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High-performance anion-exchange chromatographic separations of carbohydrates on a macrocycle-based stationary phase with eluents of relatively low pH and concentration

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Abstract

A high-pH anion-exchange chromatography stationary phase was prepared by adsorbing cryptand *n*-decyl-[2.2.2] (D222) onto polymeric resins. Anion-exchange sites were composed of complexes of D222 with K^+ or Na^+ from the mobile phase. Alditols, and mono- and disaccharides were separated isocratically with 13 m*M* KOH. Gradient separations of oligogalacturonic acids and maltooligosaccharides were achieved by allowing K^+ or Na^+ to bleed from the column, resulting in a decrease in the number of anion-exchange sites. Advantages of these capacity gradients include short column regeneration times, low baseline drift, and the use of eluents that are simple in composition and of low ionic strength and pH. © 1998 Elsevier Science B.V.

Keywords: Capacity gradients; Cryptand *n*-decyl-[2.2.2]; Carbohydrates; Maltooligosaccharides; Polygalacturonic acids

1. Introduction

Crown ethers and cryptands are noted for their ability to selectively form complexes with alkali and transition metals and have applications in chromatography. To our knowledge, the first reported use of macrocycles in chromatography was by Cram and co-workers [1–3] who employed chiral analogs of 18-crown-6 to separate enantiomers of racemic amine salts. Since that time, the use of macrocycles in chromatography has been a moderately active area of research, and is described in recent reviews [4,5]. In our laboratory, macrocycle-based ion-exchange chromatography has been used to separate cations [6], inorganic anions [7–12], nucleosides, and nucleotides [13]. We performed these separations on columns containing either tetradecyl-18-crown-6, cryptand *n*-decyl-[2.2.1], or cryptand *n*-decyl-[2.2.2] (D222) adsorbed onto polymeric resins.

Typical high-performance anion-exchange chromatography (HPAEC) columns used in carbohydrate analysis contain quaternary amine exchange sites covalently bound to polymeric resins [14]. On these resins, analyte retention can be modified by including various eluting agents in the eluent, varying the pH, or changing the temperature; however, the capacity of these columns is fixed. With macrocycle-based HPAEC columns, the capacity can be altered during separations to modify analyte retention, as has been demonstrated for inorganic anions [7]. This ability results from the labile nature of the anion-exchange sites created when eluent cations form complexes with stationary-phase macrocycles. At any given time, column capacity is determined by

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the number of cation–macrocycle complexes. The number of complexes present depends on the concentration of macrocycle on the stationary phase, the concentration of metal cations in the eluent [12], and the stability constants of the macrocycle with eluent cations. Stability constants are influenced by temperature [10,11], eluent composition [9], type of macrocycle on the stationary phase [8,9], and type of metal cation included in the eluent [8,10]. With other factors held constant, the column capacity follows the trend of the selectivity of the ligand among alkali metal ions. For D222, the binding constant trend in ascending order is $Li^+ < Cs^+ < Na^+ < Rb^+ < K^+$ [4]. In the experiments discussed in this paper, column capacity was modified by changing the concentration and type of alkali-metal cation included in the eluent.

The capacity of a column can be decreased during the course of a separation by switching from a cation that has a high affinity for the stationary phase macrocycle to one that has a lower affinity, or by cutting off the supply of cations altogether. The cations already bound to the stationary phase bleed slowly from the column and the capacity of the column drops. The gradual loss of column capacity, called a capacity gradient, affects separations in a manner similar to other types of gradients. The concentration of the complexing cation entering the column may be reduced by either applying a concentration gradient or by applying a step [7]. However, even a step results in gradient behavior because it takes time for a cation with high affinity for D222 to be removed from the column.

