognition of biomolecules, such as proteins, is more complex, however. The folding of the amino acid chain of a protein into a native three-dimensional tertiary structure may create one or more binding sites that have stereochemical preference for analytes. The most commonly used proteins for chiral stationary-phase ligands have been bovine serum albumin (BSA) and α_1-acid glycoprotein (AGP). BSA-silica was developed by Allenmark et al. [8]

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and is now commercially available as Resolvosil-BSA. According to retention studies of aromatic pharmaceuticals and amino acid derivatives, varying mobile-phase composition, hydrophobic and electrostatic interactions contribute significantly to retention, and hydrogen-bonding and charge transfer interactions are of secondary importance [9–12]. Subtle changes in mobile-phase composition and column temperature cause dramatic differences in the performance of BSA as a chromatographic ligand. Gilpin et al. [13] related the change in selectivity of L-tryptophan versus D-tryptophan in binding to BSA-silica as a function of temperature to a complex phenomenological local conformational change of the protein. AGP-silica, introduced by Hermansson [14] and now commercially available as Enantiopac, has been most commonly used for enantiomeric separations of racemic mixtures of cationic pharmaceuticals. Chromatographic investigations using aqueous buffer mobile phases in the pH range 4–7 have revealed that this acidic protein retains species based on ion-pairing, hydrophobic (related to its hydrophobic core) and hydrogen-bonding interactions [15–17], as have other studies [18,19]. Changes in retention and enantioselectivity with pH suggest that AGP is conformationally dynamic in nature [17].

More recently, Armstrong et al. [20] have introduced a new class of chiral selectors for liquid chromatography, macrocyclic antibodies. These LC stationary phases are multimodal in nature, demonstrating high enantioselectivity in both the normal- and reversed-phase modes. The macrocyclic antibodies possess the chemical diversity of the glycoproteins without irreversible denaturation of structure in nonpolar mobile-phase media. The enantioselectivity of these stationary phases was related to hydrophobic interactions (possibly inclusion into hydrophobic clefts of the antibodies) in the reversed-phase mode and related to hydrogen-bonding interactions in the normal-phase mode.

In this paper, we report the investigation of homopolypeptides as stationary-phase ligands for liquid chromatography. Homopolypeptides are analogous to proteins in their primary structure, existing as linear polymers of covalently bonded amino acids. Under the appropriate experimental conditions, proteins and peptides exist in ordered secondary structures, such as α-helices and extended β-strands or β-sheets. In contrast to proteins, homopolypeptides consist of a repeating sequence of the same amino acid, and tend to exist in predominantly one conformation. For this reason, synthetic homopolypeptides have been used extensively as physicochemical models of protein folding [21–23]. In separation science, polylysine-coated glass beads have been used in biochemistry to isolate cell membranes [24]. Spherical particles of poly-γ-methyl-L-glutamate have been used as packing material for gel permeation separations of polysaccharide and polystyrene standards [25]. Alpert has extensively investigated amino acid homopolymers, polymerized in situ on the activated silica surface, as cation exchange stationary phases for the separation of peptides, proteins, nucleic acids and polar compounds [26–29]. In liquid chromatography, the homopolypeptide stationary phases may demonstrate the effect of protein secondary structure on mechanisms of solute retention. To our knowledge, this is the first report relating the conformation of the homopolypeptide ligands to retention and selectivity in liquid chromatographic separations. In fundamental studies of retention and selectivity, a knowledge of the chemical nature and corresponding conformational properties of the homopolypeptide should aid in understanding the mechanism of retention of solutes by the stationary phases. Since synthetic homopolypeptides are also chiral, they may show enantioselectivity for racemates in LC separations.

2. Experimental

2.1. Chemicals

HPLC-grade methanol (MeOH), chloroform ($CHCl_3$), tetrahydrofuran (THF), 1,4-dioxane, electronic-grade isopropanol (IPA), hexane, methylene chloride, ethyl ether, toluene, nitric acid, potassium hydroxide (KOH), molecular

sieves (Davison, 4 Å), triethylamine, phosphorus pentoxide (P_2O_5), 85% phosphoric acid and dibasic anhydrous potassium phosphate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Poly-L-lysine (M_r 4600, $n = 22$), poly-β-benzyl-L-aspartate (M_r 13 900, $n = 68$), poly-L-tyrosine (M_r 13 900, $n = 85$ and M_r 22 500, $n = 138$) and 4-dimethylaminopyridine (4-DMAP) were obtained from Sigma (St. Louis, MO, USA). Deuterium oxide, β-mercaptoethanol and solid sodium were obtained from Aldrich (Milwaukee, WI, USA). Flavanones were obtained from Indofine (Belle Meade, NJ, USA). γ-Glycidoxypropyltrimethoxysilane, γ-glycidoxypropyldimethylethoxysilane and trimethylchlorosilane were obtained from Huls (Bristol, PA, USA). THF was dried over and distilled from solid sodium immediately before use. Methylene chloride was dried over and distilled from P_2O_5 immediately before use. Toluene was dried and stored over oven-dried 4-Å molecular sieves. All of the above chemicals were reagent-grade and used without further purification, unless otherwise noted. Distilled water obtained in-house was further purified using a Barnstead Nanopure II system (Barnstead, Boston, MA, USA) fitted with a 0.45-μm filter. Buffers were prepared with Barnstead water, dibasic potassium phosphate and 85% phosphoric acid. The pH was adjusted using a Corning pH meter (Model 125; Corning Glass Co., Corning, NY, USA). Solvents used in mobile-phase compositions that were not HPLC-grade were filtered before use with 0.45-μm filters (Micro Separations). Mobile-phase solvents were degassed by sparging with helium (Wright Brothers, Cincinnati, OH, USA) and mixed in the appropriate proportions by a ternary pump. Bare silica was obtained from Phase Separations (Norwalk, CT, USA) and epoxide-activated silica (Hydropore EP) was obtained from Rainin Instrument Co. (Woburn, MA, USA).

2.2. Synthesis equipment

Glassware for silanization reactions was pretreated with 5% (by volume) trimethylchlorosilane in chloroform for at least 4 h. Reactions requiring an anhydrous atmosphere were carried out in an air-tight glove box, constructed in-house from 1/4-inch-thick Plexiglas (Cincinnati Plastics, Cincinnati, OH, USA). The glove box was purged with dry nitrogen (Wright Brothers, Cincinnati, OH, USA) for at least 1 h prior to mixing the reagents. Nitrogen was dried by passing the gas through a tube filled with Drierite desiccant (W.A. Hammond Drierite, Xenia, OH, USA) just prior to entering the glove box. Distillation of methylene chloride, gentle agitation of trimethylsilanization reactions and acid washing of silica were accomplished using a rotary evaporator (Model RE121, Buchi Laboratoriums Technik, Flawil, Switzerland). Epoxide-blocking reactions were agitated using a laboratory stirrer (Model 106, T-Line Co., Emerson, NJ, USA) with an attached variable voltage regulator (Talboys Engineering Corp., Emerson, NJ, USA) and an adapter constructed in-house to hold the reaction vessel. Glassware, drying agents, silicas and some reactants were dried in Isotemp vacuum ovens (Model 281A, Fisher Scientific, Fair Lawn, NJ, USA). Epoxidations and peptide coupling reactions were heated in a mineral oil bath. Silicas were efficiently rinsed in polyallomer and PTFE bottles (Nalge, Rochester, NY, USA) of 250 ml capacity using a Sorvall centrifuge (Model Dupont RC5C, Sorvall Instruments). Volumes less than 1 ml were measured using an automatic electronic digital Pipetman (Rainin Instrument Co., Woburn, MA, USA).

