SEPARATION OF HYDROPHILIC OLIGOMERS AND POLYMERS USING MONODISPERSE POLY(2,3-DIHYDROXYPROPYL METHACRYLATE-*co*-ETHYLENE DIMETHACRYLATE) BEADS *via* NORMAL-PHASE AND HYDROPHILIC-INTERACTION HPLC

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Microparticulate, monosized, and macroporous poly(2,3-dihydroxypropyl methacrylate-*co*-ethylene dimethacrylate) beads have been used as a stationary phase for HPLC separations of hydrophilic oligomers and polymers. Homogeneous coverage of the sorbent surface with a large number of chemically equivalent diol functionalities affords suitable retentivity in both normal-phase and hydrophilic-interaction chromatographic modes and enables the separations of water-soluble oligomers and polymers. Chromatographic properties of this stationary phase are demonstrated on a variety of separations of poly(oxyalkylene)s, polyvinylpyrrolidones, and polysaccharides.

Keywords: Normal-phase HPLC; Hydrophilic-interaction chromatography; Stationary phases; Polymeric-diol beads; Poly(glycidyl methacrylate-*co*-ethylene dimethacrylate); Poly(2,3-dihydroxypropyl methacrylate); Poly(oxyalkylene)s; Polysaccharides; Cyclodextrins; Polymers.

Normal-phase chromatography (NPLC) in which the stationary phase is more polar than the mobile phase is known since the inception of chromatography almost 100 years ago[1–3]. NPLC is well suited for the separation of a number of organic compounds including those only slightly differing in their structures such as positional isomers or enantiomers[4–6]. Many of such compounds featuring minute differences, which may have great effects on organisms, are currently produced by pharmaceutical, agrochemical, as well as food industry and request sensitive analytical methods allowing their separation. This appears to be the trigger that revived interest in this "classical" chromatographic mode[7].

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The remarkable selectivity in NPLC separation results from a host of highly specific interactions of functionalities of the analytes with the adsorption sites located on the surface of the separation medium. Understanding of the general mechanism of NPLC based on adsorption phenomena has been laid by Snyder in the 1960s (refs[8–10]). NPLC and its modification – hydrophilic-interaction chromatography[11] have already been used for the separations of a broad spectrum of compounds including both small molecules and polymers[7,12–23]. For example, Jandera studied in detail mechanism of NPLC of polar polymers and his theoretical treatment allowed predicting and optimizing separations in both isocratic and gradient modes[24].

Silica and a few of its derivatives are currently mostly marketed separation media for NPLC (refs[25,26]). They provide excellent mechanical resistance, large surface area, and high column efficiency[27,28]. For example, polar silica-based columns afford excellent separations of poly(oxyalkylene)s both with typical normal-phase mobile phases[13–17] and with supercritical liquids[29] as an eluent.

Macroporous rigid polar organic beads are commonly used for size-exclusion chromatography in aqueous media, as well as for hydrophobic-interaction and affinity chromatography of biopolymers[30]. However, they are rarely used under normal-phase chromatography conditions although these separation media may feature versatility that enables their use in several chromatographic modes[31–36]. In our previous studies, we have demonstrated the use of macroporous monodisperse poly(2,3-dihydroxypropyl methacrylate-*co*-ethylene dimethacrylate) beads for the NPLC separations of low molecular weight compounds[37] and non-polar polymers[38]. In this report, we evaluate the performance of these polar beads in the normal-phase and hydrophilic-interaction HPLC separations of hydrophilic oligomers and polymers. Although similar studies using silica based stationary phases have already been described in the literature (*vide infra*), this is to our best knowledge the first use of synthetic polymer beads as stationary phase for the normal phase separation of a wide variety of polar water-soluble polymers.

