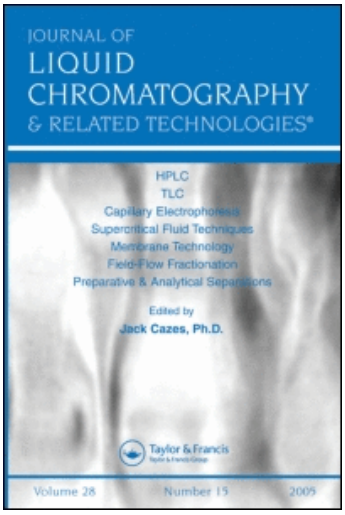

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A Novel Polar Separation Medium for the Size Exclusion Chromatography of Small Molecules: Uniformly Sized, Porous Poly(vinylphenol-*co*-divinylbenzene) Beads

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A NOVEL POLAR SEPARATION MEDIUM FOR THE SIZE EXCLUSION CHROMATOGRAPHY OF SMALL MOLECULES: UNIFORMLY SIZED, POROUS POLY(VINYLPHENOL-*CO*-DIVINYLBENZENE) BEADS

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ABSTRACT

A novel separation medium based on uniform size beads of 4-hydroxystyrene-divinylbenzene can be used for both the size exclusion chromatography of small molecules and their separation by reversed-phase chromatography. The new 5 μm material is prepared by the controlled swelling of monodispersed polystyrene particles, that serve both as porogen and shape template, with a polymerization mixture consisting of the monomers and dibutylphthalate, followed by a suspension polymerization. Removal of the acetoxy groups by hydrolysis with aqueous base leads to the final poly(4-vinylphenol-*co*-divinylbenzene) monodispersed beads. Polymerization conditions that favor the formation of very small pores were

developed to optimize the beads for the SEC separation of small molecules. The SEC calibration curve confirms that the optimized beads contain a large volume of pores suitable for the separation of solutes with a molecular weight of up to about 1000. The ability to separate alkylbenzenes according to their hydrodynamic sizes has been demonstrated using a column packed with this material. In addition, the phenol chemistry used in combination with a hydrophobic crosslinking monomer provides this separation medium with an unusual versatility that allows both reversed phase and normal phase chromatography to be run in the same column after a simple change of the mobile phase.

INTRODUCTION

The range of commercially available separation media and packed columns for HPLC. as well as the number of new chromatographic techniques, has grown rapidly in recent years. This development is stimulated by the need to achieve quickly and reliably the complete separation of an ever increasing variety of mixtures. The major share of the market for HPLC packings for both reversed phase and normal phase chromatography is accounted for by silica based materials, while polymeric media prevail for both the size-exclusion and the ion-exchange modes.[1] Although synthetic polymers with many different chemistries have been explored in order to obtain separation media with desired selectivity and efficiency, the vast majority of present day HPLC column packings are still based on only a few types of monomers, most frequently a combination of non-polar styrene and divinylbenzene.[2]

More polar packings have been prepared from monomers that contain charged groups or aliphatic hydroxyl groups. While the former are widely used for ion-exchange chromatography, the latter have found limited applications mainly in the aqueous size-exclusion chromatography of biopolymers. Crosslinked polymers with polar functionality derived from 2-hydroxyethyl methacrylate, 2,3-dihydroxyethyl methacrylate, vinyl alcohol, vinyl pyrrolidone and other hydroxylated monomers are examples of such materials.[1]

In contrast to these aliphatic monomers, vinylphenol is a good candidate for the preparation of styrenic-type polymers that contain hydroxyl groups. However, until recently, porous beads of vinylphenol remained inaccessible because this monomer is difficult to handle and does not polymerize well.[3] The situation changed dramatically when styrenic monomers containing protected phenolic groups became commercially available. We first demonstrated six

