ELSEVIER

Journal of Chromatography A, 685 (1994) 213–220

JOURNAL OF CHROMATOGRAPHY A

Synthesis of a polymeric-based stationary phase for carbohydrate separation by high-pH anion-exchange chromatography with pulsed amperometric detection ☆

Claudio Corradini[a,*], Danilo Corradini[a], Christian G. Huber[b], Gunther K. Bonn[c]

[a] *Istituto di Cromatografia del C.N.R., Area della Ricerca di Roma, P.O. Box 10, I-00016 Monterotondo Stazione (Rome), Italy*

[b] *Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University, A-6020 Innsbruck, Austria*

[c] *Department of Analytical Chemistry, Johannes-Kepler University, A-4040 Linz, Austria*

First received 9 May 1994; revised manuscript received 15 July 1994

Abstract

Spherical highly cross-linked styrene–divinylbenzene copolymer particles were chemically modified by direct nitration, followed by reduction with tin metal and quaternization with iodomethane to produce a superficial layer of quaternized amino functions. Besides being simple and economical, the proposed method of synthesis leads to an anion exchanger that allows the use of shorter columns (75 × 4.6 mm I.D.) than those currently employed for carbohydrate separation by high-performance anion-exchange chromatography with pulsed amperometric detection under alkaline conditions. The new sorbent has proved to be highly selective for isomeric disaccharides and individual oligomers of an homologous series of maltooligosaccharides of up to 21 glucose residues. Extended exposure of columns packed with this sorbent to high pressure and strong alkaline solutions did not have any untoward effect on mechanical stability and chromatographic performance.

1. Introduction

The pK_a values of neutral carbohydrates usually fall in the range 12–14, and at high pH their hydroxyl groups are either partially or completely ionized, enabling this class of compounds to be separated as anions by high-performance anion-exchange chromatography (HPAEC) [1]. Furthermore, alkaline conditions allows the detection of carbohydrates by pulsed amperometric detection (PAD) at a gold electrode. PAD is characterized by high sensitivity, relative specificity for compounds with hydroxyl groups, it allows gradient elution techniques and no sample derivatization is requested [2]. This renders HPAEC–PAD a powerful tool for the highly selective separations of carbohydrates [3–8]. Silica-based columns cannot be used for these applications, due to their poor stability at high pH. Under these conditions separations are performed on polystyrene–divinylbenzene (PS–DVB)-based columns.

Most of the anion-exchange columns currently employed for carbohydrate separations by HPAEC–PAD are packed with electrostatically

* Corresponding author.

☆ Presented at the *16th International Symposium on Column Liquid Chromatography, Baltimore, MD, 14–19 June 1992*, paper L 127.

0021-9673/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(94)00665-2

latex-coated pellicular polymeric-based anion-exchange sorbents [9–11]. These sorbents are prepared by a proprietary process, which permits the independent manufacture of polymeric nonporous pellicular sulphonated resins and anion-exchange latex particles functionalized with quaternary ammonium compounds that are brought together only at the last steps of preparing a column. Another anion exchanger, specifically designed by Dionex for the separation of weakly ionizable carbohydrates such as mono- and disaccharides, sugar alcohols, and aldoses consists of a 8.5 μm diameter macroporous poly(vinylchloro–divinylbenzene) with quaternary ammonium functional groups [12]. Furthermore, the use of a macroporous poly(N,N,N,-trimethylammoniummethylstyrene–divinylbenzene) strong anion exchanger manufactured by a proprietary process [13] has also been reported [14,15].

Several other methods have been reported to produce surface-functionalized polystyrene-based anion exchangers, mainly for the separation of organic or inorganic ionic species [16,17], and proteins and nucleic acids [18,19].

The aim of this study was to develop a new, simple method for preparing a strong anion exchanger by chemical modification of highly cross-linked PS–DVB microspheres and to examine the potential of this new sorbent for the separation of carbohydrates by HPAEC–PAD under alkaline conditions. Chemical functionalization of the support was obtained by direct nitration, followed by reduction with granulated tin metal and quaternization with iodomethane. Details of this synthesis and applications of the resultant sorbent for the separation of isomeric disaccharides and individual oligomers of maltooligosaccharides by HPAEC–PAD in short columns of 75 × 4.6 mm I.D. are reported.

