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High-Performance Anion-Exchange Chromatography of Carbohydrates Using a New Resin and Pulsed Amperometric Detection

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Key Words

Column liquid chromatography
Polymeric bonded phase
Anion-exchange chromatography
Pulsed amperometric detection
Carbohydrates

Summary

This paper reports the results of a study on the use of a new polymer-based, strong anion-exchange, stationary phase for rapid and selective separation of carbohydrates and related compounds by high-pH, anion-exchange chromatography with pulsed amperometric detection. The new adsorbent has been obtained by direct nitration of 2.8 μm, spherical non-porous highly cross-linked, styrene-divinylbenzene copolymer beads, followed by reduction of superficially introduced nitro groups with nascent hydrogen and quaternization of the resultant amino groups with iodomethane. It is reported that by optimizing the ionic strength of the mobile phase, columns packed with the new anion-exchanger can be successfully employed to separate, either in isocratic or gradient elution mode, oligosaccharides, positional isomers of gluco-disaccharides, as well as uronic acids and sugar monophosphates.

Introduction

Carbohydrates can be separated under alkaline conditions as anions by high-performance anion-exchange chromatography (HPAEC) and detected with pulsed amperometric detection (PAD) [1–2]. The detection of carbohydrates by PAD at a gold electrode is highly sensitive, is relatively specific for compounds containing hydroxyl groups, it allow gradient elution methods and no sample derivatization is required [3].

Columns currently used for carbohydrate separation by HPAEC-PAD are packed with polymer-based, anion-exchangers produced by proprietary manufacturing processes. These packings include electrostatically latex-coated, polymer-based, anion-exchangers [4–6] and a macroporous polymer-based resin functionalized with quaternary ammonium groups [7]. Furthermore, the use of a macroporous, poly(N,N,N,-trimethylammonium methylstyrene-divinylbenzene), strong anion-exchanger manufactured by a proprietary process [8] has also been reported [9–10].

Recently, we have proposed a simple and straightforward method to produce a surface-functionalized, polystyrene-based, strong anion-exchanger for carbohydrate separation by HPAEC-PAD. This sorbent has been prepared by direct nitration of microspheres of highly cross-linked, styrene-divinylbenzene copolymer beads, followed by reduction of the superficially introduced nitro groups with metalic tin and quaternization of the resultant amino groups [11].

The present study further investigates the suitability of this sorbent for the separation of carbohydrates and related compounds by HPAEC-PAD under alkaline conditions.

Experimental

Materials

All saccharides used as authentic standards were from Sigma (St. Louis, MO, USA). Sodium hydroxide (50 % w/w) solution and anhydrous sodium acetate were from J. T. Baker (Deventer, Netherlands). HPLC-grade water, and other reagent-grade solvents and salts were supplied by Carlo Erba (Milan, Italy).

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Equipment

The experiments were conducted using a Dionex (Sunnivale, CA, USA) Model 4000i, quaternary advanced gradient pump, equipped either with a Dionex pulsed electrochemical detector (PED) or with a Model PAD-2 pulsed amperometric detector, both with a gold working electrode and a silver-silver chloride reference electrode. Using the PAD, detector the applied potentials were E_1 = 0.10 V, E_2 = 0.60 V, and E_3 = –0.60 V with pulses of 300, 120, and 300 ms, respectively. The program for PED in the pulsed amperometric mode was E_1 = 0.10 V, t_1 = 0–500 ms, integration period 200 ms (beginning at 300 ms); E_2 = 0.60 V, t_2 = 510–590 ms; E_3 = –0.60 V, t_3 = 600–650 ms. A Dionex DXP single-piston pump was used to add 0.3 M sodium hydroxide to the column effluent through a tee connection at 0.2 mL min^{-1} before the cell of the detector during elution with sodium hydroxide at concentrations lower than 20 mM. Samples were injected using a Rheodyne Model 9125 non-metal (PEEK) injection valve with a PEEK sample loop of 10 µL (Cotati, CA, USA). The Dionex eluent degas module was employed to sparge and pressurize the eluent with helium. This unit both degasses the eluents and prevents absorption of carbon dioxide and subsequent production of carbonate which would act as displacing ion and shorten retention times. Chromatographic data were collected and plotted using the Dionex Auto Ion 450 chromatography workstation.