years ago that the standard suspension polymerization of 4-tert-butoxycarbonyloxystyrene[3] or its ring substituted derivatives with divinylbenzene, followed by removal of the hydroxyl protecting group, provides separation media that are suitable for the separation of amines by normal phase chromatography.[4,5] A very significant improvement of this earlier work was accomplished through a preparative method that afforded uniformly sized beads. In this method, small, monodisperse shape-template polymer particles are swollen with a mixture of 4-*tert* butoxycarbonyloxystyrene, divinylbenzene, and cyclohexanol (porogenic solvent), then subjected to suspension polymerization and thermal deprotection.[6] This uniformly sized phenolic separation medium, which has a broad pore size distribution with a maximum centered at about 50 nm, is very versatile in the separation of both small molecules and proteins using different chromatographic modes. Because the phenol group is a powerful hydrogen bond donor, the beads were also used as a very efficient tool in the measurement of relative hydrogen bond basicities of dilute compounds, which is a process that cannot be carried out using typical silica based phases due to silanophilic interactions.[7]

This report describes a novel approach to the preparation of uniformly sized porous poly(vinylphenol-*co*-divinylbenzene) beads that are uniquely suited for the hitherto elusive process of size-exclusion chromatography of small molecules, using commercially available acetoxystyrene.

EXPERIMENTAL

Materials

Styrene (99%, Aldrich) and divinylbenzene (91%, Dow Chemical) were extracted with 10% aqueous sodium hydroxide and water, dried over anhydrous magnesium sulfate, and distilled under vacuum. Azobisisobutyronitrile (AIBN) was obtained from Kodak, and 4-acetoxystyrene from Hoechst Celanese. Dibutylphthalate, benzoyl peroxide, 1-chlorodecane, and sodium dodecyl sulfate were purchased from Aldrich. All solvents were HPLC grade.

Preparation of Soluble Polystyrene Shape Templates

Monodisperse polystyrene shape templates obtained by emulsion polymerization[8] (1.0 μm, 22% solid in water, 0.3 mL) were swollen by adsorption of an emulsion of 1-chlorodecane (0.6 mL) in 0.25% aqueous

sodium dodecyl sulfate (SDS) solution (30 mL). After the droplets of the emulsified solvent had completely disappeared (about 24-30 h as verified by optical microscopy), a solution of benzoyl peroxide (0.13 g) in styrene (2.6 mL) emulsified in 0.25% aqueous SDS (30 mL) was added to the dispersion resulting from the previous step and the mixture was stirred slowly for 6 h. After transfer of the monomer to the shape templates was completed, sodium nitrite (0.01 g) and 5% solution of poly(vinyl alcohol) (PVA, MW 85,000-146,000, 88% hydrolyzed) was added to adjust the total concentration of PVA in the mixture to 1%, and the system was purged with nitrogen for 20 min. The polymerization was carried out in sealed 500 mL Erlenmeyer flasks placed in a shaker bath (Lab-Line) at 240 rotations/min and 70°C for 24 h. The resulting enlarged monodisperse particles of linear polystyrene porogen were used in the next step without further purification.

Preparation of Insoluble Porous Beads

A mixture containing p-acetoxystyrene (6 mL), divinylbenzene (4 mL), dibutylphthalate (3.8 mL), and AIBN (0.1 g) was emulsified by sonication in 0.25% aqueous SDS solution (30 mL), and added to the aqueous dispersion of polymer porogen particles. The mixture was stirred at room temperature until the emulsified liquid was completely transferred to the porogen particles. Sodium nitrite (0.009 g) and 5% aqueous PVA solution was added to adjust the total concentration of PVA to 1%, and the dispersion was purged with nitrogen for 20 min. The flask was sealed and the polymerization was carried out at 70°C and 240 rotations/min for 24 h. The resulting beads were decanted repeatedly with water and tetrahydrofuran till the supernatant liquid remained clear. The polymeric porogen was removed by extraction with toluene in a Soxhlet apparatus for 36 h, and the porous beads were dried under vacuum. The yield was 92%.

Hydrolysis of Porous Beads

A solution of potassium hydroxide (85%, 3.0 g) in water (15 mL) and methanol (15 mL) was added dropwise to poly(4-acetoxystyrene-*co*-divinylbenzene) porous beads (3.0 g) suspended in methanol (30 mL). After stirring at room temperature for 24 h, the suspension was diluted with water, and filtered through a 4-8 μm sintered glass filter. The beads were washed several times with water, followed by methanol, then dried under vacuum.

