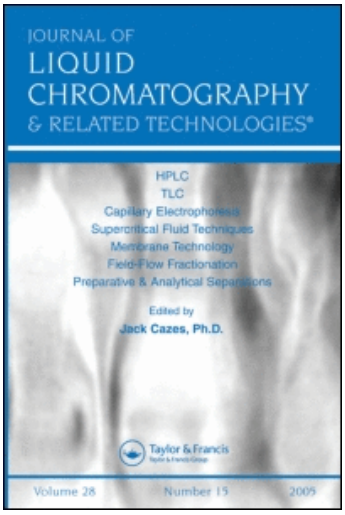

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Uniformly Sized Poly(Vinylphenol) Porous Beads: A Versatile HPLC Separation Medium Allowing Fast Switching Between Size-Exclusion, Normal Phase, and Reversed Phase Chromatography

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UNIFORMLY SIZED POLY(VINYLPHENOL) POROUS BEADS: A VERSATILE HPLC SEPARATION MEDIUM ALLOWING FAST SWITCHING BETWEEN SIZE-EXCLUSION, NORMAL PHASE, AND REVERSED PHASE CHROMATOGRAPHY

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ABSTRACT

Uniformly sized, 10 μm porous poly(4-*tert*-butoxycarbonyloxystyrene-co-divinylbenzene) beads with controlled porosity were prepared by polymerization of uniform size droplets consisting of small polymeric particles that were used as shape templates and enlarged with monomers and porogens. The *tert*-butoxycarbonyl groups were removed by a thermal treatment that affords the desired poly(4-hydroxystyrene-co-divinylbenzene) separation medium. The pore size of the beads does not change when immersed in THF as the beads are additionally crosslinked upon heating in the deprotection step. The beads were tested in size-exclusion, normal phase, and reversed phase chromatography. The size-exclusion calibration curve is almost linear in the range of molecular weights from 100 to about 480 000. Columns packed with the polymeric phenol beads can be switched between normal and reversed-phase chromatography simply by changing the solvent without removing from the chromatograph and their chromatographic properties do not change even after several hundred injections *in different chromatographic modes* including also multiple washing with aqueous sodium hydroxide. The retentions in separations of series of homologous alkylbenzenes, N-substituted, and ring substituted anilines were found to be linear functions of the number of carbon atoms, while interactions with the phenolic hydroxyls increased the retention times of N-alkylanilines. Separation of a protein mixture using a column packed with the beads has also been demonstrated and very high protein recoveries have been obtained.

INTRODUCTION

In spite of large number and great variety of separation media that are available for use in HPLC (1), the search for new, non-conventional packings suitable for demanding and specialized applications continues. Two broad categories of columns are the object of much development. First are the specialized packings, developed and optimized for a very specific type of separation. Among other applications, these columns are particularly well suited for routine or repetitive analyses in which a large number of similar samples are processed or for the monitoring of mixtures of defined compositions. Typical examples include columns for size-exclusion chromatography (SEC) with narrow exclusion limits, or beads with immobilized antibodies. The second type of columns are more versatile and may be used for different modes of chromatography without tedious repacking by simple adjustments in the chromatographic conditions. These packings are ideally suited for research and development laboratories where a wide variety of separations are performed frequently.

Current separation media may be divided into two large classes: inorganics and polymers. The most widely used inorganic packings are based on silica which, in its native state, contains siloxane groups with pK_a close to 7 (2). The acidity of siloxane hydroxyl groups of silica makes it similar to weak cation exchangers and limits its direct use in some chromatographic processes (1). While still less widely used that silica columns, polymeric stationary phases have attracted much interest lately, as they may offer a broader variety of chemistry and chemical stability even under extreme conditions of pH or solvents. In some modes of chromatography, such as ion-exchange chromatography (IEC) and SEC, polymeric stationary phases have assumed a dominant position.

The first textbook example of use of a macroporous polymer in HPLC-like liquid chromatography was described by Moore almost 30 years ago (3). Since their introduction in 1964, the styrene-divinylbenzene resins are still the most extensively studied polymeric phases for HPLC. However, their direct use is limited by their hydrophobic nature though this is clearly not a problem for their application to SEC in non-aqueous solvents such as tetrahydrofuran, and to reversed-phase chromatography (RPC) with acetonitrile or propanol as one of the components of the mobile phase. Overall, the styrene-divinylbenzene resins are considered to be typical nonpolar packings.

Chemical modification of styrene-divinylbenzene copolymers may be used to increase their polarity. For example, strongly acidic ion-exchangers are produced by direct sulfonation of the styrene matrix. Another route to increasing the acidity of the styrene-divinylbenzene matrix involves copolymerization with unsaturated aliphatic acids. For example, the incorporation of aliphatic carboxyls with pK_a values of about 5 results in weakly acidic ion-exchange resins. Ion- exchange chromatography is based on electrostatic interactions between the charged groups of both the dissolved compounds and the surface of the packing material.