EXPERIMENTAL

Chromatographic Column

Monodisperse poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) 10 μm beads were prepared from a polymerization mixture consisting of glycidyl methacrylate (24 wt.%), ethylene dimethacrylate (16 wt.%), cyclohexanol (60 wt.%), and azobisisobutyronitrile (1 wt.% with

respect to monomers) using a modified staged templated suspension polymerization procedure described in detail elsewhere[39,40]. Their epoxide groups were hydrolyzed using 0.5 M aqueous sulfuric acid at 60 °C for 3 h. Complete hydrolysis was confirmed by IR spectroscopy[41]. The pore size distribution of the beads was determined using an Autopore III 9400 mercury intrusion porosimeter (Micromeritics, Norcross, GA). Specific surface areas were calculated from data obtained by nitrogen adsorption/desorption (ASAP 2010, Micromeritics) using the BET equation.

The poly(2,3-dihydroxypropyl methacrylate-*co*-ethylene dimethacrylate) beads were slurry-packed into 15 cm × 4.6 mm i.d. and 30 cm × 8 mm i.d. stainless steel columns with a 50 : 50 (v/v) toluene–cyclohexanol mixture under a constant pressure of 20 MPa.

Equipment

Chromatography was carried out using a Waters system consisting of two or three 510 pumps and a 717plus autosampler, equipped with a PL-EMD 950 evaporative light scattering detector (Polymer Laboratories). The data were acquired and processed with Millenium 2010 software (Waters). Matrix-assisted laser-desorption ionization time-of-the-flight mass spectroscopy (MALDI-TOF MS) was performed using Voyager-DE instrument and datastation (Perseptive Biosystems).

RESULTS AND DISCUSSION

Characteristics of the Stationary Phase

The poly(2,3-dihydroxypropyl methacrylate-*co*-ethylene dimethacrylate) beads used throughout this study were prepared by staged templated suspension polymerization followed by hydrolysis of the epoxide groups to afford packing with up to 5.4 mmol/g hydroxyl functionalities. This very high polarity of our polymeric-diol beads significantly contributes to a strong retention for small molecules under normal-phase conditions that we demonstrated earlier[37].

Our polymerization process provides 10 μm monosized beads with well-controlled porous properties[39,40,42]. Specifically, the pore volume and median pore diameter determined by mercury intrusion porosimetry were 1.1 ml/g and 40 nm, respectively, and the specific surface area calculated from the BET isotherm for nitrogen was 120 m^2/g. These characteristics allow us to predict that macromolecules may penetrate the pores and interact with the inner surface of the stationary phase. Due to the size uniformity of the beads, flow resistance of the packed columns is only modest. For example, a back pressure of only 6 MPa was observed for a 30 cm × 8 mm i.d. column packed with the hydrophilic separation medium using hexane at a rather high flow rate of 10 ml/min. Although this flow rate is not practical for HPLC separations, it demonstrates very good permeability of columns

packed with our uniformly sized beads. Additionally, no problems related to swelling or shrinking were observed using any of the common HPLC solvents typical of normal-phase and hydrophilic-interaction chromatographic modes.

Poly(oxyalkylene)s and Their Derivatives

The separations of poly(oxyalkylene)s according to their size and chemical composition have been achieved in both reversed-phase and normal-phase mode or using supercritical fluid[13–19,29,43–45]. Normal-phase HPLC has been found to be the suitable method for the separation of poly(oxyalkylene)s according to their chain lengths. Many of these separations were considerably improved by using gradient of the mobile phase[46,47]. Therefore, we also used a gradient mode and our "diol" column for the separation of poly(oxyalkylene)s. These molecules, oligomers and polymers of ethylene oxide, contain both terminal hydroxyl groups and internal ether links. Both of these types of functionalities can interact with the diol moieties located within the stationary phase. This interaction is stronger in a non-polar mobile phase and therefore poly(oxyethylenes) are adsorbed on the hydrophilic surface of the beads when the initial mobile phase is rich in hexane. Figure 1 shows a separation of a poly(oxyethylene) standard with a nominal weight average molecular weight of 590 on a 15 cm long column. The individual homologues elute successively in a mobile phase containing an increasing percentage of tetrahydrofuran. Since a smaller number of interacting ether functionalities is present in the shorter oligomers than in higher oligomers, the former are less retained and elute first. While only a partial separation can be obtained at flow rate of 1 ml/min and a gradient time of 10 min (gradient volume 10 ml, Fig. 1a), a tenfold increase in the gradient time representing an increase in the gradient volume to 100 ml results in the nearly baseline separation of 21 poly(oxyethylene) homologues differing only in their number of repeat units. This separation is achieved in less than 70 min (Fig. 1b).