2. Experimental

2.1. Materials

All saccharides used as authentic standards, including maltooligosaccharides with degrees of polymerization 2–7 were obtained from Sigma (St. Louis, MO, USA). Dextrin 10 and dextrin 20 (maltodextrin) starch hydrolysates were purchased from Fluka (Buchs, Switzerland). Sodium hydroxide (50%, w/w) solution and anhydrous sodium acetate were purchased from J.T. Baker (Deventer, Netherlands). Anhydrous N,N-dimethylformamide, 1,2,2,6,6-pentamethylpiperidine and iodomethane were from Aldrich (Milwaukee, WI, USA). Reagent-grade fuming nitric acid, concentrated sulphuric acid, granulate tin metal, hydrochloric acid, HPLC-grade water, and other reagent-grade solvents and salts were purchased from Carlo Erba (Milan, Italy).

2.2. Equipment

The experiments were performed on a Dionex (Sunnyvale, CA, USA) Model 4000i gradient pump module equipped with a Model PAD II pulsed amperometric detector consisting of an amperometric flow-through cell with a gold working electrode and a silver–silver chloride reference electrode. The following working pulse potentials and durations were used for detection of analytes: $E_1 = 0.10$ V ($t_1 = 300$ ms); $E_2 = 0.60$ V ($t_2 = 120$ ms); $E_3 = -0.60$ V ($t_3 = 300$ ms). The response time of the PAD was set to 1 s. A Dionex DXP single-piston pump was used to add 0.3 M sodium hydroxide to the column effluent through a tee at 0.2 ml/min before the PAD cell during elution with sodium hydroxide concentrations lower than 40 mM. The sample loop volume was 10 μl. The Dionex eluent degas module was employed to sparge and pressurize the eluents with helium. This both degases and prevents adsorption 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.

2.3. 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 were prepared and characterized as previously reported [20]. The

PS–DVB beads were superficially nitrated and reduced by a modification of the method proposed by Maa et al. [18], and then reacted with iodomethane to produce a superficial layer of quaternized amino groups.

Nitration

Dry PS–DVB beads (5 g) were dispersed in a 6-fold excess (v/w) of N,N-dimethylformamide by sonication for about 10 min. The suspension was added dropwise under stirring at 2–5°C to 60 ml of a 75% (v/v) mixture of fuming nitric acid and sulphuric acid contained in a 250-ml three-neck flask equipped with a stirrer and a thermometer, and cooled by an ice bath. Stirring of this mixture was protracted for 3 h. The reaction mixture was then heated to 60°C and maintained at this temperature ($\pm$ 2°C) under stirring for an additional 6 h. The resulting nitrated PS–DVB beads were filtered on a sintered-glass filter and washed successively with water, 0.15 *M* sodium hydroxide, water to neutrality, and N,N-dimethylformamide. Then the particles were air-dried overnight.

Reduction

The nitrated PS–DVB beads were placed in the three-neck flask and dispersed in 30 ml of N,N-dimethylformamide by sonication for about 5 min. A 2-g amount of granulated tin metal was added to the reaction mixture cooled in an ice bath. From a dropping funnel, 60 ml of concentrated hydrochloric acid were added dropwise under stirring maintaining the temperature at 5–10°C. Thereafter, the reaction mixture was slowly warmed up to room temperature, stirred for an additional 2 h, then heated at 90°C and left at this temperature overnight under continuous stirring. The reduced product was filtered on a sintered-glass filter and washed successively with water, 1.0 *M* sodium hydroxide, water and then methanol. At the end, particles were left overnight under vacuum in an oven at 60°C.

Quaternization

The resulting amino groups were methylated following the procedure reported by Rounds and Regnier [19]. A 700-μl volume of 1,2,2,6,6-pentamethylpiperidine was added to the particles, which were previously suspended in 35 ml of dry N,N-dimethylformamide. The reaction mixture was sonicated for a few minutes, then 3 ml of iodomethane were added. The suspension was heated at 60°C for 10 h under stirring. After cooling, the reaction mixture was filtered on a sintered-glass filter and washed successively with water, methanol, triethylamine, methanol and then dried at 60°C under vacuum.

2.4. HPLC columns and procedures

Columns were packed using the following high-pressure slurry packing technique. The polymeric sorbent (1.25 g) was suspended in 20 ml of HPLC-grade water, sonicated for 3 min and packed into a 75 $\times$ 4.6 mm I.D. polyether ether ketone (PEEK) column (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).