To our knowledge, there is only one other instance of a macrocycle-mediated HPAEC separation of carbohydrates in the literature. Simms et al. [15] separated oligogalacturonic acids on a β-cyclodextrin bonded-phase HPLC column with sodium phosphate-buffered mobile phases. They state that the ''separations appeared to be governed by a classical anion-exchange-type mechanism'', and hypothesized that ''a cationic component from the mobile phase . . . might have become included in the cyclodextrin cavity, forming a dynamic anion-exchange site''. Our research suggests that their proposed mechanism is possible. There are other instances of the use of β-cyclodextrin stationary phases to separate carbohydrates [16,17]. However, the retention mechanisms in these instances appear to be principally hydrogen bonding and dipolar interactions, not ion exchange [16].

The column preparation approach we used in our research, the adsorption of hydrophobic macrocycles to reversed-phase materials, was pioneered by Kimura et al. [18,19]. Columns were prepared by the adsorption of D222 to the surface of SMP and MPIC polymeric resins, both of which are manufactured by Dionex. These resins are copolymers of ethylvinylbenzene and divinylbenzene. The main difference between these resins lies in their morphology. The MPIC resin is porous and has a large surface area (400 m^2/g). This resin is used in commercial ion-pair chromatography columns. The SMP resin, a precursor to ion-exchange resins, is also porous, but the pores are larger and the resin has a much smaller surface area (25 m^2/g). Here we show our results of the separation of carbohydrates, including polygalacturonic acids and maltooligosaccharides, accomplished with capacity gradients on D222-based columns.

2. Experimental

2.1. Materials

Maltooligosaccharide standards, degree of polymerization (DP) 2–7 (92–96%), polygalacturonic acid (98%), gluconic acid (98%), glucose, glyceraldehyde, and *myo*-inositol were obtained from Sigma (St. Louis, MO, USA). Maltodextrin (Dextrin 10) was purchased from Fluka (Buchs, Switzerland). Mannitol was obtained from Baker (Phillipsburg, NJ, USA). Sucrose was obtained from Mallinckrodt (Paris, KY, USA). Fructose (98%) was obtained from Spectrum (Gardena, CA, USA). Cryptand *n*-decyl-[2.2.2] (50% in toluene), was purchased from EM Science (Gibbstown, NJ, USA). Metal hydroxide eluents were prepared by adding the following chemicals to deionized water; reagent-grade 50% NaOH solution, reagent-grade 45% KOH solution, and reagent-grade $LiOH \cdot H_2O$ crystal.

Oligogalacturonic acids were prepared by autoclave hydrolysis of polygalacturonic acid [20]. Hydrolysis involved autoclaving 0.25% polygalacturonic acid in deionized water (pH adjusted to 4.0–4.5 with NaOH) for 40 min at 121°C. An acetate

buffer of pH 5 was also added. The solution was allowed to sit and was then filtered. Maltooligosaccharide sample solutions were prepared by dissolving 0.05 g maltodextrin in 50 ml deionized water. The other carbohydrate solutions were also prepared with deionized water. Polyether ether ketone (PEEK) column bodies (250×4 mm) and end fittings were obtained from Dionex (Sunnyvale, CA, USA). The following polymeric resins were kindly provided by Dr. Y. Agroskin at Dionex: SMP resin with a surface area of 245 m^2/g (average diameter of 11.5 μm), SMP resin with a surface area of ~25 m^2/g (average diameter of 8 μm) that is a precursor to the packing used in the Dionex AS-12A column, and a macroporous MPIC resin with a surface area of ~400 m^2/g (average diameter of 10 μm) used in the NS-1 column.

2.2. Column preparation

Resin slurries were prepared in a beaker by mixing 0.1–0.15 g of D222 with 2–3 g of the appropriate resin in approximately 15 ml of methanol:water (~2:1) and then evaporating off the methanol at ambient temperature while stirring. The slurry was diluted to ~25 ml with 0.1 *M* NaOH. The slurry was then packed into a chromatography column at a pressure of 5000 p.s.i. (1 p.s.i.=6894.76 Pa). The packing solution was 0.1 *M* NaOH and the packing jacket was heated to 55°C. The column used for maltooligosaccharide separations was prepared with the 245-m^2/g SMP resin. The columns used in the separation of mono- and disaccharides were prepared with the MPIC resin. The column used for the separation of oligogalacturonic acid was packed with the lower-surface area SMP resin.