Numerous polar packings for HPLC have been developed based on crosslinked polymers of 2-hydroxyethyl methacrylate, 2,3-dihydroxypropyl methacrylate, vinyl alcohol, hydroxylated polyethers or other monomers containing aliphatic hydroxyls. They are used directly in aqueous SEC, or, after derivatization, in the retentive modes of chromatography (1). These packings are not designed to interact in an electrostatic fashion with the compounds to be separated since their aliphatic hydroxyls have pK_a values of only approximately of 15-18.

The pK_a values of phenols being close to 10, their acidity is intermediate between organic acids and aliphatic hydroxyls. Polymeric phenols showed little potential for use as chromatographic media (4,5) until a method of polymerization of 4-*tert*-butoxycarbonyloxystyrene followed by deprotection was developed (6) and used in standard suspension polymerization to afford defined crosslinked porous beads (7). Both the pK_a of the phenol moiety and its shielding by adjacent *ortho* ring-substituents proved to be important variables for the control of the chromatographic properties of this novel medium. Its good performance in normal-phase chromatography was documented recently in a series of separations of substituted aromatic and aliphatic amines (8).

We have now vastly improved the capabilities of this new phenolic medium both with the preparation of highly efficient and uniformly sized beads of crosslinked poly(vinylphenol) and with their application in SEC, and in the direct and reversed-phase modes of chromatography of low molecular weight compounds and proteins.

EXPERIMENTAL SECTION

Preparation of Monodispersed Porous Beads

Uniformly sized shape-template latex particles 1.4 μm were prepared by emulsifier-free emulsion polymerization of styrene as described in detail elsewhere (9). The solid content in the aqueous dispersion was 29.2 wt%. To 0.80 mL of this dispersion was added 2.38 mL dibutyl phthalate emulsified by sonication in 12 mL of 0.25 wt% aqueous solution of sodium dodecyl sulfate (SDS). After all the tiny droplets of emulsified dibutyl phthalate had disappeared, a mixture of 21.7 g 4-*tert*-butoxycarbonyloxystyrene and 14.4 g divinylbenzene (80% DVB, Dow Chemical Co.)(monomers), 54 g cyclohexanol (porogenic diluent), and 0.36 g azobisisobutyronitrile (free radical polymerization initiator) emulsified in 220 mL of aqueous 0.25 wt% SDS was added. The resulting mixture was stirred slowly in a 500 mL round-bottomed glass reactor (Büchi, Uster, Switzerland) at room temperature until it contained only the swollen polymer particles. The contents of the reactor was supplemented with 90 mL of 4 wt% aqueous poly(vinyl alcohol) (Polyviol W 25/140, Wacker Chemie, Burghausen, Germany). The polymerization was then allowed to proceed under stirring with an anchor type stirrer (100 RPM) at 70 °C for 15 hours. The polymer beads were transferred to a 1000 mL beaker and washed by repeated decantation in water and methanol to remove the suspension stabilizer, surfactant, and the porogenic diluent. The original polystyrene shape-templates

were removed by extraction in toluene, the beads were washed with ethanol and finally dried in air.

Thermal Cleavage of the Protecting Groups

The deprotection of the phenol hydroxyls by removal of *tert*-butoxycarbonyl groups was done according to the method described in detail earlier (7,8). The beads were heated to 220-230°C in a flask immersed in an oil bath for 90 min. The flask was connected to a high-vacuum pump and the pressure inside the flask was kept under 25 Pa. The complete removal of the *tert*- butoxycarbonyl groups after the thermal cleavage was confirmed by the loss of the strong carbonyl band near 1760 cm^{-1} in the IR spectrum.

Chromatographic Experiments

The chromatography was carried out using an IBM LC/9560 ternary gradient liquid chromatograph equipped with a Rheodyne 7125 loop injector valve. The chromatographic bands were monitored by a HP 1050 UV detector (Hewlett-Packard, Avondale, Penn., USA) at 254 and 218 nm for low molecular weight compounds and polystyrene standards, and proteins, respectively. The beads were slurry packed with 50% aqueous acetonitrile into stainless steel columns of different sizes under constant pressure (11 MPa).