Eluents were prepared by suitable dilution of 50% sodium hydroxide solution with HPLC-grade water. After each run the sodium hydroxide concentration was ramped to 300 m*M* in 5 min and then maintained at this level for a further 15 min to clean the column. The column was then reequilibrated to the starting conditions for 15 min.

3. Results and discussion

3.1. Characterization of the column

The complete synthetic route to the strong-anion-exchange packing material is depicted in Fig. 1. In order to evaluate the mechanical stability of the polymeric-based sorbent, the pressure drop of a 75 $\times$ 4.6 mm I.D. column packed with the above stationary phase was measured upon eluting it with water at various flow-rates. The results are presented in Fig. 2 as a plot of column pressure drop versus flow-rate, which shows good linearity up to column inlet pressure of 27.6 MPa (4000 p.s.i.), the practical upper limit of the pumping system used.

The resistance of the PS–DVB-based sorbent

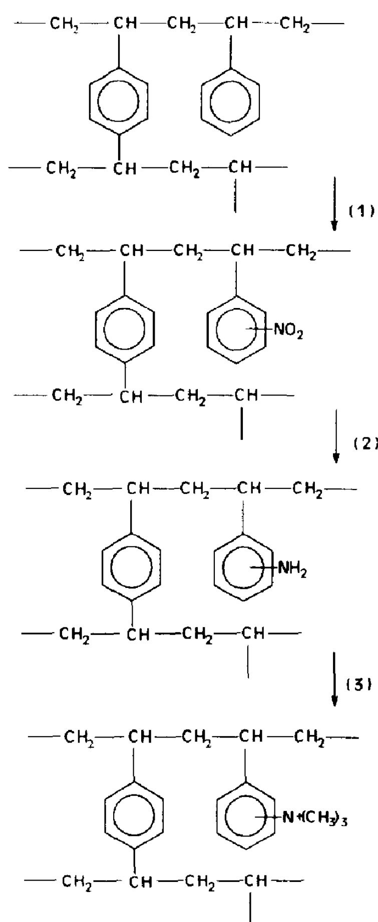

Fig. 1. Synthetic route to the quaternized amino phase on highly cross-linked poly(styrene–divinylbenzene) 2.8-μm particles. (1) Direct nitration, (2) reduction of nitro groups and (3) quaternization of amino groups.

to prolongate exposure to alkaline mobile phase and pressure was investigated by measuring the column performance during a test consisting of alternative elution of the column with aqueous solutions containing (A) sodium hydroxide (50 m*M*) and (B) a mixture of sodium acetate (400 m*M*) and sodium hydroxide (50 m*M*). At the end of the test (84 h) more than 2000 column volumes (over 2500 ml) of the two eluents were pumped through the column with no noticeable effect on retention and peak shape of a carbohydrate test mixture. Furthermore, the column used for this test withstood over 600 sample

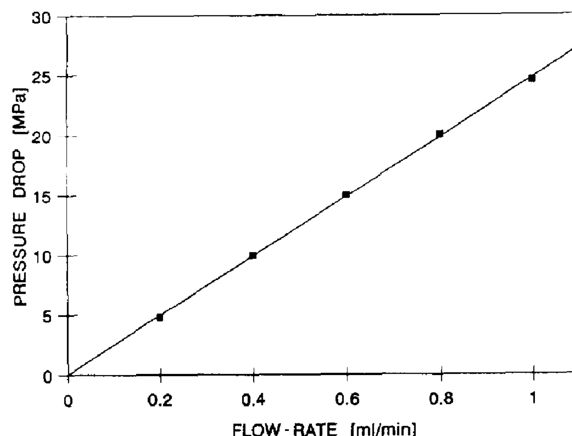

Fig. 2. Plot of pressure drop versus flow-rate of water at 25°C. Column, 75 × 4.6 mm I.D. packed with 2.8-μm highly cross-linked PS–DVB-based strong anion exchanger.

injections under various mobile phase conditions without loss of performance.

The loading capacity of the 75 × 4.6 mm I.D. column packed with the microparticulate sorbent was examined by injecting increasing levels of an equimolar mixture of maltose and turanose. Elution was performed under isocratic conditions with 50 m*M* sodium hydroxide as the mobile phase. Under these conditions, 10 μg (ca. 30 nmol) of each disaccharide was the upper loading limit for resolution. Plots of the peak area generated by the pulsed amperometric detector versus the concentration were constructed for these two saccharides and were found to be linear in the range from 4 to 32 μg/ml (see Fig. 3).