2.3. Chromatography

The chromatograph used was the Dionex DX500, equipped with vacuum degassing and an ED40 electrochemical detector. The eluent bottles were pressurized with helium. The pulsed amperometric detection (PAD) cell contained a gold working electrode and an Ag/AgCl reference electrode. The following pulse potentials and durations were used for detection of the carbohydrates: E_1=0.05 V (t_1=400 ms); E_2=0.75 V (t_2=200 ms); E_3=−0.15 V (t_3=400 ms). Integration began at 200 ms in t_1 and lasted until the end of t_1. A 10-μl sample loop was used. All chromatograms were obtained at ambient temperature (21–23°C).

Analysis for K^+ was carried out via suppressed ion chromatography with the 4000i system (Dionex). The eluent was 10 m*M* methanesulfonic acid. The CS14 column and guard column were used (Dionex).

3. Results and discussion

3.1. Alditol, and mono- and disaccharide separations

As a part of our initial exploration of the carbohydrate separation capabilities of a D222-based MPIC column, we experimented with the separation of arbitrarily selected alditols, and mono- and disaccharides. We compared the elution capabilities of NaOH and KOH. As expected on the basis of the $\log K$ values for complexation of Na^+ and K^+ for cryptand [2.2.2], KOH eluents gave longer carbohydrate retention times than NaOH eluents of the same concentration. For this reason we did no further work with NaOH. Fig. 1 shows a separation of eight different mono- and disaccharides on two D222-based MPIC columns in series, with 13 m*M* KOH eluent. We used two columns in series because they gave better resolution than one column alone. We were not able to separate mono- and disaccharides on the other columns described in this paper.

We compared the retention times of these eight mono- and disaccharides on a D222-MPIC column at various KOH eluent concentrations (Fig. 2). The best separation of these particular carbohydrates was obtained with 10–15 m*M* KOH. At KOH concentrations between 15 and 30 m*M*, sucrose and fructose coeluted, and at KOH concentrations greater than 30 m*M* KOH, maltose and gluconic acid coeluted. Of all the carbohydrates, only *myo*-inositol, mannitol, and glyceraldehyde maintained the same elution order at all concentrations of KOH. The elution order of the other carbohydrates depend on a number of competing factors, and the actual order is hard to predict [21]. In Fig. 2, the long elution times of gluconic acid as compared to the other carbohydrates at concentrations below 20 m*M* KOH was likely due

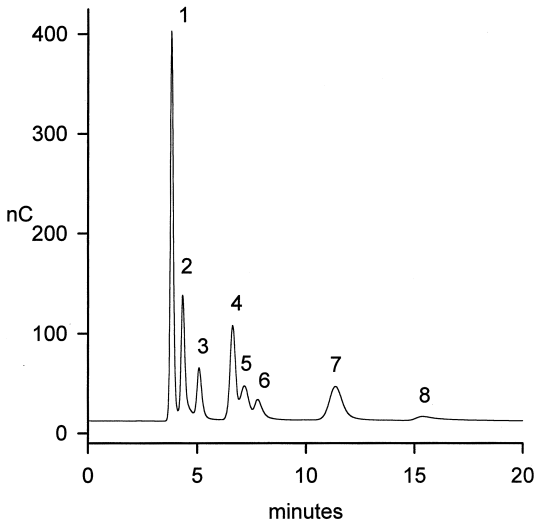

Fig. 1. Chromatogram of eight carbohydrates obtained with two D222-based MPIC columns in series. Analyte concentrations, 0.3 m*M*. Peaks: 1, *myo*-inositol; 2, mannitol; 3, glyceraldehyde; 4, glucose; 5, sucrose; 6, fructose; 7, maltose; and 8, gluconic acid. Eluent, 13 m*M* KOH; flow-rate, 1 ml/min; PAD detection, post column addition of 0.5 *M* NaOH at 0.8 ml/min.

to the fact that the other carbohydrates are not completely ionized at those hydroxide concentrations, whereas gluconic acid was almost completely ionized. As the KOH concentration increased, the eluting capability of the eluent increased and the retention of the gluconic acid dropped dramatically. The corresponding decrease in retention for the other carbohydrates was not as large because the increased fraction of carbohydrates ionized at the higher pH offset the increased eluting ability of the eluent.