The size-exclusion chromatography was performed in 50 mm x 8 mm i.d. and 250 mm x 7 mm i.d. columns in THF with toluene and polystyrene standards with molecular weights ranging from 1250 to 2 950 000 (Polymer Laboratories). The normal phase, reversed-phase, and gradient elution chromatography experiments were carried out in a 50 mm x 8 mm i.d. column. Switching between the different chromatographic modes was done simply by changing the original mobile phase in the column to methanol followed directly by pumping the new mobile phase. The new mobile phase was pumped at 0.5 mL/min through the column for 2 hours to allow good equilibration. Detailed chromatographic conditions are described in the captions to Figures. All solvents used were of HPLC grade (Fisher Scientific). The proteins, i.e. cytochrome C (from bovine heart, MW 12 400), ribonuclease A (from bovine pancreas, MW 12 600), lysozyme (from chicken egg white, MW 13 900), carbonic anhydrase (from bovine erythrocytes, MW 29 000), chicken egg albumin (MW 44 000), and human serum albumin (MW 69 000), were purchased from Sigma. Protein recovery was calculated from the ratio between the protein peak area measured under standard chromatographic conditions and the peak area of the same amount of protein injected into the chromatographic system from which the column was removed and the inlet and outlet capillaries were connected with an empty column having a volume close to the V_i of the column tested.

Characterization of the Beads

Specific surface areas were determined from nitrogen sorption-desorption measurement and calculated according to the B.E.T. method. Pore size distributions in dry beads were calculated from mercury intrusion volumes (Combined BET Sorptometer and Mercury Porosimeter, Porous Materials, Inc., Ithaca, NY, USA). The pore size distribution and specific pore volume were also determined in the swollen state by inverse size exclusion chromatography (10) with polystyrene standards as probes.

RESULTS AND DISCUSSION

Porous copolymers of 4-vinylphenol, or its 2,5-disubstituted derivatives, with divinylbenzene have been shown to be very efficient separation media in the separation of amines by normal phase chromatography. Alkyl substitution in the positions ortho to the phenolic hydroxyl proved to be a powerful tool for the fine control of access to this phenolic hydroxyl by the compounds to be separated (8).

The first beads containing phenolic hydroxyls were prepared by standard suspension polymerization, therefore, they had a broad particle size distribution (7,8). Despite the lack of size classification, the columns packed with the novel stationary phase were very efficient except for their excessive back-pressure at linear flow rates exceeding about 10 cm/min (8). In order to improve on our earlier work we applied a preparation method which results in monodispersed beads as demonstrated in the preparation of packings based on copolymers of styrene and divinylbenzene (11-13).

The preparation method is based (14-16) on controlled swelling of small monodispersed size-templates (1.4 μm latex particles) followed by a suspension polymerization in the presence of porogenic substances (11-16) while the coalescence of the droplets is excluded by an extensive stabilization. This technique is only efficient when monomers with low water solubility, such as styrene, methyl methacrylate, glycidyl methacrylate, divinylbenzene, or ethylene dimethacrylate, are used. Since 4-*tert*-butoxycarbonyloxystyrene is a nonpolar, hydrophobic monomer that is poorly soluble in water, it is well suited for this kind of polymerization.

Physical Properties of the Packing

Monodispersed porous beads 10 μm in diameter were prepared by the method described in the experimental section. Their porous properties as checked in both the dry state and in a liquid medium are presented in Table 1. The specific surface area amounting to more than 150 m^2/g is sufficiently large for retentive modes of chromatography. The polymerization conditions, i.e. 40% of crosslinking monomer in the monomer mixture and 60% of porogenic diluent contained in the overall organic phase, were

TABLE 1
Properties of Porous Poly(Vinylphenol-co-Divinylbenzene) Beads

particle size, μm	10
specific surface area, m^2/g [a]	151
pore volume, mL/g [b]	1.45
average pore size, nm	
B.E.T.	11.1
size exclusion chromatography	13.5
mercury porosimetry	15.0
exclusion limit, MW [b]	480 000

[a] According to B.E.T. measurement. [b] According to inverse size exclusion chromatography.

chosen to obtain a chromatographic medium with moderately high exclusion limit and a large pore volume with good mechanical stability. The thermolyzed *tert*-butoxycarbonyl groups represent 33% of the original weight of monomers added in the polymerization. Their removal contributes to an increase in the pore volume which then amounts to 1.45 mL/g. This value compares favorably to standard polymeric separation media and represents a porosity of over 60%. Moreover, since the content of divinylbenzene units in the copolymer does not change in the deprotection step, its actual percentage in the final beads increases to 60 wt%.

The pore size distribution measurements performed both with the beads in THF by means of reverse size-exclusion chromatography, and in the dry state using mercury porosimetry coincide unusually well (Figure 1). This confirms the rigidity of the beads and the negligible swelling of the polymer chains within the beads when used in THF. The almost linear dependency of back pressure on flow rate in the whole range from 0 to 5 mL/min documents the pressure stability of the packing and the lack of particle deformation at high flow rates. It should be pointed out that even at a flow rate of 5 mL/min the back pressure does not exceed 14 MPa (Figure 2) and, owing to its monodispersity, the packing compares favorably to commercial styrene-divinylbenzene packings (17).