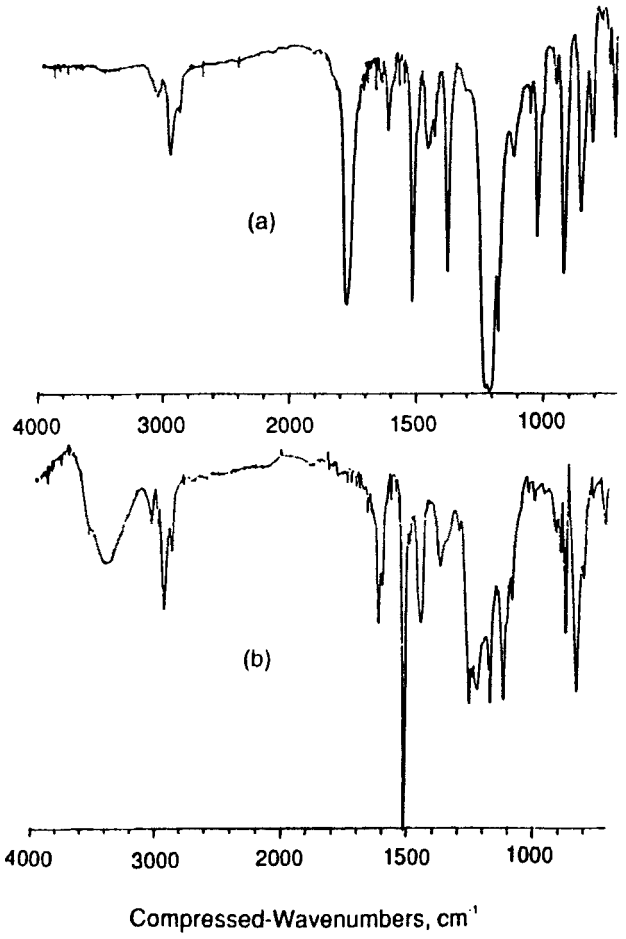


Figure 1. IR spectra of poly(acetoxystyrene-*co*-divinylbenzene) (a) and of the polymer after reaction with aqueous base (b).

Characterization of Porous Properties

The specific surface area of the beads was calculated from the BET isotherm of nitrogen. The pore size distribution in the dry state was determined by mercury porosimetry using an automated custom-made combined BET-sorptometer and mercury porosimeter from Porous Materials Inc., Ithaca, NY, USA.

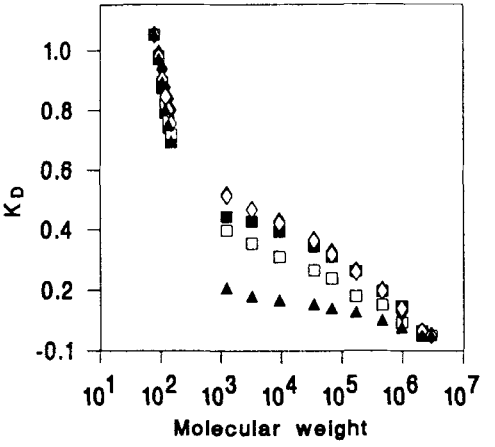


Figure 2. Size-exclusion calibration curve of poly(vinylphenol-*co*-divinylbenzene) beads with polystyrene standards and alkylbenzenes in tetrahydrofuran; co-porogen, 4-methyl-2-pentanol (■), decyl alcohol (□), 1-chlorodecane (♦), heptane (◊), dibutylphthalate (Δ). Conditions: column 150 x 4.6 mm i.d.; flow rate 1 mL/min.; UV detection at 254nm.

Chromatography

A Waters HPLC system consisting of two 510 HPLC pumps, a 717 plus autosampler, and a 486 UV detector, was used to carry out all the chromatography. The data was acquired and processed with Millenium 2010 software (Waters). Particles were packed from a tetrahydrofuran suspension into 150 x 4.6 mm i.d. and 300 x 7.8 mm i.d. stainless steel columns.