Poly(oxyethylene) chains are also part of many industrial non-ionic surfactants. These surfactant molecules most often contain only one terminal hydroxyl group while the other is substituted with a hydrophobic moiety such as lauryl, cetyl, stearyl, or oleyl. This hydrocarbon portion of the macromolecule does not interact with the stationary phase under normal phase conditions. For each of these surfactants, the molecular weight distribution of the poly(oxyethylene) units is relatively broad as a result of the manufacturing technique used for the preparation and an exact knowledge

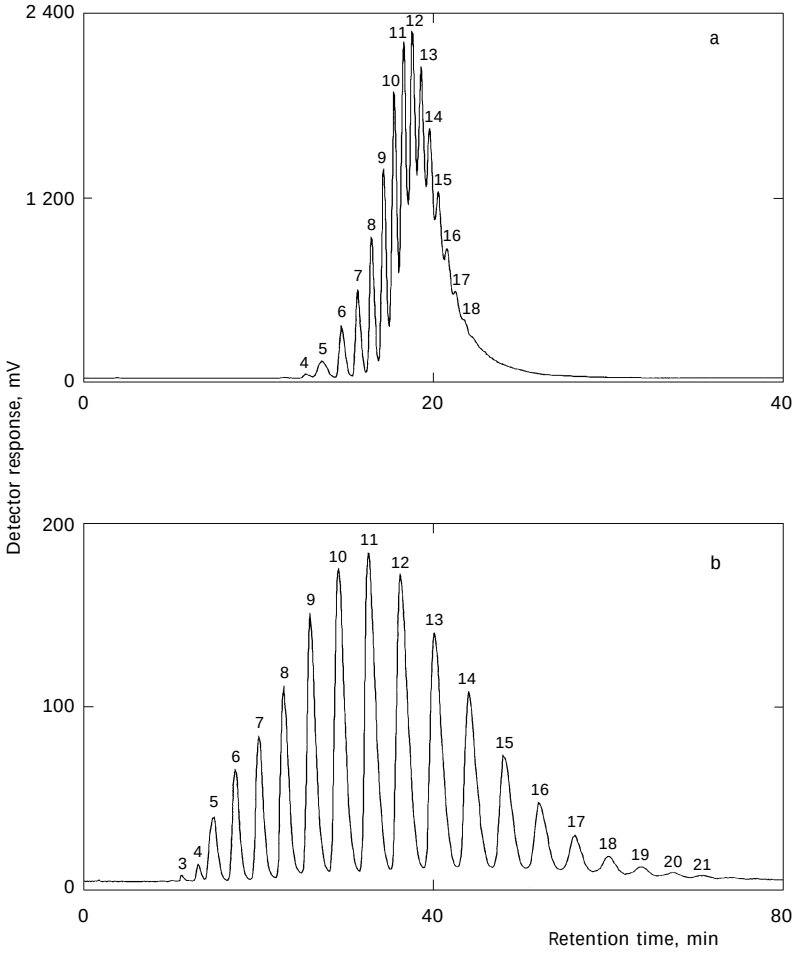

FIG. 1

Normal-phase HPLC separation of oligomers of polyoxyethylene. Conditions: column, 150 mm × 4.6 mm i.d.; packing material, 10 μm poly(2,3-dihydroxypropyl methacrylate-*co*-ethylene dimethacrylate) beads; mobile phase, linear gradient from 30 to 50% tetrahydrofuran in hexane in 10 min (a) and 100 min (b); flow rate, 1 ml/min; detection, evaporative light scattering at 50 °C; analyte, poly(ethylene glycol) standard ($\langle M \rangle_w$ = 590, $\langle M \rangle_n$ = 530); concentration, 20 mg/ml; injection volume, 20 μl. The numbers correspond to the degree of polymerization