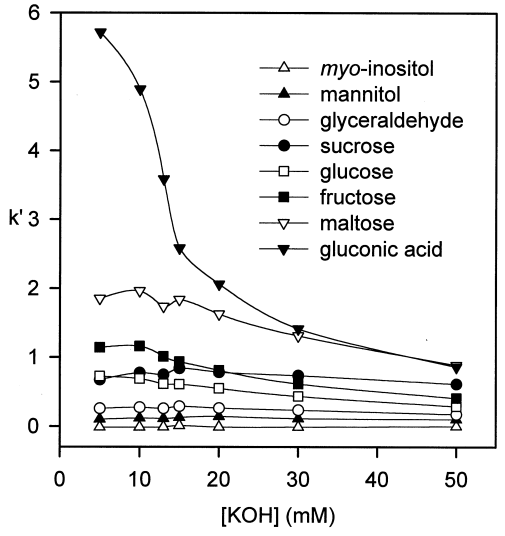

Fig. 2. Dependence of retention time of carbohydrates on [KOH]. Separation column, MPIC D222-based column; flow-rate, 1 ml/min.

3.2. Oligogalacturonic acid separations

Fig. 3 shows a chromatogram of oligogalacturonic acids obtained with the D222-based column prepared with the lower-surface area SMP resin. The separation was carried out with a step from 100 m*M* NaOH to 50 m*M* LiOH at 1 min. The separation conditions are described in the figure caption. Twenty peaks were resolved. A galacturonic acid standard eluted at 3 min in the broad band. Excellent separations of oligogalacturonic acids were obtained by Hotchkiss et al. [20] with the Dionex CarboPac PA-1 column. Their methods employed buffered gradients of acetate and oxalate. They separated up to DP 19 with a gradient of 0.3–1 *M* acetate and up to DP 50 with a gradient of 0–0.35 *M* oxalate.

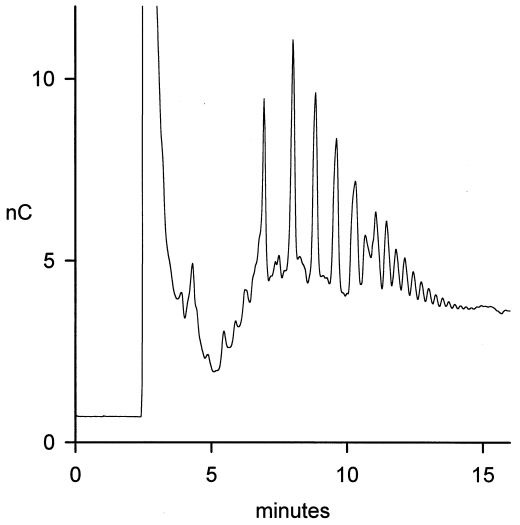

Fig. 3. Separation of oligogalacturonic acids on low-surface area SMP D222-based column. Flow-rate, 1.0 ml/min; column regeneration, 5 min of 100 m*M* NaOH; eluent step at 1 min from 100 m*M* NaOH to 50 m*M* LiOH; PAD detection, post column addition of 0.3 *M* NaOH at 0.5 ml/min.

3.3. Maltooligosaccharide separations: a pH 12.0 eluent

We separated maltooligosaccharides on the lower-surface area D222 SMP-based column used to separate the oligogalacturonic acids; however, resolution was less than optimal and some early peaks coeluted. Furthermore, the column capacity of the D222-based columns prepared from the low-surface area SMP resins decreased from run to run and extended use was not possible.