Size Exclusion Chromatography

The calibration curve measured in the determination of pore size distribution (Figure 3) already indicated that the porous polymer may be a good packing for SEC. The curve is almost linear in the entire range, from molecular weight of about 100 to 500 000. The pore volume in the 50 mm x 8 mm i.d. column (V_t = 2.5 mL) used for testing was V_i = 0.95 mL. As a low interstitial volume results from the monodispersity of the beads as well as the good packing technique (V_o = 0.76 mL, 30% of column volume),

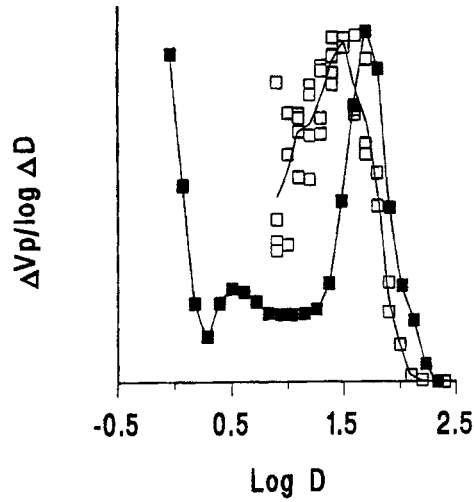

FIGURE 1. Pore size distribution curves of the beads calculated from inverse size exclusion chromatography (■) and from the mercury porosimetry (❑).

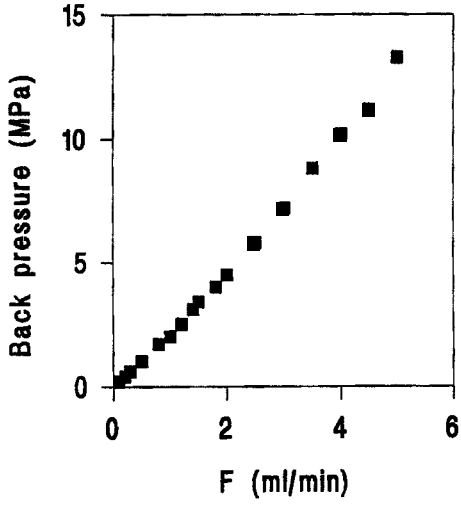

FIGURE 2. Effect of the flow rate on back-pressure. Conditions: column 50 mm x 8 mm i.d.; mobile phase, water-acetonitrile (1:1); analyte, toluene.

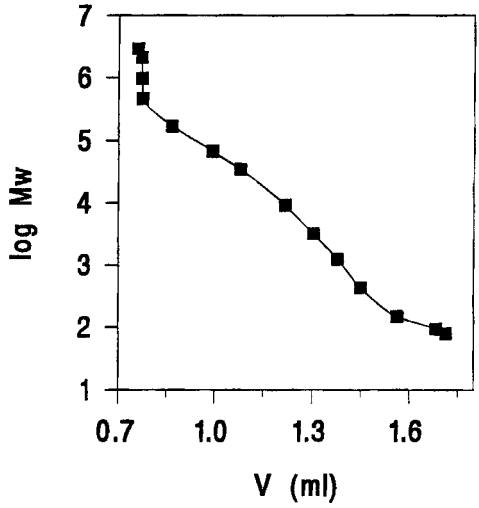

FIGURE 3. Molecular weight calibration with polystyrene standards and toluene in tetrahydrofuran with porous poly(vinylphenol-co-divinylbenzene) beads. Conditions: column 50 mm x 8 mm i.d.; flow rate 0.5 mL/min.

the phase ratio $f = V_i/V_o = 1.26$ is well within the range typical for SEC supports (1). The column efficiency measured with toluene in THF is 27 000 plates/m at a flow rate 0.5 mL/min.

The beads were repacked in a longer column (250 x 7 mm i.d.) and used in the separation of a mixture of polystyrene standards. Figure 4 shows very good resolution between the peaks of toluene and polystyrene with MW 1250, while the other peaks are also separated quite well. The characteristics of the column are similar to those of the shorter one: $V_t = 9.62$ mL, $V_o = 3.30$ mL (34%), $V_i = 3.98$ mL, $f = 1.21$. Column efficiency calculated for toluene was 19 300 plates/m at a flow rate 1 mL/min, 18 200 plates/m at 0.5 mL/min, and 12 800 plates/m at 0.2 mL/min.

Normal Phase Chromatography

The phenol groups on the surface of the beads increase the polarity of the separation medium making it useful for separations in the normal phase mode. The uniformly sized phenolic beads were tested in the separation of more complex mixtures (Figure 5) containing aniline and dimethylaniline (basic compounds), phenol (acidic compound), nitrobenzene (electron acceptor compound), and toluene (nonpolar compound), which is eluted first in hexane containing 5% of ethyl acetate, 2% methanol and 0.1% diethylamine used as the mobile phase. The peaks exhibit some fronting but the separation selectivity is good.