of this distribution is essential for quality control. NPLC using silica based stationary phases has been found very useful for their fractionation and assays[48,49]. The presence of only one highly interactive hydroxyl group gives chance to the internal ether links located between the repeating units to contribute more to the adsorption, thus making the chromatographic separation more sensitive to the number of these ether groups and, consequently, to the overall length of the poly(oxyethylene) chains. Indeed, Fig. 2 shows an example of the gradient separation of a commercial surfactant BRIJ 58 (polyoxyethylene(20) cetyl ether). An almost baseline separation into more than 30 peaks is achieved in 25 min. The exact structure of the individual peaks cannot be assigned because well-defined standards are not available. The first tall peak of the chromatogram in Fig. 2 most likely represents an unresolved mixture of surfactant molecules with very short poly(oxyethylene) chains. The separation indicates that BRIJ 58 is a mixture of more than 30 components, each of which is present in an amount sufficient to allow its detection by means of evaporative light scattering detector. The number of components and the overall shape of the molecular weight distribution of BRIJ 58 is confirmed by MALDI-TOF MS. The mass spectrum shown in Fig. 3 reveals that the major part of the BRIJ 58 molecules includes poly(oxyethylene) chains with a degree of polymer-

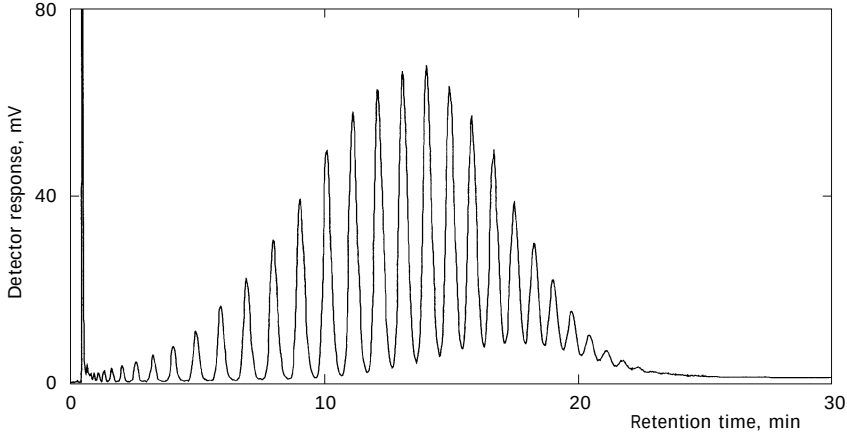

FIG. 2
Normal-phase HPLC separation of nonionic surfactant BRIJ 58. Conditions: analyte, [polyoxyethylene(20) cetyl ether]; concentration, 12.5 mg/ml; mobile phase, linear gradient of 20 to 50% tetrahydrofuran in hexane in 30 min; flow rate, 4 ml/min. For other conditions see Fig. 1

ization around 20, a value that matches well the nominal length claimed by the manufacturer. Due to the large unresolved peak at the beginning of the chromatographic separation and lack of well-defined standards, it is currently impossible to use MALDI-TOF MS data for peak identification in the chromatogram. Figures 2 and 3 indicate that HPLC and MALDI-TOF MS afford similar resolution. However, the chromatographic technique has the potential to collect substantial amounts of the individual constituents.