To help resolve the problems of low capacity and of column stability we prepared a D222-based column with an SMP resin having a surface area of 245 m^2/g. Fig. 4 shows a separation of maltooligosaccharides with this column. In 35 min, 23 peaks were resolved. Maltooligosaccharides with DP values 1–7 were positively identified by spiking with standards. This separation demonstrates the simultaneous use of a capacity and a salt gradient (see Fig. 4 caption for details). A capacity gradient occurred with a step at time zero from 10 m*M* KOH to 10 m*M* LiOH. The K^+ initially present in the eluent provided the capacity necessary to retain the smaller, early eluting maltooligosaccharides. The step to LiOH resulted in a reduction in the column capacity allowing elution of the larger maltooligosaccharides. A salt gradient of $LiNO_3$ was added which also helped elute the larger maltooligosaccharides; however, this salt gradient differed from those typically applied in the separation of maltooligosaccharides. The concentration of $LiNO_3$ in the eluent was never greater than 6.75 m*M*. This concentration is much lower than the concentration of eluting ions usually added in the separation of maltooligosaccharides. Typically, maltooligosaccharide separations require eluents containing as high as 100 m*M* NaOH and 300 m*M* NaOAc [22]. In the separations of maltooligosaccharides shown in Fig. 4, the total eluent strength was never greater than 17 m*M* and the pH was maintained constant at 12. The ability to reduce the column capacity during the course of a run permitted the separation of both large and small maltooligosaccharides under these mild eluent conditions.

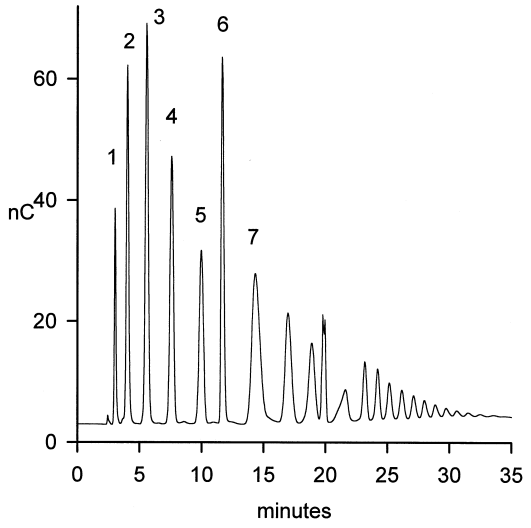

Fig. 4. Separation of linear maltooligosaccharides, up to DP 20, on a 245-m^2/g SMP D222-based column. Column regeneration, 5 min of 100 m*M* KOH followed by 10 min of 10 m*M* KOH. Step and gradient: at 0 min, step from 10 m*M* KOH to 10 m*M* LiOH; from 1 to 15 min gradient, from 0 to 0.3 m*M* $LiNO_3$/10 m*M* LiOH; from 15 to 30 min gradient, from 0.3 to 6.75 m*M* $LiNO_3$–10 m*M* LiOH. Other conditions as for Fig. 3.

We demonstrated the dependence of column capacity on the presence of K^+ in the eluent. If only KOH was included in the eluent and no step made to LiOH, only the first few maltooligosaccharides were eluted from the column in a reasonable time. If the eluent contained only LiOH and no KOH, many smaller maltooligosaccharides were not retained on the column and coeluted. To demonstrate the importance of D222 in separations, a column with no adsorbed D222 was prepared from the 245-m^2/g SMP resin, and a run was performed with the same conditions described in the caption of Fig. 4. No retention of maltooligosaccharides was observed.