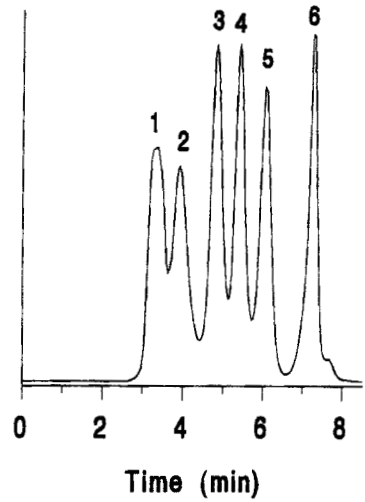

FIGURE 4. Separation of polystyrene standards with molecular weight 295 000 (1), 170 000 (2), 34 000 (3), 9 200 (4), 1 250 (5), and toluene (6). Conditions: column 250 mm x 7 mm i.d.; flow rate 1 mL/min; solvent THF.

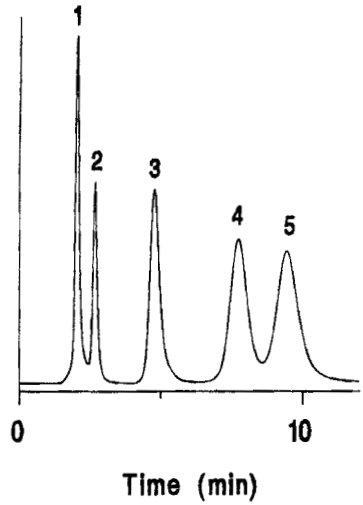

FIGURE 5. Separation of toluene (1), N,N-dimethylaniline (2), nitrobenzene (3), phenol (4), and aniline (5) by normal phase chromatography. Conditions: column 50 mm x 8 mm i.d.; flow rate 1 mL/min; mobile phase: hexane-ethyl acetate-methanol (93:5:2) with 0,1% diethylamine added.

TABLE 2
Capacity Factors k', Separation Selectivities α, and Asymmetry Factors A_s of Poly(Vinylphenol- co-Divinylbenzene) Column as Measured with Chemical Homologs and Other Test Compounds

Compound	k'	α	A_s
benzene	2.74		1.13
		1.33	
toluene	3.64		1.39
		1.32	
ethylbenzene	4.80		1.52
		1.37	
propylbenzene	6.58		1.59
		1.40	
butylbenzene	9.25		1.62
		1.38	
pentylbenzene	12.72		1.78
aniline	1.58		1.61
		1.40	
4-methylaniline	2.21		1.64
		1.39	
3,5-dimethylaniline	3.08		1.75
N,N-dimethylaniline	5.42		1.39
		1.33	
N-ethyl-N-methylaniline	7.23		1.36
		1.39	
N,N-diethylaniline	9.78		1.35
acetone	0.23		1.50
		2.65	
phenol	0.60		1.50
		1.69	
2-methylphenol	1.01		1.34
		1.49	
pyridine	1.50		1.43
		2.03	
nitrobenzene	3.05		1.63

Conditions: column 50 mm x 8 mm i.d.; mobile phase, acetonitrile-water 1:1; flow rate 1 mL/min.

Reversed Phase Chromatography

Since reversed phase HPLC is the most widely used mode of chromatography, it was useful to determine the capabilities of the packing in this chromatographic mode. The hexane based solvents in the column used in the previous tests were simply replaced by methanol, then by a mixture of water-acetonitrile without removing the column from the chromatograph. Several separations of mixtures, each containing very similar compounds, were made in an isocratic mode to confirm the usefulness of the packing medium in reversed phase chromatography. Table 2 summarizes the retention data, selectivities and peak symmetry factors obtained in some model separations. The peak symmetry factor of all separated compounds ranges from 1.13 to 1.78 and is generally good. An excellent peak symmetry ranging from 1.35 to 1.39 is characteristic of the separations of N-substituted anilines.

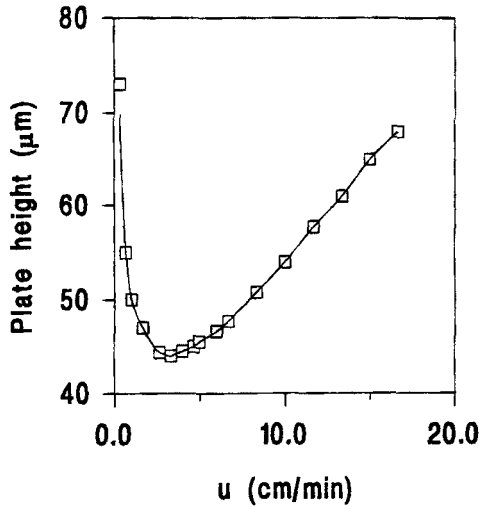

FIGURE 6. *Effect of the linear flow velocity on the efficiency of poly(vinylphenol-co-divinylbenzene) column. Conditions: column 50 mm x 8 mm i.d.; mobile phase, water acetonitrile (1:1); analyte toluene.*

A plot of plate height *vs.* linear flow velocity (Figure 6) obtained for the monodisperse 10 μm poly(vinylphenol) beads from a measurement in 1:1 v/v acetonitrile-water, shows that the plate height achieved with the beads is comparable to that for poly(styrene-co-divinylbenzene) stationary phases (17,18) with the optimum linear velocity at about 1 ml/min.