The linear diblock copolymers of ethylene oxide and propylene oxide are finding numerous applications as fluids for specific applications and lubricants. These molecules are terminated by a short alkyl chain at one end and by hydroxyl group at the other which makes them structurally similar to the BRIJ surfactants. Since both blocks are similar, normal-phase chromatography in a gradient of the mobile phase can be used to assess the number of individual components or, at least, the overall molecular weight distribution of these copolymer molecules. Fig. 4a shows overlaid separations of three commercial UCON copolymers produced by Union Carbide Company that incorporate both blocks in a 1 : 1 weight ratio but differ in their average molecular weights. UCON 50-HB-100 is said to have an average molecular weight of 520 which may represent two blocks each composed of 5 ethylene oxide and 5 propylene oxide repeat units. A near-baseline separation of 10 individual peaks is achieved using a mixture

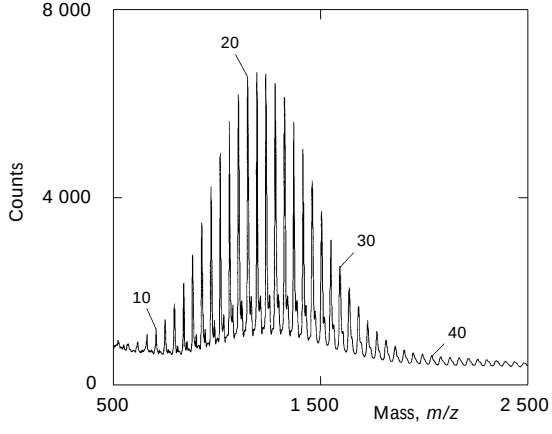

FIG. 3
MALDI-TOF MS trace of nonionic surfactant BRIJ 58. Conditions: compound, [polyoxyethylene(20) cetyl ether]; matrix, *trans*-3-indoleacrylic acid. The numbers correspond to the degree of polymerization of polyoxyethylene chains. Split peaks represent species with attached sodium and potassium ion, respectively

of acetone and hexane as the mobile phase. Again, the exact assignment of these peaks is impossible due to lack of individual standards. Similarly, UCON 50-HB-660 is said to have an average molecular weight of 1 590 which represents a total of about 30 repeat units of both types. While some separation can be observed in the range of lower molecular weights at the front of the peak, the larger molecules are no longer separated. This trend is even more obvious in UCON 55-HB-5100 with a claimed average molecular weight of 3 930. The number of individual compounds in this copolymer that consists of about 80 repeat units clearly exceeds the resolving ability of the chromatographic system. Therefore, only an envelope profile that actually represents the molecular weight distribution can be obtained. Despite its lack of ability to separate individual compounds in the UCONs of higher molecular weights, the polymer based stationary phase can easily separate all three UCONs from each other while the trace for each component retains the features found in separate injections (Fig. 4b). Since these block

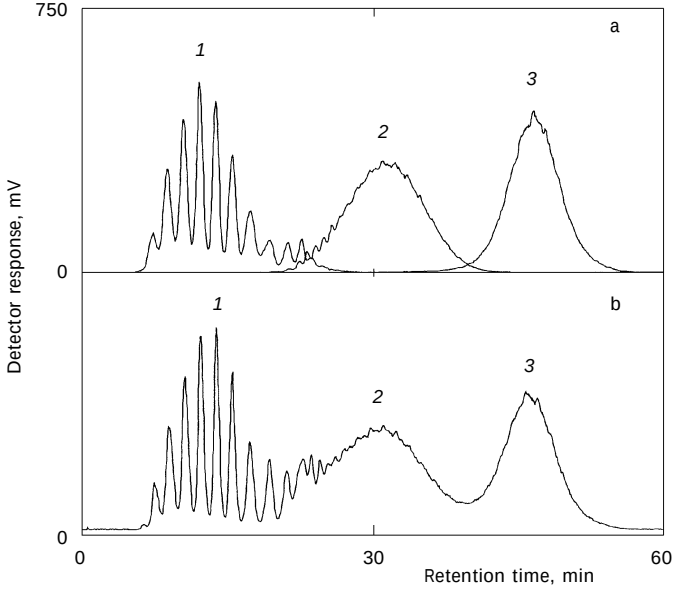

FIG. 4

Normal-phase HPLC separation of polyoxyalkylene-based UCON fluids. Conditions: column, 300 mm × 8 mm i.d.; mobile phase, linear gradient from 0 to 50% acetone in hexane in 60 min; flow rate, 3 ml/min; analytes: UCON 50-HB-100 (*1*), UCON 50-HB-660 (*2*), and UCON 50-HB-5100 (*3*), with average molecular weights 520, 1 590, and 3 930, respectively, injected separately (a) or as a mixture (b); concentration, 20 mg/ml each; injection volume, 30 μl. For other conditions see Fig. 1