RESULTS AND DISCUSSION

In contrast to silica based porous packings, typical porous polymers are characterized by bimodal pore size distribution curves.[2,8-11] In addition to mesopores (2-50 nm) and macropores (over 50 nm), the polymers always contain some micropores with a diameter smaller than 2 nm.[10,11] Due to these micropores, the size-exclusion calibration curves exhibit a plateau for molecules with a molecular weight of less than about 500, instead of a sharp lower exclusion limit.

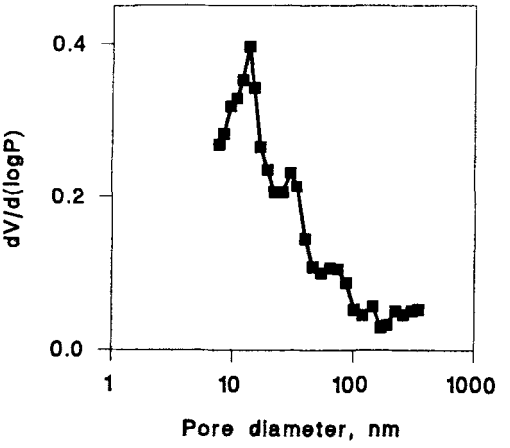


Figure 3. Differential pore size distribution curve of the poly(vinylphenol-*co*-divinylbenzene) beads measured by mercury porosimetry.

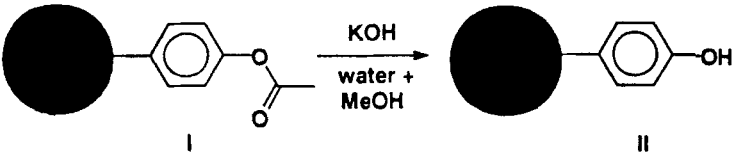
These pores do not play any role in the separation of large molecules;[8] however, it is believed that the micropores, which cannot be avoided, are the major difference between polymeric and silica based separation media in reversed phase chromatography of low molecular weight compounds, and affect both the column efficiency and the separation selectivity.[8,10]

Since it is impossible to prepare porous polymer beads without these micropores, we explored an unusual approach designed to enhance this formation particularly in a size range suitable for the size-exclusion chromatographic separation of small molecules.

Preparation of Beads

The method used for the preparation of uniform, porous poly(4-acetoxystyrene-*co*-divinylbenzene) beads I includes (i) the preparation of relatively large monodisperse polymer porogen beads from uniform latex shape-template particles, (ii) their controlled swelling with a polymerization mixture consisting of 4-acetoxystyrene, divinylbenzene and dibutylphthalate, and finally (iii) the suspension polymerization of the swollen polymeric porogen beads. This technique is well suited for monomers with a low water solubility, such as styrene and divinylbenzene, and also for 4-acetoxystyrene, which is relatively non-polar and hydrophobic. The extent of swelling, and the

ratio of the volumes of the various components of the polymerization mixture used in step (ii) to the volume of the polymer porogen beads, define the size of the final beads, and their porous characteristics.[12-15] Once the polymerization is complete, the porous poly(4-vinylphenol-*co*-divinylbenzene) beads II are obtained by base hydrolysis of the acetate groups (Scheme 1). The hydrolysis



reaction is readily monitored by IR spectroscopy because hydrolysis is accompanied by loss of the strong ester carbonyl band near 1765 cm^{-1}, and the appearance of a broad hydroxyl band centered at about 3400 cm^{-1} (Figure 1).

Physical Properties

Current separation media for HPLC are beads with a diameter in the range of 3-10 μm. The size uniformity of these beads contributes to both better efficiency and lower back pressure. Therefore, typical manufacturing processes involving beads prepared by suspension polymerization require a size fractionation step that is tedious, and a significant part of the batch ends in waste. Our method directly provides a high yield of monodisperse beads. The size of the beads used in this study was measured from scanning electron micrographs, and found to be 5 μm.