2.3. Stationary-phase synthesis

Homopolypeptides were covalently bonded to epoxide-activated microparticulate silica, both pre-activated and activated in-house, under the appropriate solvent and temperature conditions as shown in Fig. 1. Stationary-phase identification and reaction conditions are given in Table 1. Further details of the synthesis of these stationary phases are described below. Percent elemental analyses were performed by Robertson Microlit Laboratories (Madison, NJ, USA).

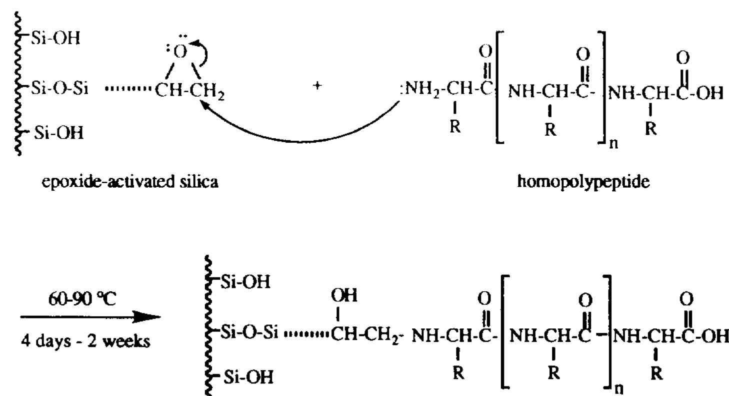


Fig. 1. The coupling of a homopolypeptide to epoxide-activated silica, where R is the amino acid sidechain group and $n + 2$ is the mean degree of polymerization of the homopolypeptide.

Silica pretreatment

Microparticulate silica (10 g) was added to 0.1 *M* nitric acid (100 ml) and heated at 90°C for 24 h under atmospheric conditions on a rotary evaporator. The silica was rinsed to neutrality (tested using pH paper) with Barnstead-purified water and then with two volumes of methanol to facilitate air-drying. The silica was dried under vacuum (30 inches of Hg) at 240°C for at least 4 h immediately prior to monomeric silanization reactions. Molar excesses of reagents for silanization reactions were calculated according to Eq. 1, assuming 5 μmol/m^2 sterically accessible silanol groups:

$$(5 \cdot 10^{-6})(\text{S.A.})(g)(M_r) = \text{one molar excess} \quad (1)$$

where S.A. is the specific surface area of the silica (m^2/g), g is grams of silica reacted and M_r is the molecular mass of the silane (g/mol).

Monomeric epoxide silanization

The following reagents were added to a 250-ml round-bottom flask under a dry nitrogen atmos-

Table 1
Reaction conditions for the covalent attachment of homopolypeptides to epoxide-activated silicas

Stationary phase identification	Ligand	Epoxide-activated Silica	Reaction conditions		
			Time (days)	Solvent	Temperature (°C)
PL22-R	PL, $n = 22$	Hydropore EP[a]	5	pH 7.5, phosphate buffer	60
PBA68-R	PBA, $n = 68$	Hydropore EP[a]	5	$CHCl_3$	65
PT85-R	PT, $n = 85$	Hydropore EP[a]	5	THF	65
PT138-M	PT, $n = 138$	Phase Separations[b] with monomeric activation[c]	15	THF	65
PT138-P	PT, $n = 138$	Phase Separations[b] with polymeric activation[d]	15	THF	65

[a] Particle diameter = 12 μm, nominal pore diameter = 300 Å, surface area = 74 m^2/g.
[b] Particle diameter = 5 μm, nominal pore diameter = 300 Å, surface area = 109 m^2/g.
[c] 1.97 μmol epoxide/m^2.
[d] 1.94 μmol epoxide/m^2.

phere in order: vacuum-dried, acid-washed silica (5 g); a four-fold molar excess of γ-glycidoxypropyldimethylethoxysilane, 8 μl triethylamine (dried over oven-dried KOH for 24 h); and 50 ml toluene (dried over 4-Å oven-dried molecular sieves for 24 h). The mixture was swirled, quickly connected to a continuously nitrogen purging condenser apparatus and heated at 90°C in an oil bath with magnetic stirring for 16 h. The mixture was removed and rinsed with three 50-ml volumes each of toluene, tetrahydrofuran and methanol, then air-dried overnight. Percent elemental carbon was determined and the surface density (μmol/m^2) of epoxide functionalities was calculated using Eq. 2:

$$\frac{\mu\text{mol}}{\text{m}^2} = \frac{(\%\text{C})(10^6)}{(\text{S.A.})(n_c)(12.001)\left[100 - \frac{\%\text{C}}{(n_c)(12.001)}(M_r)\right]} \quad (2)$$

where S.A. is the specific surface area of the silica, n_c is the number of carbon atoms in the ligand and M_r is the molecular mass of the ligand minus the molecular mass of the leaving group. Epoxide-activated silica was vacuum-dried (30 inches of Hg) at 125°C for at least 4 h immediately prior to endcapping.

Polymeric epoxide silanization

A 5% (by volume) aqueous solution of γ-glycidoxypropyltrimethoxysilane was prepared by simultaneous dropwise addition of the silane and 0.01 *M* KOH into Barnstead-purified water, maintaining solution pH between 5.0 and 6.5 (tested using pH paper). This solution (10 ml) was then added to air-dried, acid-washed silica (5 g) in a 250-ml round-bottom flask and heated at 90°C under atmospheric conditions in an oil bath with magnetic stirring for 16 h. The mixture was removed and thoroughly rinsed with Barnstead-purified water and then with methanol to facilitate air-drying overnight. The epoxide-activated silica was dried under vacuum (30 inches of Hg) at 125°C for at least 4 h immediately prior to endcapping. Percent elemental carbon was determined and the surface density of epoxide functionalities was calculated using Eq. 2.

Deactivation of residual silanol groups

The following reagents were added to a 250-ml round-bottom flask under a dry nitrogen atmosphere in order: vacuum-dried silica; a two-fold molar excess of trimethylchlorosilane; a four-fold molar excess of 4-dimethylaminopyridine; and 100 ml methylene chloride (dried over and distilled from P_2O_5, under dry nitrogen). The mixture was swirled and quickly connected to a continuously nitrogen-purging condenser and heated in a mineral oil bath at 28°C for 24 h. The mixture was then removed and rinsed with three 100-ml volumes each of methylene chloride, methanol, 50:50 (v/v) methanol–water, methanol and ethyl ether and allowed to air-dry.