copolymers are often used as heavy-duty lubricants and hydraulic fluids, this selectivity may complement the other methods available to monitor their degradation.

Polyvinylpyrrolidones

The polymeric-diol stationary phase was also found to be useful for the separation of poly(*N*-vinylpyrrolidone)s, another family of hydrophilic commodity polymers. The diol stationary phase is rather retentive and some polar polymers may be very strongly adsorbed. Therefore their elution might be difficult using the solvents typical of NPLC such as isopropyl alcohol and acetone. However, the polymeric diol beads can be used with essentially any solvent ranging from non-polar hexane to mixtures of water with polar organic solvents such as tetrahydrofuran and acetone. The elution power of these aqueous mobile phases exceeds significantly those of mixtures of pure organic solvents, thus making them suitable for elution of even strongly adsorbed polymer molecules. Figure 5 shows overlaid chromatographic traces for poly(*N*-vinylpyrrolidone)s with an average molecular weight of 10 000, 40 000, and 360 000 obtained in water–acetone gradient using the hydrophilic-interaction separation mode. The elution

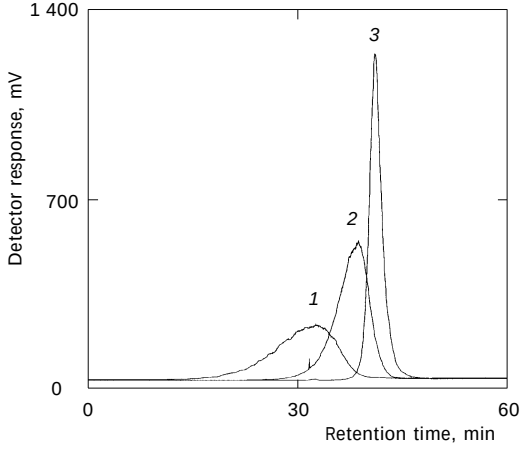

FIG. 5
Hydrophilic-interaction HPLC separation of polyvinylpyrrolidones. Conditions: column, 300 mm × 8 mm i.d.; mobile phase, linear gradient from 0 to 50% water in acetone in 50 min; flow rate, 2 ml/min; detection, evaporative light scattering at 90 °C; analytes: polyvinylpyrrolidones with average molecular weights 10 000 (*1*), 40 000 (*2*), and 360 000 (3); injection volume, 60 µl. For other conditions see Fig. 1

order in adsorption chromatography is opposite to that obtained in size-exclusion process: the smaller macromolecules elute first followed by polymers with higher molecular weights. The overall retention time is controlled by the percentage of water in the mobile phase. Since the same gradient conditions were used in all three separations, poly(*N*-vinylpyrrolidone) with a molecular weight of 360 000 elutes last in the mobile phase that contains the highest concentration of water. Obviously, this method could be calibrated with narrow standards and used as an alternative to size-exclusion chromatography for the determination of molecular weight distribution.