3.2. Retention behaviour and selectivity

The chromatogram of a mixture of authentic standards of glucose, turanose, maltose, panose and maltotriose is reported in Fig. 4. This mixture was used to evaluate the retention behaviour of the stationary phase in relation to the ionic strength of the mobile phase. The standard mixture was eluted isocratically with mobile phases containing sodium hydroxide at various concentrations ranging from 40 to 60 m*M*. The retention times of all carbohydrates

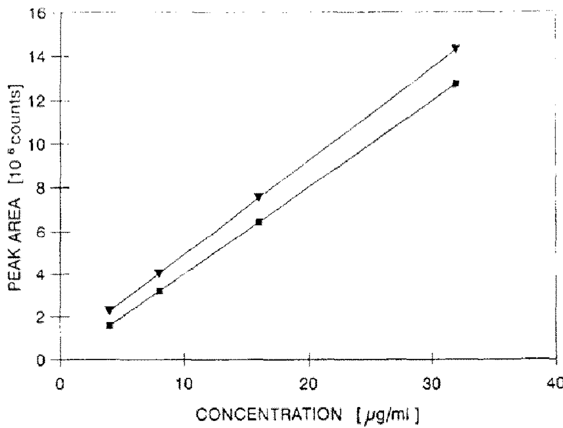

Fig. 3. Dependence of peak area generated by PAD on the concentration of injected sample. Column, 75 × 4.6 mm I.D. packed with 2.8-μm highly cross-linked PS–DVB-based strong anion exchanger; eluent, 50 m*M* sodium hydroxide; flow-rate, 0.5 ml/min; temperature, 25°C; sample: ▼ = turanose, ■ = maltose. Detector, PAD 2; attenuation, 1000 nA.

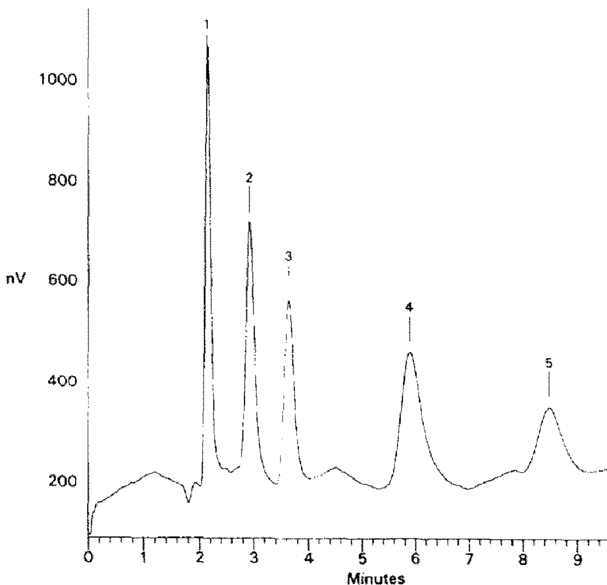

Fig. 4. Separation of (1) glucose, (2) turanose, (3) maltose, (4) panose and (5) maltotriose on column 75 × 4.6 mm I.D. Chromatographic conditions as in Fig. 3, except isocratic elution with 40 m*M* sodium hydroxide.

decreased with increasing sodium hydroxide concentration as shown in Fig. 5. Glucose was eluted first, while the disaccharides turanose and maltose and the trisaccharides panose and maltotriose showed higher retention times, which can be explained on the basis of an increasing number of negative charges on the oligosaccharides owing to the increasing number of hydroxyl groups. Fig. 6 demonstrates that the oligomers of the homologous maltooligosaccharides glucose, maltose and maltotriose, showed a linear relationship between the logarithmic retention time and the number of glucose residues in the homologous series. However, besides chain