Synthesis of the Packing Material

Spherical, highly cross-linked, PS-DVB particles having a mean diameter of 2.8 µm, and surface area of 7 m^2 g^{-1} were prepared and characterized as previously reported [12]. The PS-DVB bead was chemically modified by direct nitration, followed by reduction with metallic tin and quaternization with iodomethane to produce a superficial layer of quaternized amino functions following the same procedures described earlier [11].

Figure 1 shows the structural characteristics of the micropellicular stationary phase. In addition to the quaternary ammonium groups, ethyl- and vinyl groups are present due to polymerization of technical divinylbenzene monomer which consists of approximately 60 % divinylbenzene and 35 % ethylstyrene; nitro groups are also present because of incomplete reduction of the nitrated particles.

HPLC Columns and Procedures

Columns were packed using the following high-pressure slurry packing technique. The polymeric sorbent (1.25–1.50 g) was suspended in 20–24 mL HPLC-grade water, sonicated for 3 min and then packed into either 75 × 4.6 mm I.D. (column 1) or 90 × 4.6 mm I.D. (column 2) polyether ether ketone (PEEK) columns (Alltech, Deerfield, IL, USA). Water was used as the driving solvent at a constant pressure of 40 MPa, by using a Model DSTV 122 pneumatic pump (Haskel, Burbank, CA, USA).

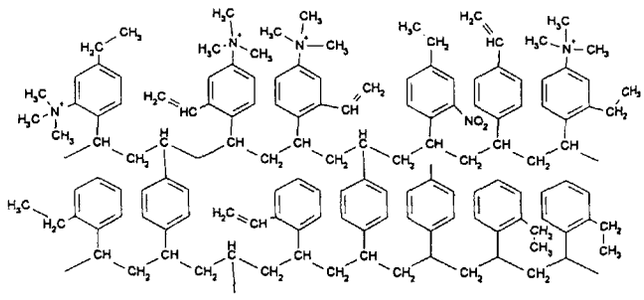

Figure 1
Structure of quaternized amino phase based on highly cross-linked, poly-(styrene-divinylbenzene) 2.8 µm particles.

Eluents were obtained from stock solutions of sodium hydroxide and sodium acetate in sodium hydroxide which were prepared by addition of the required amount of anhydrous sodium acetate and 50 % (w/w) solution of sodium hydroxide to HPLC-grade water, which was degassed with helium prior to addition of these compounds. After each run the sodium hydroxide concentration was ramped to 300 mM in 5 min and then maintained at this level for further 15 min to clean the column. The column was then equilibrated for 15 min with the eluent composition used under the initial conditions. All saccharides, acidic sugars and sugar phosphates were chromatographed at a flow-rate of 0.5 mL min^{-1} at room temperature.

Results and Discussion

The mechanical stability, the resistance to prolonged exposure to alkaline mobile phases, and loading capacity of the columns packed with the synthesized, polystyrene-based, quaternary amine stationary phase were investigated and reported previously [11]. In this study, the potential of the new sorbent for carbohydrate separation by HPAEC-PAD was first evaluated by eluting, over a wide range of mobile phase ionic strength, a standard mixture of a monosaccharide (glucose), two disaccharides (turanose and maltose), three trisaccharides (panose, maltotriose, laminaritriose), the tetrasaccharide maltotetraose, and the pentasaccharide maltopentaose. In a first experiment, chromatography was carried out by isocratic elution with 50 mM sodium hydroxide containing 5 mM sodium acetate. Under these conditions the monosaccharide glucose was eluted near the void volume of the column, turanose and maltose were only partially resolved, the trisaccharides were much more retained and eluted as rather broad peaks, whereas the tetra and pentasaccharides were strongly retained. An effective separation of the three trisaccharides, as well as the tetrasaccharide maltotetraose and the pentasaccharide maltopentaose was achieved in less than 10 min using gradient elution,

Table I. Optimized gradients A, B, and C for carbohydrate separation by HPAEC-PAD.