Polysaccharides

Aqueous mobile phases are also suitable for the hydrophilic-interaction chromatography separation of polysaccharides. In this chromatographic mode, gradients of water in acetonitrile are used almost exclusively[20]. We replaced in our study acetonitrile with acetone, which is less toxic and enables the use of evaporative light scattering detection at lower temperatures while enhancing selectivity for polysaccharide separations. For example, Fig. 6 shows the baseline separation of 10 oligosaccharides with 3–12 glu-

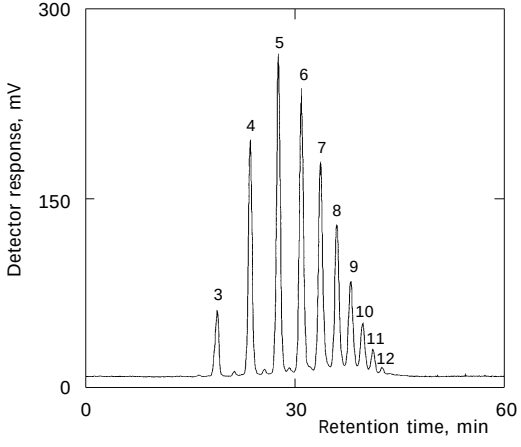

FIG. 6

Hydrophilic-interaction HPLC separation of dextran oligomers. Conditions: column, 300 mm × 8 mm i.d.; mobile phase, linear gradient from 10 to 40% water in acetone in 60 min; detection, evaporative light scattering at 90 °C; analyte, dextran standard ($\langle M \rangle_w$ = 1 270, $\langle M \rangle_n$ = 1 010); injection volume, 30 μl. The numbers correspond to the degree of polymerization. For other conditions see Fig. 1

cose units that are present in a dextran standard with a narrow molecular weight distribution using a shallow gradient of the mobile phase. The assignment of peaks is made on the basis of injections spiked with isomaltotriose and isomaltotetraose. Using the molecular weights of the individual oligomers and their peak areas in the chromatogram, number average molecular weights $\langle M \rangle_n$ and weight average molecular weights $\langle M \rangle_w$ of 1 030 and 1 160, respectively, can be calculated. The size-exclusion chromatography values claimed by the manufacturer (Pharmacosmos) for this standard are $\langle M \rangle_n$ = 1 010 and $\langle M \rangle_w$ = 1 270. Although these two determination methods rely on rather different properties of the macromolecules (adsorption and hydrodynamic volume), the values for the molecular weights are in good agreement, demonstrating that hydrophilic interaction chromatography can also be used for the molecular weight characterization of oligo- and polysaccharides.

Figure 7 shows the hydrophilic interaction chromatography separation of several dextran standards. Although a steeper linear gradient of water in acetone is used for this separation, the standard with a weight average molecular weight of 1 270 used above is again well separated. In contrast, only a

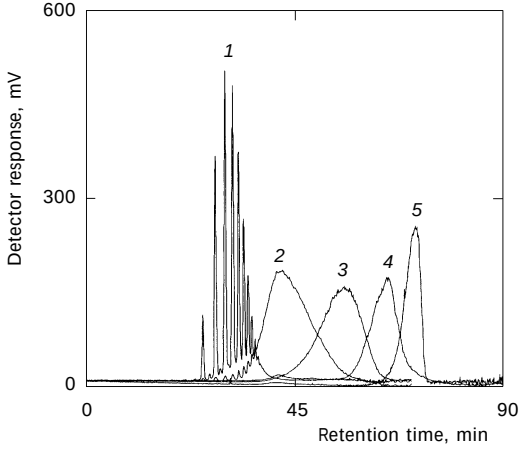

FIG. 7

Hydrophilic-interaction HPLC separation of dextran polymers. Conditions: column, 300 mm × 8 mm i.d.; mobile phase, linear gradient from 0 to 60% water in acetone in 60 min; detection, evaporative light scattering at 90 °C; analytes, dextran standards ($\langle M \rangle_w$ = 1 270, $\langle M \rangle_n$ = 1 010 (*1*), $\langle M \rangle_w$ = 5 220, $\langle M \rangle_n$ = 3 260 (*2*), $\langle M \rangle_w$ = 11 600, $\langle M \rangle_n$ = 9 890 (*3*), $\langle M \rangle_w$ = 23 800, $\langle M \rangle_n$ = 21 400 (*4*), and $\langle M \rangle_w$ = 147 600, $\langle M \rangle_n$ = 123 600 (*5*)); injection volume, 30 μl (*1*) and 60 μl (*2–5*). For other conditions see Fig. 1

portion of the next standard ($\langle M \rangle_w$ = 5 220) containing fractions with molecular weight lower than *ca* 2 000 (dodecamer) is separated while the remaining higher molecular weight components do not separate into individual peaks. Only envelope-type elution curves can be obtained with all other standards. Once again, the retention times of individual standards can be used for calibration and, eventually, determination of the molecular weight distributions of unknown dextrans.