Polypeptide coupling

A solution of polypeptide and organic solvent or buffer was added to Rainin Hydropore EP silica or epoxide-activated Phase Separations silica (5 μm in diameter) to create a concentrated slurry (approximately 2 ml solvent or buffer per gram of silica) in a dry-nitrogen purged glove box. Each silica was previously vacuum-dried (30 inches of Hg) at 125°C for at least 4 h. The mixture was heated to the appropriate temperature in an oil bath for 4 days to 2 weeks to achieve the desired ligand coverage with continuous nitrogen purging and occasional manual agitation. Percent elemental nitrogen was determined and the surface density (μmol/m^2) of homopolypeptide was calculated using Eq. 3:

$$\frac{\mu\text{mol}}{\text{m}^2} = \frac{(\%\text{N})(10^6)}{(\text{S.A.})(n_\text{N})(14.0067)\left[100 - \frac{\%\text{N}}{(n_\text{N})(14.0067)}(M_r)\right]} \quad (3)$$

where S.A. is the specific surface area of the silica, n_N is the number of nitrogen atoms in the ligand and M_r is the molecular mass of the ligand minus the molecular mass of the leaving group.

Deactivation of residual epoxide functionalities

Polypeptide-bonded silicas were agitated on a rotary evaporator with 1 *M* β-mercaptoethanol (adjusted to pH 8.0 with phosphoric acid, tested using a pH meter) under atmospheric conditions for 2 h at 25°C. The reaction mixture was then rinsed thoroughly to neutrality (using pH paper) with three 50-ml volumes of Barnstead-purified water and then rinsed with two 50-ml volumes of methanol to facilitate air-drying.

Column packing procedure

Unreacted Rainin Hydropore EP silica and homopolypeptide-bonded silicas were suspended in $CHCl_3$ (approximately 2 g of silica per 50 ml $CHCl_3$) and the slurry was degassed by immersing in an ultrasonic bath for 15 min. The slurry was then transferred to the precolumn assembly of an air-driven fluid pump. Compressed air (Wright Brothers, Cincinnati, OH, USA) was applied at 60 p.s.i. to deliver 300 ml 50:50 (v/v) $CHCl_3$–MeOH, 300 ml each of MeOH and 200 ml 50:50 (v/v) MeOH–H_2O sequentially through the stainless-steel column and then to waste. Chiraspher (EM Science, Darmstadt, Germany), a commercial chiral stationary phase, and Rainin Hydropore EP epoxide-activated silica were used as reference stationary phases. Ligand coverages of the stationary phases and dimensions of the columns used are shown in Table 2.

2.4. Chromatographic equipment and analysis

The chromatographic system consisted of a Spectra-Physics ternary pump (Model SP8800, ThermoSeparations, San Jose, CA, USA), a VICI 6-port injector (Valco Instruments Co., Houston, TX, USA) fitted with a 20-μl injection loop and a TosoHaas variable-wavelength detector (Model TSK-6041, Philadelphia, PA, USA). Two stainless-steel columns were positioned in series before the injector; a static mobile-phase mixer column (80 × 4.6 mm, packed with stainless-steel ball bearings) and a 12 cm × 4.6 mm I.D. column hand-packed with 10 μm, 300 Å PhaseSep silica (Phase Separations, Norwalk, CT, USA). The latter column maintained system back pressure above 300 p.s.i. for optimal pump performance. Both columns before the injector and the analytical column placed after the injector were water-jacketed and were temperature-controlled using a Fisher Scientific Isotemp refrigerated circulator (Model 9100, Fisher Scientific, Fair Lawn, NJ, USA) circulating a mixture of 50:50 (v/v) methanol–water. The column was allowed to equilibrate to temperature for 1 h prior to data collection. Flavanones were dissolved in hexane for normal-phase separations and dissolved in HPLC-grade methanol and mixed with Barnstead-purified water to match the mobile-phase composition for reversed-phase separations. Sample solutions (10^{-4} *M*) were

Table 2
Stationary-phase identification, surface coverage for homopolypeptide stationary phases and column dimensions for LC experiments

Stationary phase identification	Surface coverage ($\mu mol/m^2$)		Stationary phase deactivated?[a]	Column dimensions (mm)
	Homopolypeptide	Amino acid		
Rainin	–	–	no	60 × 4.6
PL22-R	0.064	1.4	no	100 × 4.6
PBA68-R	0.10	6.8	no	250 × 4.6
PT85-R	0.061	5.2	no	250 × 4.6
PT138-M	0.040	5.5	yes	150 × 4.6
PT138-P	0.025	3.4	yes	150 × 4.6
Chiraspher	–	–	–	250 × 4.6

[a] Refers to deactivation of residual silanol groups and residual epoxide functionalities.

filtered through 0.45-μm porosity nylon Aerodisc filters (Gelman Sciences, Ann Arbor, MI, USA). Each solute was injected in duplicate and the average capacity factor was reported. Data were collected using Turbochrom 3.1 software (PE Nelson, Cupertino, CA, USA) installed on a 486 PC with a CTX color monitor (Integrity Computers and Electronics, Cincinnati, OH, USA) with a Series 900 PE Nelson interface (PE Nelson, Cupertino, CA, USA). For the majority of experiments, a flow-rate of 2 ml/min was used, columns were maintained at 20°C and solutes were detected at 254 nm. Deuterium oxide was injected to determine the column void time, t_0. Capacity factors, k', were calculated based on the retention time of the solute t_r relative to t_0:

$$k' = \frac{t_r - t_0}{t_0} \tag{4}$$

Selectivity values were calculated as ratios of capacity factors.

3. Results and discussion

The monomers of the three homopolypeptides chosen for this study, poly-L-lysine (PL), poly-β-benzyl-L-aspartate (PBA) and poly-L-tyrosine (PT) possessing hydrophilic, hydrophobic and amphiphilic properties, respectively, are shown in Fig. 2. The secondary structures of homopolypeptide ligands are not irreversibly denatured under polar or nonpolar solvent conditions. The application of such stationary phases under both normal- and reversed-phase mobile-phase conditions extends their usefulness in LC. Existing protein chiral stationary phases only operate in the reversed-phase mode.