Gradient	Analysis time	Percent eluent 1	Percent eluent 2
	minutes	50 mM NaOH	500 mM NaAc in 50 mM NaOH
A	0	100	0
	2	100	0
	12	80	20
B	0	98	2
	10	0	100
		20 mM NaOH	100 mM NaAc in 20 mM NaOH
C	0	100	0
	5	100	0
	30	0	100

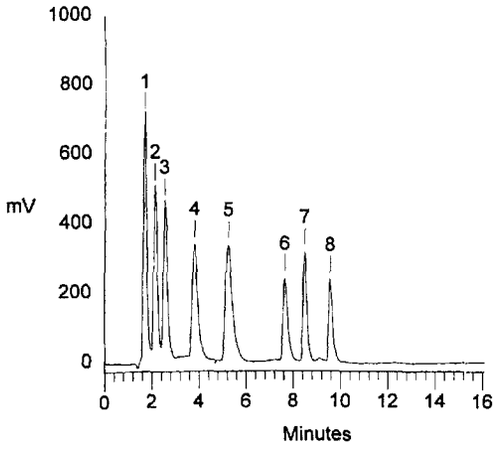

Figure 2
Separation of **1** = glucose, **2** = turanose, **3** = maltose, **4** = panose, **5** = maltotriose, **6** = laminaritriose, **7** = maltotetraose, **8** = maltopentaose on column 1. Gradient elution as Table I, A; flow rate 0.5 ml min^{-1}; room temperature; detector, PAD 2; attenuation, 1000 nA.

Table II. Reproducibility of retention of carbohydrates. Five repeated injections. Conditions as Figure 2.

Carbohydrate	Renention time (min)			R.S.D. (%)
	Individual values	Mean	S.D.	
Glucose	1.67, 1.66, 1.67, 1.70, 1.68	1.68	0.0152	0.90
Turanose	2.12, 2.13, 2.14, 2.15, 2.11	2.13	0.0158	0.74
Maltose	2.53, 2.55, 2.71, 2.56, 2.54	2.54	0.0192	0.76
Panose	3.80, 3.78, 3.81, 3.83, 3.84	3.81	0.0239	0.63
Maltotriose	5.23, 5.19, 5.26, 5.27, 5.22	5.23	0.0321	0.61
Laminaritriose	7.63, 7.52, 7.67, 7.65, 7.69	7.63	0.0665	0.87
Maltotetraose	8.47, 8.35, 8.54, 8.58, 8.55	8.50	0.0920	1.08
Maltopentaose	9.55, 9.41, 9.61, 9.71, 9.60	9.58	0.1094	1.14

which was accomplished by maintaining the sodium hydroxide concentration at 50 mM and increasing the sodium acetate concentration from 0 to 100 mM during analysis, according to the profile A of the optimized gradients reported in Table I. The separation obtained under these conditions is reported in Figure 2. In order to examine the reproducibility of the retention times, the mean value, the standard deviation (S.D.) and the relative standard deviation (R.S.D.) of the retention time were calculated from the chromatograms obtained by five repeated injections of the standard mixture. The results are reported in Table II and show that the R.S.D.s were better than 1.15 %.

The poor resolution of the slightly retained disaccharides can be improved by lowering the concentration of sodium hydroxide and/or acetate in the eluent. This decreases the elution strength of the mobile phase allowing sufficient retention of disaccharides for their selective elution.

In the preceding paper [11] we have shown that disaccharides can be selectively retained by using mobile phases containing sodium hydroxide at concentrations ranging from 6 to 45 mM. To evaluate further the selectivity of the strong anion-exchanger toward neutral disaccharides, five different positional isomers of glucodisaccharides, differing in the anomeric configuration (isomaltose and gentiobiose) or in the linkage type (threalose, nigerose, and maltose) were eluted isocratically on column 2 using 6 mM sodium hydroxide mobile phase. When performing isocratic separation at sodium hydroxide concentrations lower than 20 mM, post column addition of 0.3 M sodium hydroxide at 0.2 mL min^{-1} was necessary to maintain optimum detector sensitivity and minimize baseline drift. Under these conditions all five gluco-disaccharides were selectively retained with highly reproducible retention times, as reported in Table III. A typical chromatogram is depicted in Figure 3.

Uronic acids are widely distributed in nature and have been successfully determined by HPAEC-PAD in biological samples of both animal and plant origin [13–14]. The suitability of the polystyrene-based quaternary am-

Table III. Reproducibility of retention of glucobioses. Five repeated injections. Conditions as Figure 3.