The most common cyclic oligosaccharides, α, β, and γ cyclodextrins, consist of 6, 7, and 8 glucose units, respectively[50]. Their chemical similarity with dextrans makes them interesting target for separation by hydrophilic interaction chromatography using the polymeric diol column and acetone–water as the mobile phase. Their separation is shown in Fig. 8. In contrast to linear oligomers of dextran, the retention of cyclodextrins in-

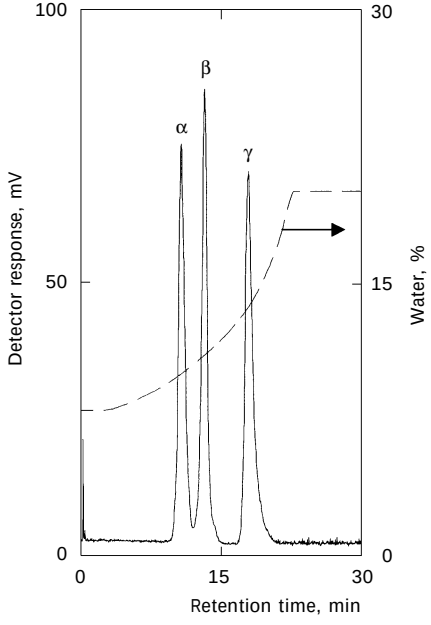

FIG. 8

Hydrophilic-interaction HPLC separation of cyclodextrins. Conditions: mobile phase, concave gradient from 8 to 20% water in acetone in 20 min; flow rate, 4 ml/min; detection, evaporative light scattering at 90 °C; analytes, α, β, and γ cyclodextrins; concentration, 10 mg/ml (total); injection volume, 20 μl. The dotted line represents a gradient profile at the column outlet. For other conditions see Fig. 1

creases rapidly with the size of their ring and a linear gradient of the mobile phase is not sufficient to elute them all in a reasonable period of time. Therefore, a concave gradient has to be used to achieve the elution.

CONCLUSION

Monodisperse poly(2,3-dihydroxypropyl methacrylate-*co*-ethylene dimethacrylate) beads appear to be well suited for the gradient HPLC of polar oligomers and polymers. Since this separation medium is compatible with mobile phases of very different polarities ranging from non-polar hexane to water, the separations under conditions typical of both normal-phase and hydrophilic-interaction chromatography can be performed on a single column without any loss of performance. As a result of the high retentivity of our diol beads, a variety of separations can be achieved in aqueous eluents, while still retaining the selectivity and resolution power of the normal-phase separation mode. In addition, the use of solvents such as acetone and water as mobile phases is more environmentally friendly than hexane, ethyl acetate, or methylene chloride typically used in NPLC. Good resolutions comparable to those typical of matrix-assisted mass spectroscopic techniques were obtained for polar oligomers. Our results also suggest that normal-phase HPLC using porous polymer beads can be used as an alternative method to classical size-exclusion chromatography for the determination of the molecular weight distribution of some hydrophilic polymers.

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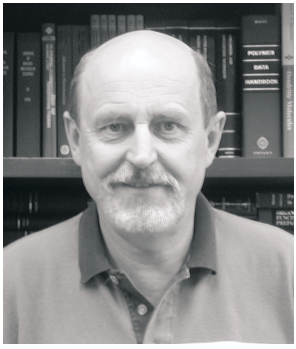

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