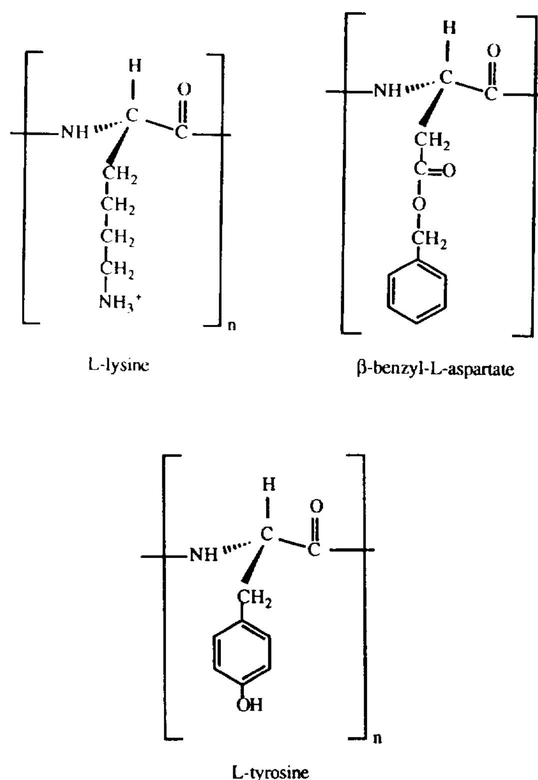


Fig. 2. The chemical structures of the homopolypeptides, where n represents the mean degree of polymerization.

Poly-L-lysine exists in three distinct conformations: β-sheet, right-handed α-helix and unordered coil. Conformational transition from unordered coil to α-helix occurs as a function of deprotonation of the ionizable amino sidechain groups ($pK_a = 10.5$) reducing electrostatic repulsion along the polypeptide [30–32]. The presence of salts shields the electrostatic repulsion and conformational transitions occur at pH values below the pK_a [30]. In aqueous solutions above 40°C, the α-helical conformation is converted to β-sheet structure [33]. High alcohol content in aqueous solutions favors the α-helical formation even when the polypeptide is in the fully charged state [34,35]. Thus, it is suggested that PL would exist as an α-helix in the normal-phase mobile-phase compositions used here. The PL ($n = 22$) lot used here may not be of sufficient length to form a stable secondary structure, however. In addition, it is likely that the PL was attached to the silica surface at multiple sites as both the amino terminus and the amino sidechain groups could react with the epoxide functionalities. The PL, therefore, would be able to undergo minor conformational transitions.

Due to steric interactions between sidechain groups and the amide backbone, poly-β-benzyl-L-aspartate may exist in a left-handed as well as a right-handed α-helical conformation. PBA also

exists in β-sheet and unordered conformations. The homopolypeptide exists in the left-handed α-helical conformation in solvents such as chloroform and carbon tetrachloride [36–38]. The unordered conformation is obtained by the addition of strong organic acids [39,40] or dimethylsulfoxide [41]. PBA exists in the right-handed α-helical conformation in the solid state at room temperature [42]. By Fourier-transform infrared (FT-IR) spectroscopic studies, we have concluded that PBA, $n = 68$, exists in a right-handed α-helical conformation under reversed-phase mobile-phase conditions used in this investigation [43]. Based on literature reports, it is suggested that PBA also would exist in a right-handed α-helix in the normal-phase mobile-phase conditions used here.

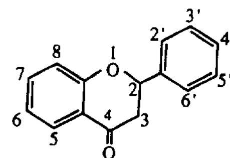
Poly-L-tyrosine (PT) contains ionizable phenolic groups in its sidechains, rendering this polypeptide amphiphilic in nature. Therefore, the polypeptide may undergo conformational transitions based on the degree of ionization of the phenolic groups (electrostatic interactions) and according to aggregation of sidechain groups in aqueous/organic solvent mixtures (hydrophobic interactions). Three prominent conformations have been observed for this polypeptide: unordered, right-handed α-helical and β-sheet. PT undergoes an unordered to intramolecular antiparallel β-sheet conformational transition as pH is decreased over a narrow range (11.2 to 11.5), related to the pK_a of the phenolic group [44,45]. In aqueous alcohol mixtures, conformational transitions between the β-sheet and the α-helical conformations are observed [46,47]. We have determined by FT-IR spectroscopic studies that PT, $n = 85$, undergoes a conformational transition between β-structure and an α-helix near 50% methanol in water [43]. It is assumed that PT, $n = 138$, would undergo the same conformational transition. Based on investigations in the literature it is suggested that each lot of PT exists in an α-helical conformation under the normal-phase mobile-phase conditions used here.

A set of flavanone derivatives was chosen as solutes to compare the fundamental retention and selectivity of the stationary phases under both normal- and reversed-phase mobile-phase conditions. The chemical structure of flavanone and identification of hydroxy- and methoxy-substituted derivatives used in this study are given in Table 3. Some flavanones are central intermediates in the synthesis of flavanoids, responsible for the coloration of plants, and others are secondary plant metabolites [48]. All the flavanones investigated here are chiral; they exist as pairs of nonsuperimposable optical isomers. Chiraspher was chosen as a reference stationary phase since a variety of optical isomers, including flavanones [49] have been resolved previously on this stationary phase in both the normal-phase mode [50,51] and the reversed-phase mode [52]. The chemical structure of the Chiraspher polymer differs from polypeptides in that it has a hydrocarbon backbone and its aromatic and amide groups exist as "sidechains" (Fig. 3).

3.1. Separation of flavanones in the reversed-phase mode

The capacity factors for the flavanones under reversed-phase mobile-phase compositions are given in Table 4. In order to obtain capacity factors of appropriate values, three different

Table 3
The structure of flavanone with positions labeled for derivative identification



Derivative	Identification
Flavanone	F
2'-Hydroxy	2'H
4'-Hydroxy	4'H
7'-Methoxy	7'M
4'-Methoxy	4'M
5-Hydroxy-7-methoxy	5H–7M
4',5-Dihydroxy-7-methoxy	4',5H–7M

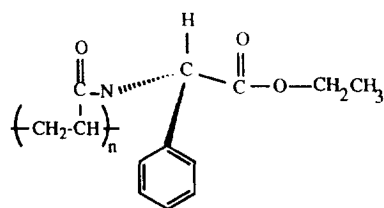


Fig. 3. The monomer of the Chiraspher stationary-phase polymer, N-acryloyl-(*S*)-phenylalanine ethyl ester.

$MeOH–H_2O$ mobile-phase compositions were used: 40:60 $MeOH–H_2O$ to compare the PBA and PT85-R stationary phases, 50:50 $MeOH–H_2O$ to compare the three PT stationary phases, and finally 60:40 $MeOH–H_2O$ to compare the PT85-R stationary phase with the Chiraspher stationary phase. A chromatogram of the seven flavanones on the PT138-M stationary phase at 50:50 $MeOH–H_2O$ is given in Fig. 4. Relative retention plots were constructed from calculated capacity factors in order to compare the stationary phases for the retention of flavanones in the same mobile-phase composition and at the same temperature. The linearity of relative retention plots suggests that each solute in the set is being retained by a similar mechanism on each stationary phase. Deviation from a slope equal to one indicates that differences exist in the relative loadings, structure or chemical nature of the two stationary-phase ligands.