The use of a polymeric porogen allows fine tuning of the porous properties of monodisperse beads within a very broad range during the process of their preparation.[12-15] Because this study was aimed at beads optimized for the separation of small molecules, preparative conditions had to be adjusted in order to incorporate as many small pores as possible. The porogen system of linear polystyrene of molecular weight 64,000[15] and a co-porogen solvent, intentionally makes up only 31% by volume of the monomer/porogen mixture in order to keep the pores as small as possible. The use of various co-porogens results in beads that contain different percentages of pores, particularly in the size range above 50 nm (Table 1). The change in volume of large pores affects the distribution coefficients for alkylbenzenes and polystyrene standards (Figure 2). An increase in the volume of these pores results in decreased separation

Table 1

Effect of Co-Porogen on Total Pore Volume, V_p, Measured by Mercury Intrusion Porosimetry, of Poly(4-vinylphenol-co-divinylbenzene) Beads

Co-Porogen	V_p (mL/g)	Pore Volume in Range, %		
		<10nm	10-50nm	50-500nm
dibutylphthalate	0.27	15	59	26
4-methyl-2-pentanol	0.31	16	52	32
decyl alcohol	0.34	18	47	35
1-chlorodecane	0.38	11	53	37
heptane	0.32	13	53	34

selectivity of compounds with molecular weight less than 1000. The polymerization in the presence of dibutylphthalate provides beads with the best selectivity for the separation of small molecules. The pore size distribution of these beads, measured in the dry state by mercury porosimetry (Figure 3) shows that the beads have a total pore volume of 0.27 mL/g, which translates to a modest porosity of about 30% and correlates well to the total volume of porogens added to the polymerizing system. Although this level of porosity is low when compared to standard polymeric separation media, over 70% of the total pore volume is located in pores smaller than 80 nm in diameter. As a result, and in contrast to typical porous polymeric separation media, almost all of the pores have sizes that are nearly ideal for the separation of small molecules. In addition, because of their lower porosity, these beads have much better mechanical stability than standard polymeric media. The scanning electron micrographs in Figure 4 document the porous structure of the beads. The specific surface area of the beads was found to be 66 m^2/g, which is mainly due to the mesopores and is consistent with the pore volume.

Suitability in Interactive Chromatography

Because the polar surface chemistry of the poly(4-vinylphenol-*co*-divinylbenzene) beads is different from that of poly(styrene-*co*-divinylbenzene), it is useful to determine the capabilities of the packing in reversed phase and normal phase HPLC, since these are the most widely used modes of chromatography.