Glucobiose	Renention time (min)			R.S.D. (%)
	Individual values	Mean	S.D.	
Trehalose	2.87, 2.88, 2.88, 2.88, 2.88	2.88	0.004	0:15
Isomaltose	3.92, 3.92, 3.87, 3.91, 3.91	3.90	0.021	0:53
Gentiobiose	4.82, 4.81, 4.90, 4.85, 4.82	4.84	0.037	0.76
Nigerose	6.00, 6.05, 6.23, 5.99, 6.01	6.08	0.100	1.64
Maltose	7.02, 6.97, 7.25, 7.03, 7.06	7.07	0.108	1.52

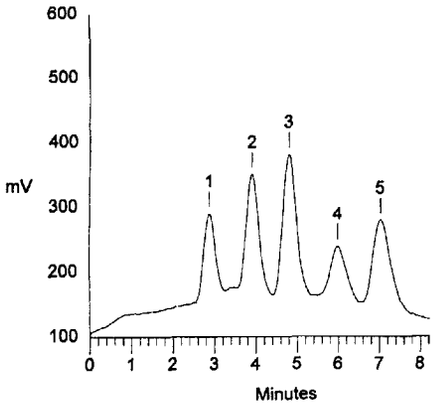

Figure 3
Separation of **1** = trehalose, **2** = isomaltose, **3** = gentiobiose, **4** = nigerose, and **5** = maltose on column 2. Isocratic elution with 6 mM sodium hydroxide; flow rate 0.5 ml min^{-1}; room temperature; detector, PAD 2; attenuation, 1000 nA.

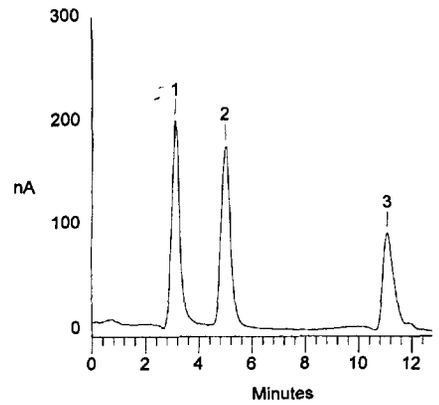

Figure 4
Separation of **1** = gluconic acid, **2** = glucuronic acid and **3** = glucaric acid on column 2. Gradient elution as Table I, B; other conditions as Figure 2.

monium adsorbent for separation of uronic acids was investigated by evaluating the gradient elution behavior of gluconic, glucuronic, galacturonic and glucaric acid over a wide range of mobile phase compositions and gradient slopes. Owing to the carboxylic acid moiety, the four uronic acids were much more retained than the corresponding neutral monosaccharides and mobile phases of higher ionic strength were required for their selective elution from the column investigated.

In a first experiment gluconic and glucuronic acids were selectively eluted by a 15 min linear gradient from 10 to 100 mM sodium acetate in 50 mM sodium hydroxide. Under these conditions the more acidic glucaric acid was strongly retained and not eluted from the column, whereas galacturonic and glucuronic acid coeluted. To elute glucaric acid the concentration of sodium acetate in the mobile phase containing 50 mM sodium hydroxide was increased by a 10 min linear gradient up to 400 mM (optimized gradient B in Table I). Under these conditions, gluconic, glucuronic and glucaric acid were successfully resolved in less than 12 min, as reported in Figure 4.

The selective elution of glucuronic and galacturonic acid was obtained by varying both the ionic strength of the eluent and the gradient slope. The dependence of the retention behavior of these two uronic acids on the gradient slope was investigated by employing either 15 or 30 mM sodium hydroxide as the starting eluent (eluent A) and 100 mM sodium acetate in eluent A as the gradient former (eluent B). All experiments were performed at constant flow rate (0.5 mL min^{-1}) by running linear gradients from 0 to 100 % B with different gradient times ranging from 10–60 min. The effect of the gradients at two different ionic strength on the retention of glucuronic and galacturonic acid is shown in Figure 5. The baseline separation of glucuronic and galacturonic acid with eluents containing 15 mM sodium hydroxide and gradient time of 40 min is reported in Figure 6.