The PBA and PT stationary-phase ligands differ from many stationary-phase ligands used in RPLC in that they contain polar functionalities in addition to hydrophobic aryl groups. A relative retention plot for the two phases for the separation of the flavanones is shown in Fig. 5. The solutes were retained significantly longer on the PT85-R stationary phase than the PBA68-R stationary phase. Differences in retention based on the chemical nature of the activation groups may be discounted here since the same preactivated silica was used in synthesizing each phase. Instead, differences in retention may

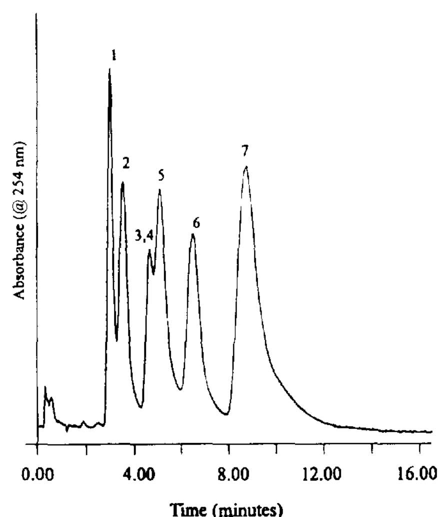


Fig. 4. The separation of the seven flavanones on the PT138-M stationary phase in 50:50 $MeOH–H_2O$ at 20°C and 2 ml/min. Peak identification: 1 = 4′-hydroxy-flavanone; 2 = 2′-hydroxy-flavanone; 3 = 4′,5-dihydroxy-7-methoxy-flavanone; 4 = flavanone; 5 = 4′-methoxy-flavanone; 6 = 7-methoxy-flavanone; 7 = 5-hydroxy-7-methoxy-flavanone.

Table 4
Capacity factors for flavanone derivative pairs for specified stationary phases and $MeOH–H_2O$ mobile-phase compositions at 20°C

Flavanone	Capacity factor						
	$PBA68\text{-}R_{40:60}$	$PT85\text{-}R_{40:60}$	$PT138\text{-}P_{50:50}$	$PT85\text{-}R_{50:50}$	$PT138\text{-}M_{50:50}$	$PT85\text{-}R_{60:40}$	$Chiraspher_{60:40}$
F	0.639	7.85	2.64	3.16	3.95	1.25	5.30, 5.52
2′H	0.618	4.71, 5.33	1.43	2.04	2.58	0.827	7.97
4′H	0.444	3.83	1.21	1.63	1.91	0.687	5.02
7M	0.743	12.1, 12.8	3.56	4.59	5.73	1.69	5.84, 6.20
4′M	0.701	11.1	3.40	4.13	5.11	1.56	5.89, 6.05
5H–7M	1.16	19.9	4.93	6.87	8.23	2.31	13.2, 13.7
4′,5H–7M	0.757	9.31	2.22	3.39	3.81	1.27	13.2

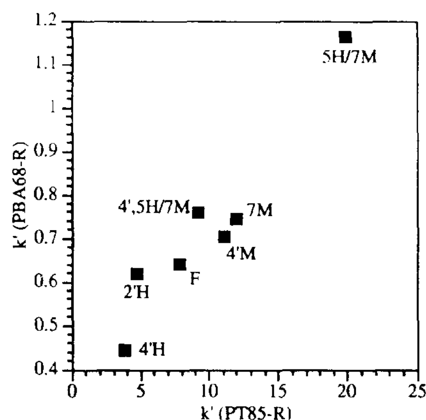


Fig. 5. Relative retention plot for flavanones on PT85-R and PBA68-R stationary phases in 40:60 MeOH–H_2O mobile-phase composition at 20°C. Linear coefficient of determination (r^2) = 0.9111, slope (m) = 0.0388 and $1/m$ = 25.8.

reflect differences in inherent hydrophobicities of the constituent amino acids of the polypeptides. The elution orders of the flavanones on the two phases are similar and a high linear correlation exists in their relative retention plot. This may be related to their common structural feature, the amide backbone. Slight differences in elution order may, however, be attributed to differences in the chemical nature of the polypeptide sidechains. Each polypeptide contains polar functionalities in its sidechains; PT contains an ionizable p-phenolic group in each sidechain, and PBA an ester linkage. The phenolic group, however, is more polar, and hence may undergo more interactions with the flavanones. Alternatively, the polypeptide ligands may differ in their hydrogen-bonding capabilities, dictated by their secondary structures. Peptide conformations differ fundamentally in the extent of intramolecular hydrogen-bonding that they possess. Therefore as a chromatographic ligand, the polypeptide will be more or less able to hydrogen-bond to solutes based on its conformation at a particular mobile-phase composition. The PT ligand exists in a more extended conformation than the PBA ligand in this mobile-phase composition [43]. Therefore, more amide bonds along the polypeptide backbone are able to participate in hydrogen-bonding with the polar solutes. Also, the PT85-R phase demonstrated enantioselectivity for both the 2′-hydroxy- and 7-methoxy-flavanone derivatives under these chromatographic conditions, yielding selectivities of 1.13 and 1.06, respectively. Higher resolution of the chiral enantiomers was observed at lower column temperatures and slower mobile-phase flow-rates. The partial chiral resolution of 2′-hydroxy-flavanone at 40:60 MeOH–H_2O, 10°C and 1 ml/min is shown in Fig. 6. The aromatic group of L-tyrosine is in closer proximity to the chiral carbon center of the amino acid compared to β-benzyl-L-aspartate, facilitating chiral interactions of the solutes with PT [53]. The lower apparent efficiency of the homopolypeptide stationary phases in general may be related to the mass transfer of the solute through a noncovalent (hydrogen-bonded) polymeric network. In addition, the chromatographic ligands used here are much longer than those typically used in liquid chromatographic coatings. Longer homopolypeptides, able to form stable secondary structures, were most desirable for this investigation, however. Slow solute desorption rates have been observed also for protein stationary-phase ligands [54].

In comparing the three PT stationary phases for the retention of flavanones in the slightly stronger mobile-phase composition, an elution order reversal is observed on the PT85-R phase relative to the other PT stationary phases (Table

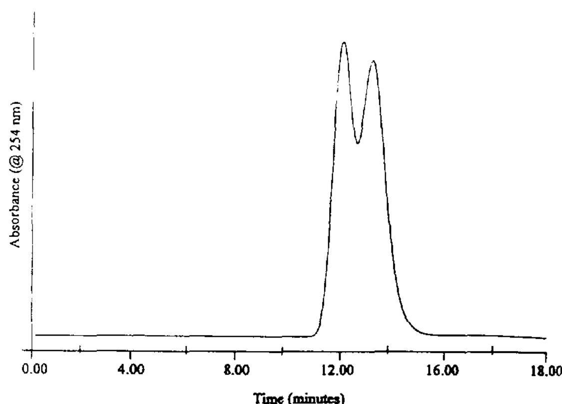


Fig. 6. The partial resolution of the 2′-hydroxy-flavanone optical isomers on the PT85-R stationary phase in 40:60 MeOH–H_2O at 10°C and 1 ml/min.