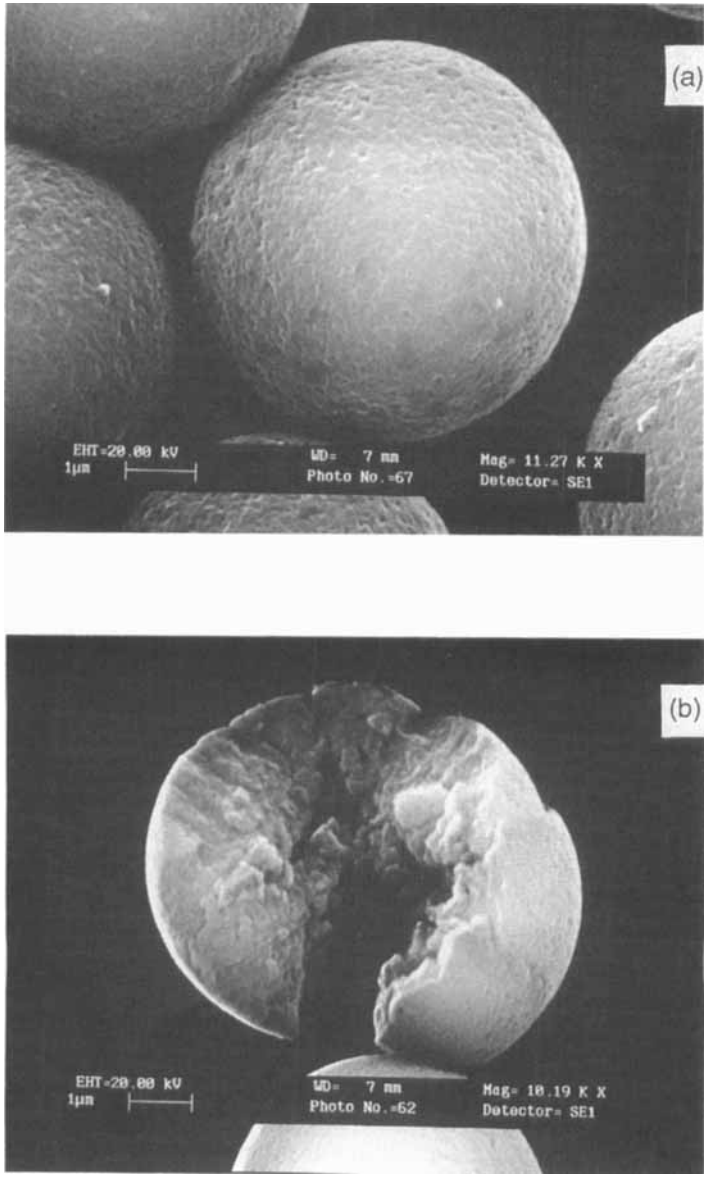


Figure 4. Scanning electron micrographs of the surface (a), and internal structure (b) of monosized poly(4-vinylphenol-*co*-divinylbenzene) beads.

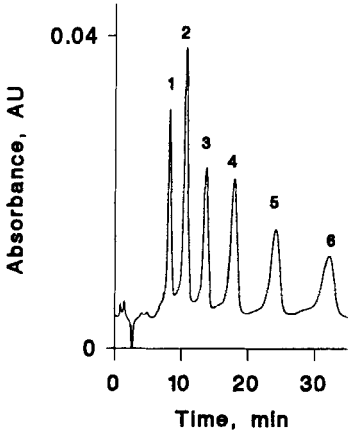


Figure 5. Separation of alkylbenzenes by reversed-phase chromatography. Conditions: 5 μm poly(vinylphenol-*co*-divinylbenzene) beads; column 150 x 4.6 mm i.d.; mobile phase: acetonitrile-water (1:1); flow rate 1 mL/min. Peaks: benzene (1), toluene (2), ethylbenzene (3), propylbenzene (4), butylbenzene (5), pentylbenzene (6).

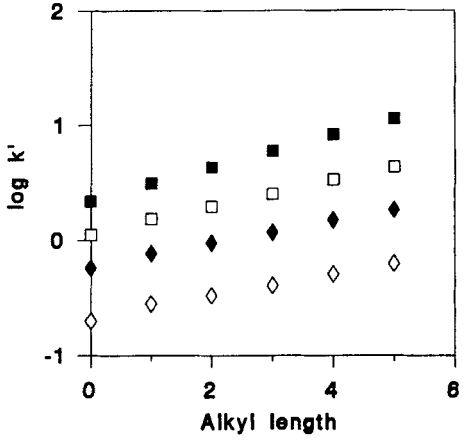


Figure 6. Variation of log *k'* with the number of carbon atoms in alkyl substituents for a homologous series of alkylbenzenes. Conditions: column 150 x 4.6 mm i.d.; flow rate 1 mL/min, mobile phase: acetonitrile/water 50/50 (■), 60/40 (□), 70/30 (♦), and 60/40 (◊).

The separation of a series of alkylbenzenes by reversed phase chromatography was investigated at different mobile phase compositions. With a mobile phase consisting of a 1:1 mixture of acetonitrile and water, the alkylbenzenes are baseline separated (Figure 5). Figure 6 shows that the standard linear relationship between the log k' and number of carbon atoms in alkylbenzenes in the series from benzene, which elutes first, to pentylbenzene is observed. As a result of the high polarity of the packing, separation is already achieved at a relatively low content of acetonitrile in the mobile phase. At a mobile phase composition of 1:1 acetonitrile and water, the line has a slope of 0.143, which is close to that found for styrene-divinylbenzene packings, but in a mobile phase containing 30% percent less acetonitrile.