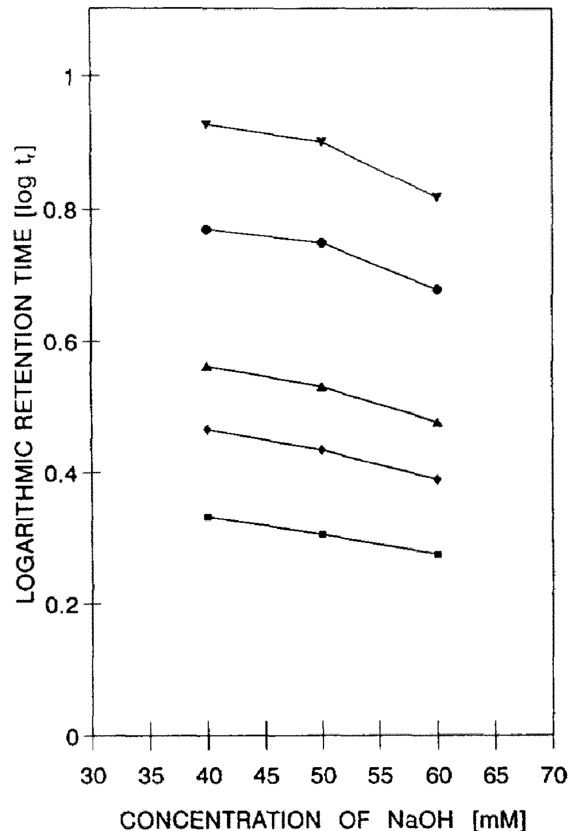

Fig. 5. Plots of the logarithmic retention time as a function of the concentration of sodium hydroxide in the eluent. Chromatographic conditions as in Fig. 3, except concentration of sodium hydroxide in the eluent as indicated in the graph. Samples: ■ = glucose; ◆ = turanose; ▲ = maltose; ● = panose; ▼ = maltotriose.

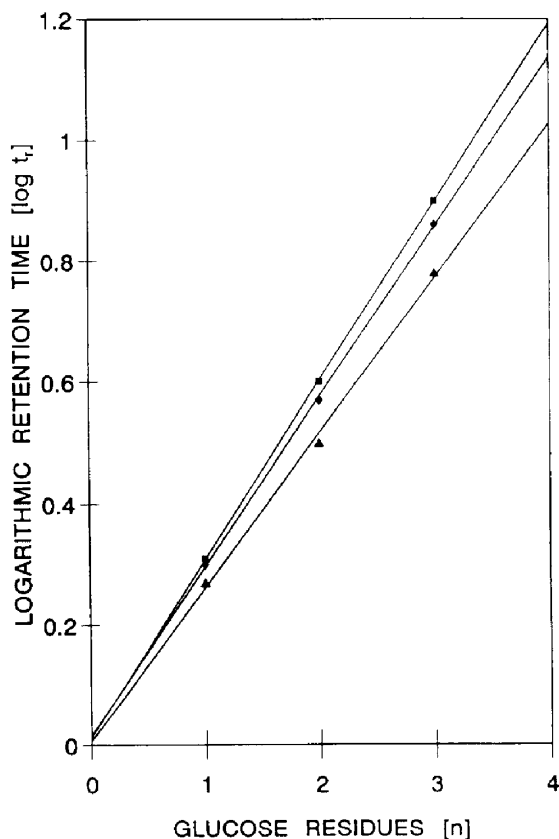

Fig. 6. Dependence of the logarithmic retention time on the chain length of homologous maltooligosaccharides. Eluent aqueous solution of sodium hydroxide at (■) 40, (◆) 50 and (▲) 60 mM. Other conditions as in Fig. 3.

length, saccharide composition and linkage position are expected to affect the chromatographic retention of oligosaccharides [21,22].

Disaccharides were selectively retained under isocratic conditions with mobile phases containing sodium hydroxide at concentrations ranging from 6 to 45 mM. When performing isocratic analysis at these low sodium hydroxide concentrations, postcolumn addition of 0.3 M sodium hydroxide at a flow-rate of 0.2 ml/min was necessary to maintain optimum detector sensitivity and minimize baseline drift. The retention times of twelve disaccharides are listed in Table 1.

Separation of oligosaccharides by AEC is strongly affected, besides their acidity, by the accessibility of oxyanions to functional groups attached to the stationary phase [23]. The disaccharides trehalose, isomaltose, gentiobiose, nigerose and maltose are similar in structure (they are all composed of two D-glucosyl residues), and differ only in the configuration of their glycosidic bonds. These disaccharides follow the elution order showed in HPAEC using a currently available strong anion-exchange sorbent [24], and the differences in the retention times are believed to be related either to the different acidity of the substituted hydroxyl groups or to the different configuration of the glycosidic bond, which causes their orientation to differ when adsorbed to the stationary phase. The greater retention time of the trisaccharide maltotriose compared to the trisaccharide isomaltotriose is an other example of the selectivity of the stationary phase for isomeric forms.