4). Flavanone elutes before its tri-substituted derivative on the PT85-R phase but the opposite elution order is observed on the other PT phases. This may be due to the different activation chemistry of this phase (pre-activated Rainin Hydropore EP silica) compared to the two PT phases of more similar activation chemistries, synthesized in-house. Despite slight differences in retention due to the activation chemistries of the PT85-R phase and the PT138 phases, the flavanones appear to be retained by a similar retention mechanism overall (Figs. 7 and 8). Despite differences in total amino acid loadings, the PT138 phases retain the polar species by a similar mechanism as well (Fig. 9). The greatest correlation does exist, however, between the PT phases of more comparable amino acid loadings, PT85-R and PT138-M (Fig. 7). These observations suggest that retention on the polypeptide stationary phases is dominated by the total amino acid content of the phase and not the nature of activation groups (polymeric versus monomeric) or ligand length. The relative capacity factors of the PT phases do not correlate directly with relative tyrosine loadings of the stationary phases. A variety of interactions, both polar and nonpolar, complicate the retention mechanism of the flavanones on these stationary phases. At this slightly stronger mobile-phase

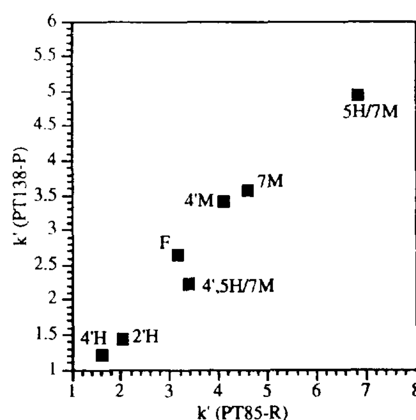


Fig. 8. Relative retention plot for flavanones on PT85-R and PT138-P stationary phases in 50:50 MeOH–H_2O mobile-phase composition at 20°C. Linear coefficient of determination (r^2) = 0.9672, slope (m) = 0.732 and $1/m$ = 1.37.

composition, the PT phases were not able to discriminate optical isomers of the flavanones.

The PT85-R stationary phase was then compared with the commercial chiral stationary phase, Chiraspher, for the retention of flavanones at an even stronger mobile-phase composition. Chiraspher preferentially retained the solutes, by a factor of approximately three. This is likely due to differences in the surface area of the base silicas or alternatively differences in the inherent hydrophobicities of the

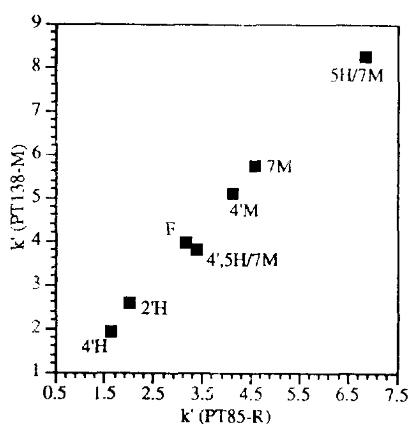


Fig. 7. Relative retention plot for flavanones on PT85-R and PT138-M stationary phases in 50:50 MeOH–H_2O mobile-phase composition at 20°C. Linear coefficient of determination (r^2) = 0.9938 and slope (m) = 1.21.

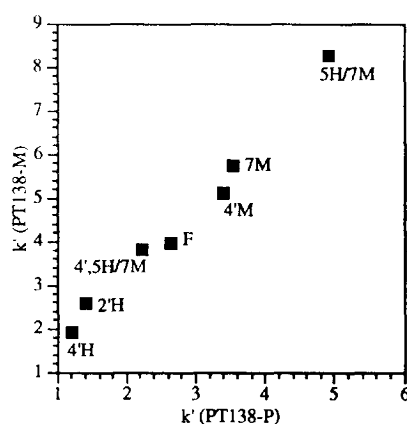


Fig. 9. Relative retention plot for flavanones on PT138-P and PT138-M stationary phases in 50:50 MeOH–H_2O mobile-phase composition at 20°C. Linear coefficient of determination (r^2) = 0.9836 and slope (m) = 1.61.

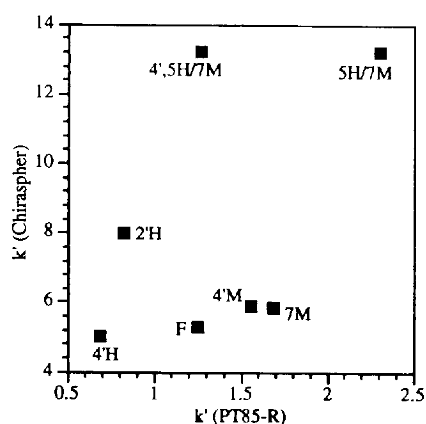


Fig. 10. Relative retention plot for flavanones on PT85-R and Chiraspher stationary phases in 60:40 MeOH–H_2O mobile-phase composition at 20°C. Linear coefficient of determination (r^2) = 0.2227 and slope (m) 3.13.

ligands. PT has a polar amide backbone, whereas the Chiraspher ligand has a more hydrophobic hydrocarbon backbone. On both the PT85-R and Chiraspher stationary phases, 2′-hydroxy-flavanone is retained least and 5-hydroxy-7-methoxy-flavanone is retained most. Otherwise, the elution orders of the flavanones on the two phases are significantly different, indicating the influence of the amide backbone structure. The differences in elution order are shown in Fig. 10, in the relative retention plot for the two stationary phases. These results suggest that the amide backbone of the PT phase may dramatically influence the separation of polar species. Therefore, significant differences would be anticipated in the retention mechanisms between PT stationary phases and traditional RPLC stationary phases containing phenyl or alkyl stationary phases.

The selectivity values calculated for pairs of flavanones give insight into the retention processes for polar aromatic solutes on these phases (Table 5). The PT85-R phase had greater methoxy- and hydroxy-selectivity than the PBA68-R phase, while comparable selectivities are found for the two phases in the separation of molecular mass isomers. All three PT phases had similar selectivities for the flavanone pairs, despite differences in length, activation chemistry, surface coverage of ligands and stationary-phase deactivation. The PT85-R stationary phase also demonstrated greater methoxy- and hydroxy-selectivities than the Chiraspher phase, but the Chiraspher was able to separate more optical isomer pairs under these experimental conditions.