As was noted earlier, the capacity of the D222-based column prepared with the low-surface area SMP resin dropped so rapidly from run to run that long-term experiments could not be performed. Although the D222-based column prepared with the 245-m^2/g SMP resin showed loss in capacity over time, the loss was not rapid after the initial days of use. Over the course of 3 weeks, the retention time of maltotriose decreased from 6.13 ± 0.02 min (number of repeat runs, $n=3$) to 5.59 ± 0.02 min ($n=5$). The retention time of maltohexaose decreased from 13.3 ± 0.2 min ($n=3$) to 11.5 ± 0.2 min ($n=5$). To further test the stability of the column, 2 l of a

solution of 0.1 *M* KOH and 0.01 *M* $LiNO_3$ was pumped through the column at a flow-rate of 1 ml/min. The retention times of maltooligosaccharides DP 1–5 decreased slightly. Maltotriose eluted at 5.54±0.07 min (n=6). The retention times of maltooligosaccharides DP 6,7 increased slightly. After pumping 2 l of solution through the column, maltohexaose eluted at 11.8±0.2 min (n=6). The retention times of larger maltooligosaccharides showed greater variability, especially between 17 and 23 min. The increased variability may have been the consequence of two types of gradient occurring simultaneously. A more robust method could likely be developed with further experimentation.

3.4. Maltooligosaccharide separations with a step to deionized water eluent

With the 245-m^2/g SMP D222-based column we separated maltooligosaccharides up to DP 19, using a step from 10 m*M* KOH eluent to deionized water prior to sample injection. Fig. 5a shows a separation of maltooligosaccharides, and Fig. 5b shows a chromatogram of 4×10^{-7} *M* maltotriose for comparison. The separation conditions are described in the caption. Although the pH of the bulk eluent was below the pK_a of glucose (12.3), the maltooligosaccharides show good retention and are well resolved. Retention occurs because neutral carbohydrates in the bulk eluent partition into the stationary phase and are ionized by reaction with KOH in the stationary phase. The ionized carbohydrates are then retained [23].

The chromatography literature contains at least two instances of HPAEC separations of monosaccharides in neutral aqueous eluent [24,25]. These separations were performed on the CarboPac PA-1 column. In both instances the column was conditioned with 0.25 or 0.3 *M* NaOH between runs. Petterson et al. [25] reported that column conditioning was required in order to obtain a good separation on the subsequent run. From the perspective of our research, it appears that the column conditioning step in their work converted the stationary phase to the hydroxide form, so that when neutral carbohydrates in the bulk eluent entered the stationary phase they were ionized and thereby retained.

The baseline drift shown in Fig. 5b was typical,

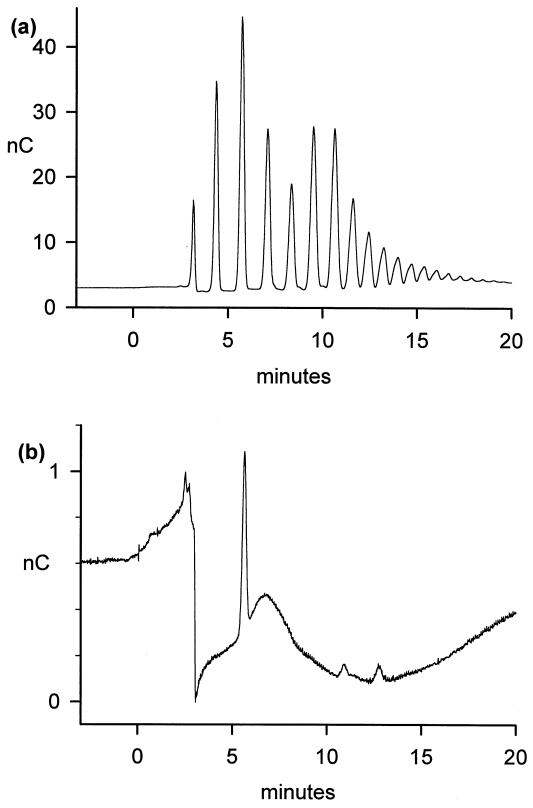

Fig. 5. Chromatograms of (a) maltooligosaccharides and (b) 4×10^{-7} *M* maltotriose on a 245-m^2/g SMP D222-based column. Samples were injected at time 0. Initial eluent, deionized water; column regeneration, from −3 to −1 min the eluent was 10 m*M* KOH, from −1 min the eluent was deionized water. Other conditions as for Fig. 3.