A characteristic of these poly(vinylphenol-*co*-divinylbenzene) beads is that once packed in a column, they can withstand rapid changes of solvents without significant loss of separation ability. This allows their use in a variety of chromatographic modes. As expected, the presence of phenolic groups on the surface of the beads increases the polarity of the separation medium, making it useful also for separations in the normal phase mode.[5,6] Therefore, the uniformly sized phenolic beads prepared in this study were tested again in the normal phase separation of a mixture containing acidic (phenol), basic (N,N-dimethylaniline and aniline), non-polar (toluene), and electron acceptor compound (nitrobenzene), using hexane containing 5% ethyl acetate, 2% methanol, and 0.1% diethylamine as a mobile phase. All of the compounds are completely separated, and the overall selectivity is good, despite some tailing (Figure 7).

Size Exclusion Chromatography

Size-exclusion chromatography is a powerful technique used to separate molecules according to their effective size in solution. Molecules larger than the largest pore size of the column packing are excluded, and are first to be eluted. Molecules that can penetrate all the pores are entrapped, and are therefore the last to be eluted. The average residence time in the pores depends on the relative sizes of the molecules. While SEC of large molecules is a well established method for characterization of polymers, few chromatographic packings are available for the separation of small molecules using size-exclusion chromatography.[16]

In this work, the pore size distribution profile of the beads has been optimized for size-exclusion chromatography of small molecules. The volume of pores with a diameter in the range 10-300 nm, measured in the dry state by mercury porosimetry, represent about 0.3 mL/g of the polymer. Because the

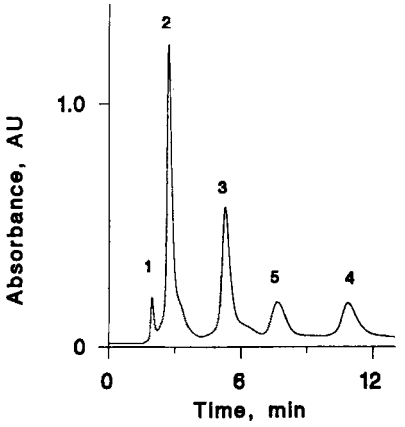


Figure 7. Separation of toluene (1), N,N-dimethylaniline (2), nitrobenzene (3), phenol (4), and aniline (5) by normal phase chromatography. Conditions: 5 μm poly(vinylphenol-*co*-divinylbenzene) beads; column 150 x 4.6 mm i.d.; flow rate 1 mL/min; mobile phase: hexane-ethyl acetate-methanol (93:5:2) with 0.1% diethylamine added.

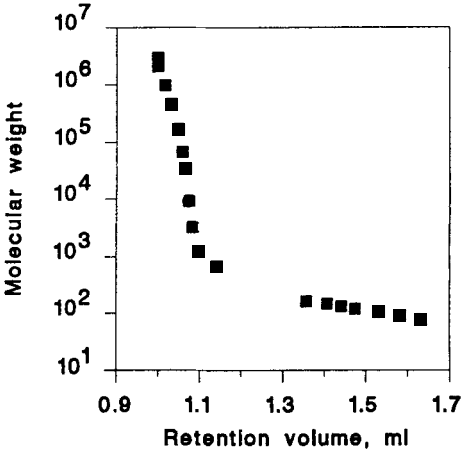


Figure 8. Size-exclusion calibration curve of poly(vinylphenol-*co*-divinylbenzene) beads with polystyrene standards and alkylbenzenes in tetrahydrofuran. Conditions: column 150 x 4.6 mm i.d.; flow rate 1 mL/min.; UV detection at 254nm.

150 x 4.6 mm i.d. column contains 1.7 g of the packing, the entire volume of pores in this column is 0.5 mL. This correlates well with the pore volume of 0.6 mL, found from the size exclusion calibration curve measured with a series of alkylbenzenes and polystyrene standards with molecular weights from 78 to 2,100,000 (Figure 8). However, about 80 % of this effective pore volume is only available for the separation of alkylbenzenes and styrene oligomer standards with molecular weight lower than 1000. The calibration curve is linear in the range of molecular weights 80-1000 and its slope is low. This translates into an excellent size-exclusion selectivity for low molecular weight solutes. The separation of larger molecules is poor as documented by the part of the calibration curve that becomes very steep at higher molecular weights. A low exclusion limit of the separation medium results from the low density of macropores, as indicated by the almost complete lack of separation of polystyrene standards above 1000 molecular weight.