3.2. Separation of flavanones in the normal-phase mode

In comparing stationary phases, separations were performed in the same mobile-phase composition (92.5:6.0:1.5 hexane–1,4-dioxane–IPA) and at the same temperature (20°C). Thus, all differences in retention and selectivity are attributable to differences in stationary-phase properties. The mobile phase chosen for this

Table 5
Selectivity values for flavanone derivative pairs for specified stationary phases and MeOH–H_2O mobile-phase compositions at 20°C

Flavanone Pair	α						
	PBA68-$P_{40:60}$	PT85-$P_{40:60}$	PT138-$P_{50:50}$	PT85-$R_{50:50}$	PT138-$M_{50:50}$	PT85$R_{60:40}$	Chiraspher$_{60:40}$
7M–F	1.16	1.54	1.35	1.45	1.45	1.35	1.10
4′M–F	1.10	1.41	1.29	1.29	1.29	1.25	1.11
2′H–4′H	1.39	1.23	1.18	1.25	1.35	1.20	1.59
7M–4′M	1.06	1.09	1.05	1.11	1.12	1.08	0.992
F–4′H	1.44	2.05	2.18	1.94	2.07	1.82	1.06
F–2′H	1.03	1.67	1.85	1.55	1.53	1.51	0.665
4′,5H–7M/5H–7M	1.53	2.13	2.22	2.03	2.16	1.82	1.00

investigation was similar to that used previously for the separation of flavanones on Chiraspher [49].

The capacity factors for the seven flavanones on the various homopolypeptide, Rainin and Chiraspher stationary phases are given in Table 6. The elution order for this set of compounds was the same on the four homopolypeptide stationary phases and two reference stationary phases. Also, there is approximately an order of magnitude difference in retention for flavanone and the 5-hydroxy-7-methoxy-, 4′-methoxy- and 7-methoxy-flavanone derivatives versus 2′-hydroxy-, 4′-hydroxy- and 4′,5-dihydroxy-7-methoxy-flavanone derivatives. A chromatogram of the separation of the seven flavanones on the PT138-M stationary phase in 92:6:2 hexane–1,4-dioxane–IPA is given in Fig. 11. This stationary phase was synthesized using silica activated in-house. The homopolypeptide stationary phases, PL22-R, PBA68-R and PT85-R were synthesized using Rainin Hydropore EP preactivated silica. Therefore, retention of the flavanones on the Rainin silica was included to determine the contribution of its hydrophilic polymeric activation groups, of proprietary chemical structure, to the retention of polar solutes under normal-phase conditions. Low retention of the flavanones is observed on the Rainin silica in comparison with the polypeptide phases; retention is more significant on the Rainin silica for the 2′-hydroxy-, 4′-hydroxy- and 4′,5-dihydroxy-7-methoxy-flavanone derivatives.

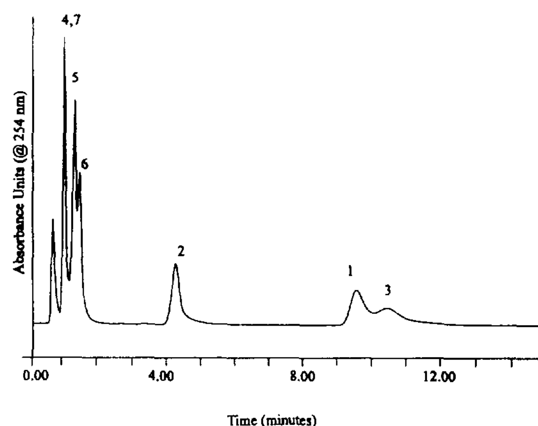


Fig. 11. The separation of the seven flavanones on the PT138-M stationary phase in 92:4:2 hexane–1,4-dioxane–IPA at 20°C and 2 ml/min. Peak identification is the same as that given in Fig. 4. The first peak is the system peak.

All of the polypeptide phases were able to retain polar species due to interactions with their polar amide backbones. Retention of the flavanones on the homopolypeptide-bonded stationary phases in the normal-phase mode is expected to increase with increasing polarity of the homopolypeptide according to the following trend, PBA < PT < PL. This trend in retention is most pronounced for the hydroxy-containing flavanones. Differences in the relative retention of polar species on the PL22-R, PBA-68-R and PT85-R stationary phases may be related to differences in the chemical structure of their sidechains. In addition, differences in their secondary structures, due to the amount of hydro-

Table 6
Capacity factors for flavanones on Rainin Hydropore EP silica, various homopolypeptide and Chiraspher stationary phases in 92.5:6.0:1.5 hexane–1,4-dioxane–IPA mobile-phase composition at 20°C

Flavanone	Capacity factor					
	Rainin	PL22-R	PBA68-R	PT85-R	PT138-M	Chiraspher
F	0.0645	0.270	0.272	0.713	0.677	1.00, 1.09
2′H	0.548	10.1	2.28	3.67	4.05	13.3, 14.3
4′H	0.935	13.6	4.00	7.75	7.84	26.5, 27.6
7M	0.129	0.492	0.503	1.50	1.33	1.88, 2.04
4′M	0.0968	0.413	0.435	1.19	1.04	1.69, 1.80
5H–7M	0.0968	0.381	0.388	0.880	0.785	1.55, 1.63
4′,5H–7M	1.06	18.8	5.35	9.41	8.77	40.3

gen-bonding along the polypeptide backbone, may affect the retention of polar species.

PL22-R demonstrated greater retention for most of the hydroxy-containing flavanones than would be predicted based on its relative loading of total amino acid. This may be related to the additional primary amino groups of strongly basic nature in the sidechains of PL. As with amino-bonded phases, there is strong affinity for the acidic hydroxy groups [55,56]. Alternatively, these primary amino functionalities may contribute to enhanced hydrogen-bonding with the solutes. As stated previously, based on reports in the literature, the PL lot used here would exist in an unordered coil in such nonpolar conditions. This more extended conformation would also contribute to the hydrogen-bonding ability of the stationary-phase ligand.

The PBA68-R stationary phase had significantly lower retention for the flavanones than would be predicted based on its high coverage of total amino acid. The ether linkage in the sidechain of PBA must only be slightly basic in nature compared to a primary amino group, lowering the overall retention for this stationary phase under normal-phase conditions [57]. In addition, charged ligands such as PL and PT tend to be more polar than uncharged ligands such as PBA. Since the chemical structure of PBA is remarkably similar to that of Chiraspher, the lower retention on PBA68-R phase may be related to a higher surface coverage of ligands or higher specific surface area for the Chiraspher phase. Alternatively, the lower retentivity of the PBA68-R stationary phase may be related to its secondary structure. As stated previously, based on reports in the literature, the PBA lot used here would exist predominantly in a right-handed α-helical conformation in the nonpolar mobile phase. This secondary structure has a significant amount of hydrogen-bonding along the polypeptide backbone, possibly contributing to the low retentive properties of this stationary phase. Such polar interactions are accentuated under nonpolar normal-phase mobile-phase conditions.

The PT85-R stationary phase contains ionizable hydroxyl groups in its sidechains. These groups are expected to be slightly acidic in nature, similar to diol-bonded phases. Therefore, one would expect lower retentivity due to charge repulsion for the potentially negatively charged PT stationary phases with potentially negatively charged flavanones. The higher retentivity of this stationary phase then may reflect the importance of hydrogen-bonding interactions. Alternatively, the relatively high retention for this stationary phase may be related to its conformation. Based on reports in the literature, PT ($n = 85$) would predominantly exist in intramolecularly hydrogen-bonded antiparallel single β-sheet structure in more nonpolar solvents. This polypeptide conformation is more extended than α-helical conformation with less intramolecular hydrogen-bonding. Therefore, its amide backbone groups would contribute more significantly to retention of polar species than that observed for the PBA stationary phase.