although the scale makes it look more severe than it is under normal concentration conditions. The baseline drift in the region between 3 and 25 min, the period in which the maltooligosaccharides elute, is less than 0.5 nC. The identities of the broad peak at 7 min and the smaller ones between 10 and 15 min are not known. They were present in the blank runs as well. These peaks may be due to impurities in the reagent-grade 45% KOH solution used to prepare the eluent. In an attempt to decrease baseline drift, phosphate buffer was added to the post-column addition solution. The baseline drift was reduced but detector sensitivity was also greatly reduced so this approach was not explored further.

A problem apparent in a comparison of Fig. 5a Fig. 5b is that glucose elutes in the region of steep

baseline change (t=3.2 min). This sharp change that appears in the baseline at 3 min was consistent from run to run. A gradient method was designed to move the glucose peak away from this region of the chromatogram. The method consisted of 10 m*M* KOH for 2 min, followed by 2 m*M* KOH for 3 min, followed by a step to water at which time the sample was injected (see Fig. 6 caption). Fig. 6a shows a chromatogram of maltooligosaccharides obtained with this method, and Fig. 6b shows a chromatogram of 5×10^{-7} *M* maltotriose. A comparison of Fig. 6a Fig. 6b shows that none of the maltooligosaccharides elute in the region of steep baseline change. The minimum detectable amount of maltotriose using this method was found to be 3×10^{-8} *M* (0.3 pmol) at three times the baseline noise. The least-squares fit was obtained with four points and the r^2 value was 1.00. The linear range extended to 8×10^{-5} *M* (800 pmol).

Fig. 7 shows $[K^+]$ at the column inlet and outlet over the course of a run using the method described in the caption of Fig. 6. Each point shows the $[K^+]$ of solution collected for a 1-min period. Fig. 7 shows that the maximum $[K^+]$ at the column outlet was about 4 m*M*, less than half of the maximum $[K^+]$ at the column inlet. The $[K^+]$ curve for the column outlet shows slow decay after the maximum. The slow loss of K^+ from the column is the basis of the capacity gradient. Fig. 7 also shows that the eluent at the column inlet at the time of injection contains less than 2 m*M* KOH. At that time the maximum of the KOH band is approximately half way through the column. Consequently, most of the larger maltooligosaccharides are never in eluent containing more 2 m*M* KOH. The smaller maltooligosaccharides, glucose and maltose, catch up with the [KOH] maximum and elute with it, but the larger, slower moving maltooligosaccharides do not.

From a practical standpoint, these capacity gradient methods provide a major advantage over other gradient methods. The time required for column regeneration is small as compared with other gradient methods. The method used to obtain the