A longer column (300 x 7.8 mm i.d.) packed with the beads was used in the separation of a mixture of benzene, ethylbenzene, amylbenzene, and two polystyrene standards. Figure 9 shows the good resolution achieved between the peaks of the low molecular weight species, which in the case of benzene and ethylbenzene, differ by only 28 g/mol. Column efficiency calculated for ethylbenzene is 32,600 plates/m at a flow rate of 1 mL/min.

A plot of plate height vs. linear flow velocity (Figure 10) obtained for the monodisperse 5 mm poly(4-vinylphenol-*co*-divinylbenzene) beads from a measurement with toluene in THF shows that the plate heights that can be achieved with these phenolic beads are comparable to those for poly(styrene-*co*-divinylbenzene) stationary phases.[3,16] The optimum linear velocity is obtained at about 0.8 mL/min. The back pressure of the column is a linear function of the flow rate and documents the pressure stability of the packing. Even at a flow rate of 3.5 mL/min, the back pressure was only 8 MPa.

CONCLUSION

The porous properties of poly(4-vinylphenol-*co*-divinylbenzene) beads prepared by the suspension polymerization of 4-acetoxystyrene and divinylbenzene within uniform particles of linear polystyrene, serving both as porogen and shape template, can be controlled. For example, to match the needs of a particular application, an optimized procedure provides monodisperse beads tailored for the separation of low molecular weight compounds. Although these chemically stable beads are a versatile packing material for both reversed phase and normal phase HPLC with great tolerance for solvent changes, their major field of application is in the size-exclusion

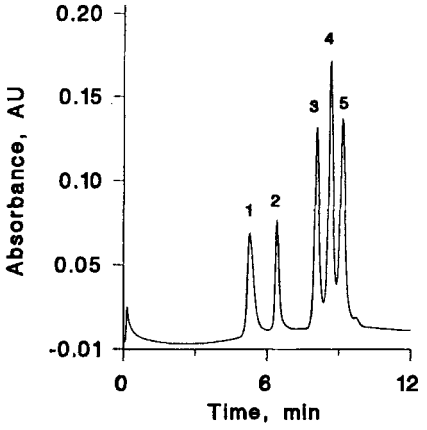


Figure 9. Separation of polystyrene standards with molecular weight 2 950 000 (1), 9200 (2), pentylbenzene (3), ethylbenzene (4), and benzene (5) by size-exclusion chromatography. Conditions: 5 μm poly(4-vinylphenol-*co*-divinylbenzene) beads; column 300 x 7.8 mm i.d.; flow rate 1 mL/min; solvent tetrahydrofuran.

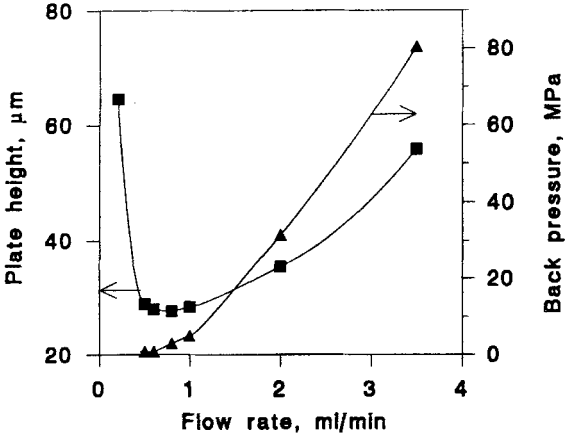


Figure 10. Effect of the linear flow velocity on efficiency and back pressure of column packed with 5 μm poly(4-vinylphenol-*co*-divinylbenzene) beads. Conditions: column 300 x 7.8 mm i.d.; mobile phase, tetrahydrofuran; analyte, benzene.

chromatography of small molecules with a molecular weight of less than 1000, which cannot be separated using reversed phase or normal phase chromatography. In this application, the separation of a homologous series of alkylbenzenes with very small differences in molecular weight is readily achieved.

ACKNOWLEDGEMENT

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