The PT138-M stationary phase was synthesized from silica activated with a monofunctional glycidoxypropylsilane under anhydrous conditions forming a monomeric structure. No significant differences were observed in retention for this stationary phase and the PT85-R stationary phase with polymeric activation. Both homopolypeptides should exist in the same secondary structure under these mobile-phase conditions. In addition, PT138-M was endcapped by deactivating the residual hydroxyl groups on the silica surface with hydrophobic trimethyl functionalities.

As noted in the literature, under similar mobile-phase conditions the Chiraspher stationary phase was able to separate six of the seven flavanones into their optical isomers [49]. The homopolypeptide phases did not demonstrate chiral selectivity, however, since their aromatic groups are more removed from the chiral center of the polymer than in the chemical structure for the Chiraspher polymer. Close proximity of an aromatic functionality to the chiral center of a selector allows more favorable chiral interactions [53].

Since an order of magnitude difference in retention existed for this set of data, probably due to large differences in solute polarity, the

data will be referred to as two sets in order to emphasize individual retention effects. Set A includes flavanone and the 5-hydroxy-7-methoxy-, 4′-methoxy- and 7-methoxy-flavanone derivatives. Set B includes the more polar 2′-hydroxy-, 4′-hydroxy- and 4′,5-dihydroxy-7-methoxy-flavanone derivatives.

Overall, the PT85-R phase is the most retentive of the four homopolypeptide stationary phases for set A flavanones. The enhanced retention of this set of solutes displayed by the PT85-R phase may be related to hydrogen-bonding interactions with the phenolic sidechain group of this amphiphilic stationary phase. The PBA68-R and PL22-R phases show parallel behavior for retention of set A flavanones. This suggests that the retention of these four flavanones is dominated by the amide bonds of the polypeptide backbone, their common structural feature. The presence of aromatic functionalities in the PBA stationary phases may not significantly affect retention of these compounds. The PL22-R stationary phase is the most retentive of the set B flavanones of the four homopolypeptide phases. The basic nature and hydrogen-bonding ability of the primary amino group sidechains of PL may be a dominant factor in retaining the more acidic polar solutes. There is a high linear correlation between the PBA68-R stationary phase and Chiraspher stationary phase for the retention of all seven flavanones (Fig. 12). This is not surprising, however, due to the great similarity in chemical structure of their corresponding ligands.

The selectivity values for flavanone pairs are given in Table 7. The PL, PBA and PT ligands had similar methoxy-selectivity that appeared to be position-dependent. In addition, all the homopolypeptide phases were able to discriminate molecular mass isomers. The selectivity is greater between the more polar pair of hydroxy-flavanone derivatives versus the methoxy-flavanone derivatives. In terms of hydroxy-selectivity, differences in stationary-phase properties became apparent. As expected, due to the basic nature and hydrogen-bonding ability of the amino sidechain groups of PL, the PL22-R phase demonstrated the greatest hydroxy-selectivity.

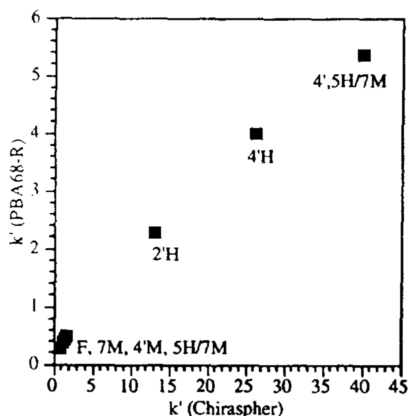


Fig. 12. Relative retention plot for flavanones on the PBA68-R stationary phases versus the Chiraspher stationary phase in 92.5:6.0:1.5 hexane–1,4-dioxane–IPA mobile-phase composition at 20°C. Linear coefficient of determination $(r^2) = 0.9921$, slope $(m) = 0.132$ and $1/m = 7.58$.

The PT phases demonstrated the least hydroxy-selectivity and this was anticipated due to the acidic nature of its phenolic sidechain groups. Even though the PBA68-R phase had the lowest retention of the three types of polypeptide phases, the PBA68-R stationary phase showed intermediate hydroxy-selectivity.

4. Conclusions

The retention mechanism for the homopolypeptide stationary phases under reversed-phase conditions primarily involves hydrophobic interactions between the solutes and the aromatic rings of the ligands. The sidechain chemistry of the homopolypeptide determines its overall hydrophobicity. Elution orders varied for the stationary phases especially between the Chiraspher and PT stationary phases and all the flavanones eluted within the same order of magnitude.

In contrast to reversed-phase conditions, the retention of the solutes was "normalized" for the overall polarity of the phases under normal-phase conditions due to the amide backbone common to each ligand. The same elution order for the flavanones was observed for all the

Table 7
Selectivity values for flavanone derivative pairs on specified stationary phases in 92.5:6.0:1.5 hexane–1,4-dioxane–IPA mobile-phase composition at 20°C

Flavanone pair	α				
	PL22-R	PBA68-R	PT85-R	PT138-M	Chiraspher
7M–F	1.82	1.85	2.10	1.96	1.88
4'M–F	1.53	1.60	1.67	1.54	1.69
4'H–2'H	1.35	1.75	2.11	1.94	1.99
7M–4'M	1.19	1.16	1.26	1.28	1.11
4'H–F	37.4	8.38	5.15	5.98	13.3
2'H–F	50.4	14.7	10.9	11.6	26.5
4',5H–7M/5H–7M	49.3	13.8	13.8	11.2	26.0

homopolypeptide stationary phases and the Chiraspher stationary phase. In addition, the separation of the flavanones under normal-phase conditions was highly sensitive to the polarity of the solutes as they eluted in two distinct groups whose capacity factors differed by an order of magnitude. Under nonpolar mobile-phase conditions, polar interactions between the ligand and the solute are enhanced. Both modes of liquid chromatography allowed the separation of flavanone molecular mass isomers. Hydroxy-, methoxy- and molecular mass isomer selectivities were higher under normal-phase conditions, however. Sidechain chemistries of the homopolypeptides played a dominant role in retention processes.

The stationary phases derivatized with PT offer great potential in future applications, especially in the separation of polar solutes. Shorter homopolypeptide ligands are recommended for separations of higher efficiency, however. In comparison with PBA and PL phases, the PT phases were able to retain solutes strongly under both normal- and reversed-phase conditions. The PT phases demonstrated chiral selectivity under reversed-phase conditions as well. These chiral recognition properties of the PT phases should be more fully investigated and applied to the separation of other racemic mixtures. In an additional practical application of these stationary phases, their low retentivity may lead to their use in hydrophobic interaction chromatography. The advantage of amphiphilic PT stationary-phase ligand is that it may be used in hydrophilic interaction chromatography as well for less polar biomolecules such as histones, membrane proteins and phosphorylated amino acids and peptides.

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