To characterize further the retention properties of the quaternary amine-stationary phase, sugar monophosphates were chromatographed. Sugar monophosphates are bivalent acidic compounds and, consequently they are strongly retained on the anion-exchange column. An effective separation of two sugar phosphates was achieved using gradient elution, accomplished by maintaining the sodium hydroxide concentration at 20 mM and increasing the sodium acetate concentration during analysis. The gradient program used for the separation of glucose, glucose 1-phosphate and fructose 1-phosphate reported in Figure 7 is summarized in Table I (C). The neutral monosaccharide glucose eluted near the

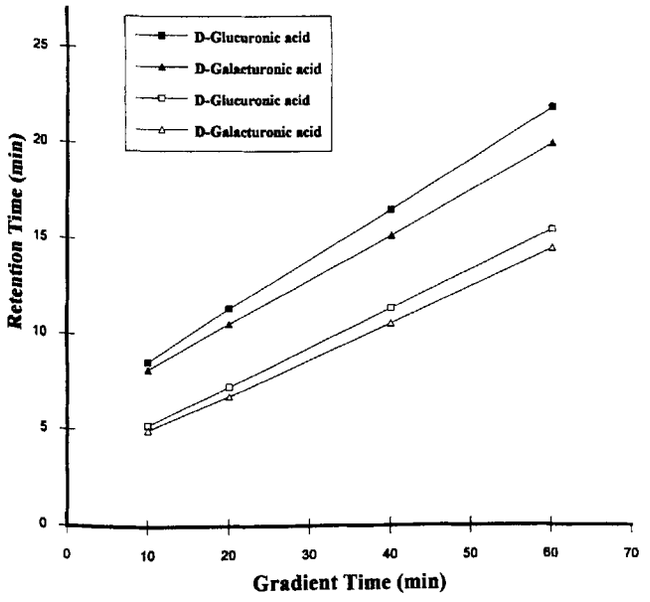

Figure 5
Retention of D-glucuronic acid and D-galacturonic acid against gradient time.

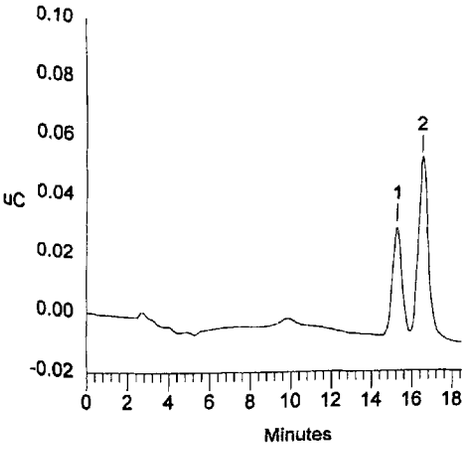

Figure 6
Separation of **1** = D-glalacturonic acid and **2** = glucuronic acid on column 2. Linear gradient 0 to 100 % B in 40 min.; mobile phase A, 15 mM sodium hydroxide; mobile phase B 100 mm sodium acetate in 15 mM sodium hydroxide. Other conditions as Figure 2.

front during isocratic elution with 20 mM sodium hydroxide, whereas, the more retained sugar phosphates were selectively separated by gradient elution in less then 30 min.

Conclusions

The poly-(styrene-divinylbenzene) based quaternary amino adsorbent used in this study has been found to be

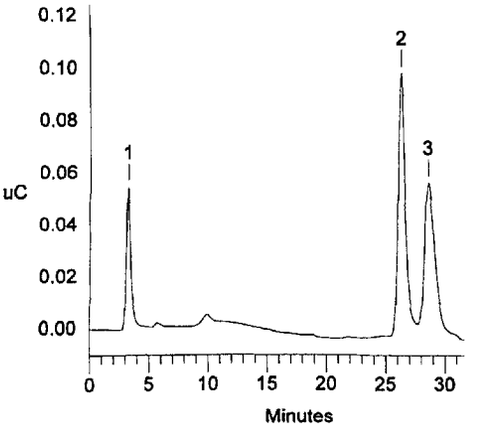

Figure 7
Separation of **1** = glucose, **2** = glucose 1-phosphate, **3** = fructose 1-phosphate on column 2. Gradient elution as Table I, C; detector, PED in PAD mode (see Experimental). Other conditions as Figure 2.

effective at separating both neutral and acidic carbohydrates by anion-exchange chromatography under alkaline conditions. Experimental data show that the retentivity of columns packed with this sorbent can be easily modulated by changing the ionic strength of the mobile phase. This is obtained either by selecting the optimum concentration of sodium hydroxide, as in the case of the isocratic elution of closely related neutral disaccharides, or increasing the concentration of sodium acetate during the analysis. Both isocratic and gradient elution modes have been shown to be highly selective and provide satisfactory reproducible retention times.

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