The separation of a mixture similar to that used in normal phase chromatography is shown in Figure 7. Obviously, toluene is now eluted last as it is the most non-polar compound in the mixture, while phenol, which is more polar, is located close to the front of the chromatogram. The inverse elution sequence is typical for the change from normal to reversed phase chromatography.

The non-polar alkylbenzenes are baseline separated within 20 minutes at a flow rate of 1 mL/min (Figure 8). The standard linear relationship between the logarithm of number of carbon atoms in alkylbenzenes in the series from benzene to pentylbenzene (Figure 9) has a slope of 0.134 which is close to that found for styrene-divinylbenzene packings (19).

The separation of ring and N-alkyl substituted anilines may be driven by both polarity and hydrogen bonding between the solutes and the stationary phase. The poly(vinylphenol) packing separates substituted anilines very well (Figure 10a). Figure 10b shows an example of separation of benzylamine and N-substituted anilines.

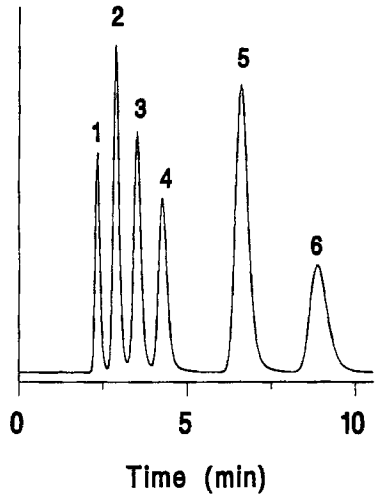

FIGURE 7. Separation of acetone (1), phenol (2), 2-methylphenol (3), pyridine (4), nitrobenzene (5), and toluene (6) by reversed-phase chromatography. Conditions: column 50 mm x 8 mm i.d.; flow rate 1 mL/min; mobile phase: acetonitrile-water (1:1).

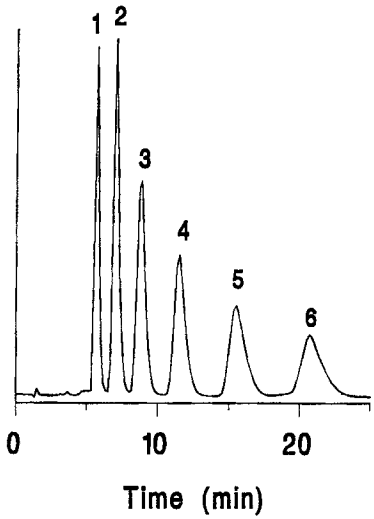

FIGURE 8. Separation of alkylbenzenes by reversed-phase chromatography. Conditions: column 50 mm x 8 mm i.d.; flow rate 1 mL/min; mobile phase: acetonitrile-water (1:1). 1- benzene, 2-toluene, 3-ethylbenzene, 4-propylbenzene, 5-butylbenzene, 6-pentylbenzene.

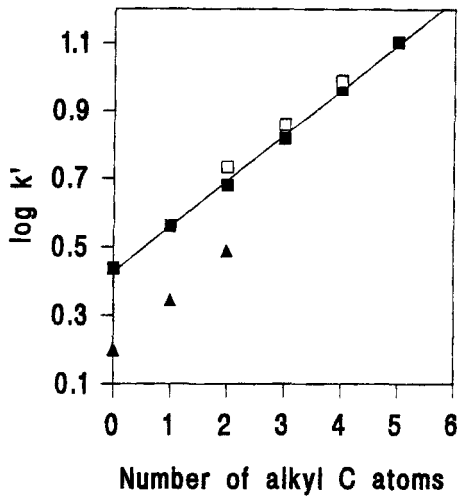

FIGURE 9. Variation of log k' with the number of carbon atoms for compounds in homologous series. Conditions: column 50 mm x 8 mm i.d.; flow rate 1 mL/min; mobile phase: acetonitrile-water (1:1). Alkylbenzenes (■), N-alkylanilines (□), ring alkyl substituted anilines (▲).