The effective separation of individual components of an homologous series of maltooligosaccharides of different provenance was achieved using linear gradient elution. The chromatogram of the mixture containing maltooligosaccharides of up to 21 glucose residues is reported in Fig. 7. The first peak in the chromatogram corresponds to glucose, the second peak to maltose, etc. The number of each peak, which corresponds to the number of glucose residues in the linear maltooligosaccharide, was confirmed by adding authentic standard of maltooligosaccharides of known degree of polymerization. Maltooligosaccharides of up to 13 glucose residues were eluted during the gradient development, whereas the homologues of greater than 13 glucose residues eluted at the end of the gradient during the following isocratic step. A plot of the adjusted elution volume versus the number of glucose residues in the maltooligosaccharide oligomers is reported in Fig. 8.

4. Conclusions

The results of this study have demonstrated that the synthesized polystyrene-based quaternary amine stationary phase is a highly selective, alkaline-resistant sorbent for high-pH AEC of oligo- and polysaccharides. Isocratic elution with

Table 1
Structures and retention times of neutral di- and trisaccharides by HPAEC–PAD

Trivial name	Structure[a]	Retention time (min)
α,α-Trehalose	α-D-Glc*p*-(1↔1)-α-D-Glc*p*	3.04
Sucrose	α-D-Glc*p*-(1↔2)-β-D-Fru*f*	3.41
Melibiose	α-D-Gal*p*-(1→6)-D-Glc	4.14
Lactose	β-D-Gal*p*-(1→4)-D-Glc	4.49
Lactulose	β-D-Gal*p*-(1→4)-Fru*f*	4.52
Xylobiose	β-D-Xyl*p*-(1→4)-D-Xyl	4.55
Isomaltose	α-D-Glc*p*-(1→6)-D-Glc	4.71
Gentiobiose	β-D-Glc*p*-(1→6)-D-Glc	5.19
Palatinose	α-D-Glc*p*-(1→6)-D-Fru*f*	5.95
Turanose	α-D-Glc*p*-(1→3)-D-Fru	6.55
Nigerose	α-D-Glc*p*-(1→3)-D-Glc	7.01
Maltose	α-D-Glc*p*-(1→4)-D-Glc	7.74
Melezitose	α-D-Glc*p*-(1→3)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*	4.03
Raffinose	α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1↔2)-β-D-Fru*f*	4.13
Isomaltotriose	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Glc	5.67
Maltotriose	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc	19.78

Isocratic elution with 6 m*M* sodium hydroxide solution at a flow-rate of 0.5 ml/min; temperature 25°C.
[a] Abbreviations as recommended by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUP) as reported in ref. [25].

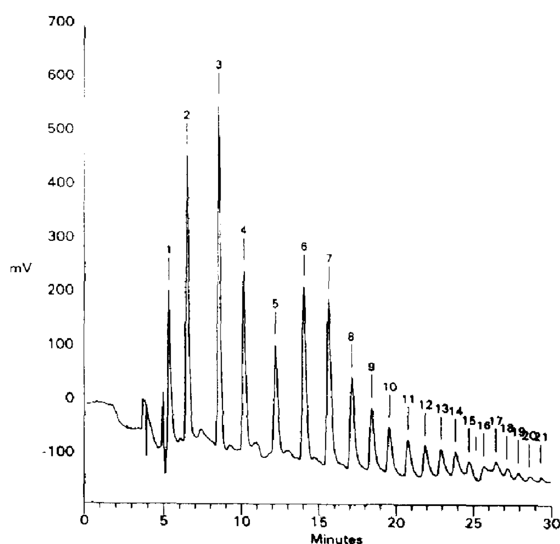

Fig. 7. Separation of linear maltooligosaccharide oligomers of up to 21 glucose residues. Chromatographic conditions as in Fig. 3, except 20 min linear gradient from 0 to 0.2 *M* sodium acetate in 50 m*M* sodium hydroxide, followed by 10 min isocratic elution with the mobile phase containing 0.2 *M* sodium acetate. Peak numbers indicate the number of glucose residues in the separated maltooligomers.