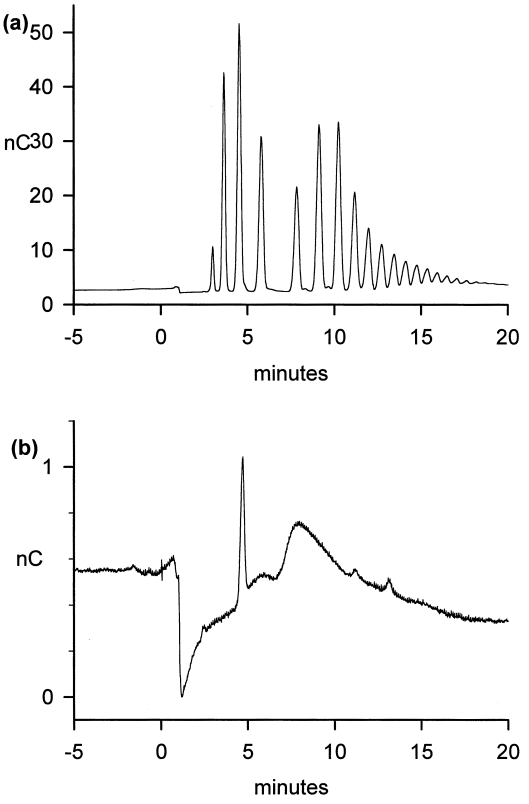

Fig. 6. Chromatograms of (a) maltooligosaccharides and (b) 5×10^{-7} *M* maltotriose on a 245-m^2/g SMP D222-based column. Samples were injected at time 0. Initial eluent, deionized water; column regeneration, from −5 to −3 min the eluent was 10 m*M* KOH, from −3 to 0 min the eluent was 2 m*M* KOH. A step to deionized water was made at time 0. Other conditions as for Fig. 3.

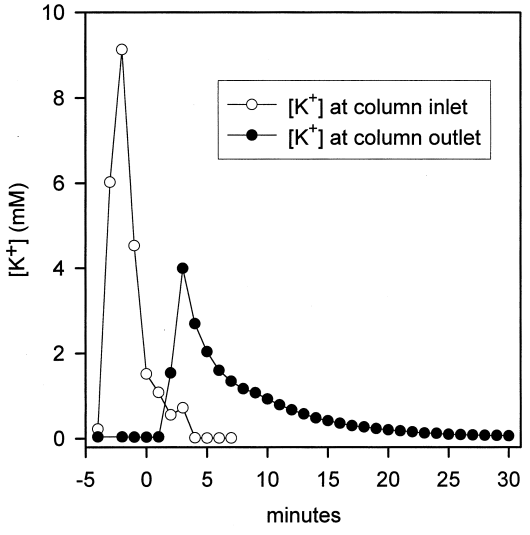

Fig. 7. The concentration of K^+ at the column inlet and outlet using the method as for Fig. 6.

chromatograms in Fig. 5 required only 3 min for column regeneration, as is described in the figure caption. No additional column regeneration was required between runs. With the method used to obtain the chromatograms in Fig. 6, 5 min was required for column regeneration between runs.

4. Conclusion

Separations of oligogalacturonic acids and maltooligosaccharides can be carried out in D222-based columns using capacity gradients and eluents of relatively low pH containing little or no added buffer. These methods can probably be extended to other types of oligosaccharides. The methods described here have several potential advantages. First, HPAEC carbohydrate separations are potentially compatible with mass-spectrometry detection since the eluent concentrations are within the operation range of electrospray ionization. Traditional HPAEC separation methods for oligosaccharides cannot be directly coupled with mass spectrometry because eluents of high buffer concentrations preclude the use of electrospray ionization. Secondly, alkali-sensitive oligosaccharides may undergo less degradation because of the relatively low pH of the bulk eluent. With other HPAEC methods, the only ways to minimize alkali-mediated side reactions is to reduce the elution time or use lower pH solutions and compensate by adding more acetate or nitrate [26]. With our methods, lower pH is achieved without the necessity of adding high concentrations of acetate or nitrate to the eluent. Although less degradation is expected with these methods, it is doubtful that alkali-mediated side reactions can be eliminated completely because the hydroxide concentration in the stationary phase must, of necessity, remain high.

Methods involving a step from KOH to water prior to, or at the time of, injection of the sample have several additional advantages. Column reconditioning times are very short (5 min or less). The separations can be performed with eluent switching alone; a pump with gradient capabilities is not required. Buffers are not required for maltooligosaccharide separations. Avoidance of buffers precludes the possibility of the formation of crystals in the pump heads and other components of the chromatograph, prolonging component life.

Our results show a correlation between the surface area of the packing material and column capacity. Mono- and disaccharides were separated with the D222-based MPIC columns but they could not be separated on the columns prepared with the SMP resins. While maltooligosaccharides were not well separated on the lower-surface area SMP column used to separate oligogalacturonic acids, they were well separated on the 245-m^2/g SMP column.

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