The values of log k' appear to be a linear function of the carbon atom number with a slope similar to that of other separations (Figure 9). In contrast to styrenic phases (19), the retention of N-alkylanilines exceeds the retention of alkylbenzenes. This may be assigned to the stronger interaction of the N-alkylaniline with the phenolic hydroxyls of the packing. The retention of ring-substituted anilines is lower. Benzene and aniline behave as the first members of the alkylbenzenes and ring substituted anilines homologous series, respectively, while aniline does not fit as the parent compound in terms of its retention behavior within a series of N-substituted anilines.

The pore size of the beads and their good efficiency in isocratic reversed-phase chromatography of small molecules also suggests the use of this chromatographic medium for the separation of proteins. The gradient elution separation of a model mixture containing 6 proteins is depicted in Figure 11. The proteins are well separated at the flow rate of 1 mL/min in a 20 min gradient (Figure 11a). Increased flow rate and decreased gradient time make the chromatography faster but at the expense of lower resolution (Figure 11b). Despite the presence of phenolic hydroxyl groups on the surface of the polymer matrix, there is no irreversible protein adsorption in the beads. Table 3 documents the very high recovery (close to 100 %) for all individual proteins tested under reversed phase conditions.

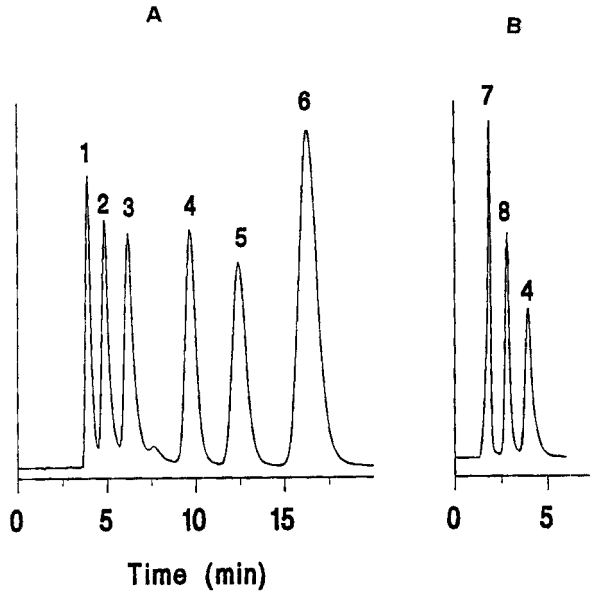

FIGURE 10. Separation of aniline derivatives by reversed-phase chromatography. Conditions : column 50 mm x 8 mm i.d.; flow rate 1 mL/min; (a) mobile phase, acetonitrile-water 1:1. 1-aniline, 2- 4-methylaniline, 3- 3,5-dimethylaniline, 4- N,N-dimethylaniline, 5- N-ethyl-N-methylaniline, 6- N,N-diethylaniline; (b) mobile phase, methanol -water-THF (70:25:5) with 0.6% acetic acid; 7- benzylamine, 8- N-methylaniline.

TABLE 3
Protein Recovery from a Poly(Vinylphenol-co-Divinylbenzene) Column

Protein	Recovery, %
Ribonuclease	98
Cytochrome C	104
Lysozyme	99
Human serum albumin	107
Carbonic anhydrase	94

Conditions: column 50 mm x 8 mm i.d.; mobile phase 0.1% v/v aqueous trifluoroacetic acid/acetonitrile (1:1 v/v); flow rate 1 mL/min; protein concentrations 10 mg/mL; injected volume 20 μL.

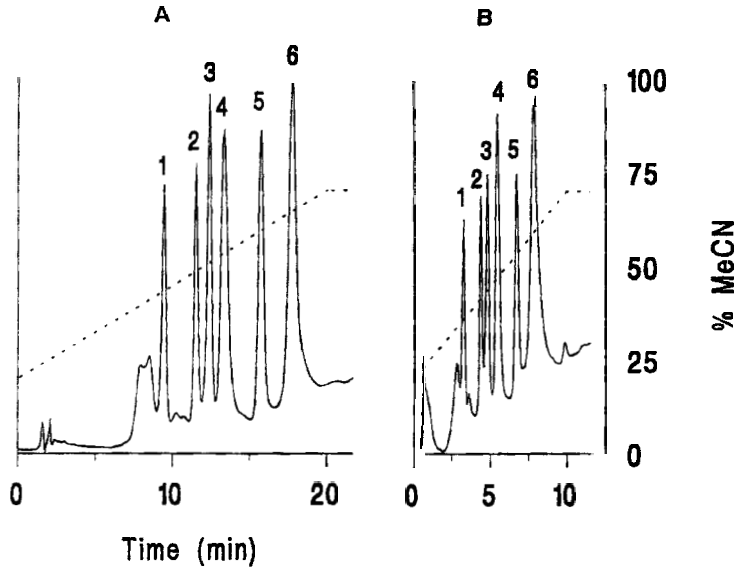

FIGURE 11. Separation of protein mixture by reversed-phase chromatography. Conditions: column 50 mm x 8 mm i.d.; mobile phase, 0.1% trifluoroacetic acid in water containing 20% acetonitrile (eluent A) or 70% acetonitrile (eluent B). 1- ribonuclease A, 2- cytochrome C, 3- lysozyme, 4- human serum albumin, 5- carbonic anhydrase, 6- chicken egg albumin. a) flow rate 1 mL/min, gradient time (from 100% eluent A to 100% eluent B) 20 min; b) flow rate 3 mL/min, gradient time 10 min.

Column Stability

As most of the measurements described above were done on a single column, the chromatographic mode had to be changed from reversed phase to normal phase and back several times. Since these changes did not appear to have any effect on the efficiency, k' values, and on the back pressure a systematic test of reproducibility of chromatographic properties was initiated. This test involved 300 injections of toluene or a mixture of benzene, toluene and ethylbenzene in acetonitrile-water (1:1 v/v) interspersed with over 100 injections in other modes (e.g. normal phase and gradient elution) within a three months period. A typical protocol for this long-term test is as follows:

(a) 25 Injections of hydrocarbon mixture, each tenth injection being toluene alone.

(b) 20 Protein injections in a gradient mode.

(c) Repeat hydrocarbon injections as in (a), each tenth injection being toluene alone until a total of 100 hydrocarbon injections (10 toluene data points).

(d) Wash the column with aqueous sodium hydroxide (1 mole/L), then acidify.

(e) 50 Hydrocarbon injections as above.

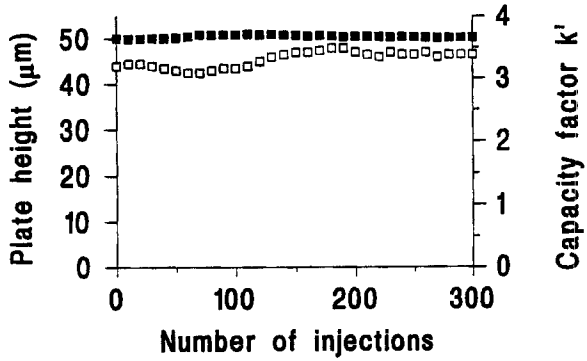

FIGURE 12. Stability of poly(vinylphenol-co-divinylbenzene) column as measured from changes of plate height (□) and toluene capacity factor k' (■) upon multiple injections. Conditions: column 50 mm x 8 mm i.d.; mobile phase, water-acetonitrile (1:1 v/v); flow rate, 1 mL/min; analyte, toluene.

(f) Switch to normal phase and perform 50 injections of a mixture of toluene, aniline, and N,N-dimethylaniline under the conditions of Figure 5.

(g) 50 Hydrocarbon injections as above.

(h) Repeat washing as in (d).

(i) Repeat (e), (f), (g), (h).

Figure 12 documents the hydrocarbon injections in reverse phase and shows that the column efficiency only decreases very slowly with the number of injections while the capacity factor does not change. It should be emphasized that Figure 12 shows only the injections of toluene which were interspersed with other injections as noted above.

No changes in selectivity was observed during the series of 300 injections. Column washing was carried out using 50 volumes of 1 mol/L aqueous sodium hydroxide to determine whether or not phenol group ionization had an effect on the reproducibility of the chromatographic separation. The original column properties were restored within 10 hours of equilibration in a flow of mobile phase containing water, acetonitrile and trifluoroacetic acid. The back pressure *vs.* flow rate dependency did not differ from that shown in Figure 2 even after using the same column for a total of more than 500 injections. These results clearly document the stability of the column properties even after numerous chromatographic mode changes and washing with sodium hydroxide. This makes the column superior to the silica based media as no silica phases can withstand the washing with NaOH used in the testing protokol of this medium.

CONCLUSION

Poly(4-hydroxystyrene-co-divinylbenzene particles prepared by a swelling of polystyrene shape template particles followed by a suspension polymerization affords uniformly sized spherical beads are highly chemically stable and show the rigidity and durability necessary to withstand rapid changes from highly polar to non polar solvents. The beads are versatile and can be used efficiently for size-exclusion, normal phase and reversed-phase chromatography of low molecular weight compounds as well as proteins. In contrast to the preparation of typical polymeric separation media, the preparation method used also includes a deprotection step at a relatively high temperature and results in a polymeric separation medium with increased rigidity and minimized swelling ability. The phenolic moieties in the beads contribute greatly to the separation process through their interaction with polar compounds. In comparison with the water compatible modified polystyrene stationary phase (20), the poly(vinylphenol) phase do not require any additional chemical modification procedure and is more flexible for solvent changes.

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