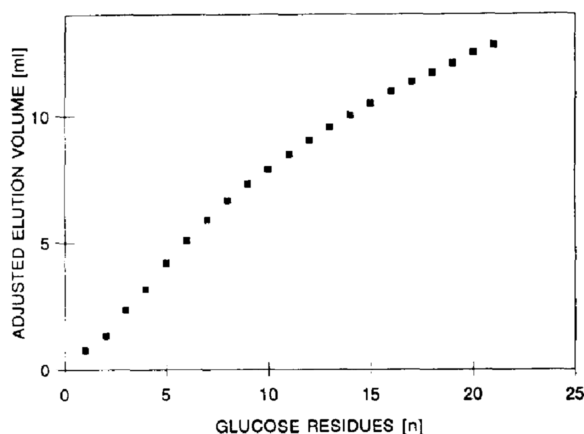

Fig. 8. Dependence of adjusted elution volume on the number of glucose residues in the homologous maltooligosaccharides. Chromatographic conditions as in Fig. 7.

mobile phases containing sodium hydroxide at low concentration has been found to be satisfactory for resolution of isomeric disaccharides, whereas oligo- and polysaccharides are selectively eluted by increasing the sodium acetate concentration during the analysis. Moreover, the use of a short column enables a considerable saving

in time, eluent consumption and instrument usage, which can be advantageous in routine analysis process monitoring and method development.

Acknowledgements

The contributions of Miss Anette Holbek and Miss Charlotte Anker to some of the experiments is gratefully acknowledged. Part of this work was supported by a grant from the Austrian National Bank (No. 4539).

References

[1] Y.C. Lee, *Anal. Biochem.*, 189 (1990) 151.
[2] W.R. LaCourse, *Analusis*, 21 (1993) 181.
[3] M.R. Hardy, R.R. Townsend and Y.C. Lee, *Anal. Biochem.*, 170 (1988) 54.
[4] K. Koizumi, Y. Kubota, T. Tanimoto and Y. Okada, *J. Chromatogr.*, 454 (1988) 303.
[5] D.A. Martens and W.T. Frankenberger, Jr., *Chromatographia*, 30 (1990) 651.
[6] J. van Riel and C. Olieman, *Carbohydr. Res.*, 215 (1991) 39.
[7] T. Hayase, M. Sheykhanazari, V.P. Bhavanandan, A.V. Savage and Y.C. Lee, *Anal. Biochem.*, 211 (1993) 72.
[8] A.T. Hotchkiss and K.B. Hicks, *Carbohydr. Res.*, 247 (1993) 1.
[9] R.D. Rocklin and C.A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577.
[10] J.D. Olechno, S.R. Carter, W.T. Edwards and D.G. Gillen, *Am. Biotechnol. Lab.*, 5 (1987) 38.
[11] *Technical Note 20*, Dionex, Sunnyvale, CA, March 1989.
[12] R.E. Majors, *LC·GC Int.*, 6 (1993) 196.
[13] D.P. Lee, *J. Chromatogr. Sci.*, 22 (1984) 327.
[14] D.P. Lee and M.T. Bunker, *J. Chromatogr. Sci.*, 27 (1989) 496.
[15] K. Mopper, C.A. Shultz, L. Chevolot, C. Germain, R. Revuelta and R. Dowson, *Environ. Sci. Technol.*, 26 (1992) 133.
[16] L.M. Warth, J.S. Fritz and J.O. Naples, *J. Chromatogr.*, 462 (1989) 165.
[17] R.C. Ludwig, *J. Chromatogr.*, 592 (1992) 101.
[18] Y.-F. Maa, S.-C. Lin, Cs. Horváth, U.-C. Yang and D.M. Crothers, *J. Chromatogr.*, 508 (1990) 61.
[19] M.A. Rounds and F.E. Regnier, *J. Chromatogr.*, 443 (1988) 73.
[20] S. Wongyai, J.M. Varga and G.K. Bonn, *J. Chromatogr.*, 536 (1991) 155.
[21] K. Koizumi, Y. Kubota, T. Tanimoto and Y. Okada, *J. Chromatogr.*, 464 (1989) 365.
[22] T.J. Paskach, H.-P. Lieker, P.J. Reilly and K. Thielecke, *Carbohydr. Res.*, 215 (1991) 1.
[23] M.R. Hardy and R.R. Townsend, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 3289.
[24] K. Koizumi, Y. Kubota, H. Ozaki, K. Shigenobu and M. Fukuba, *J. Chromatogr.*, 595 (1992) 340.
[25] J.F. Kennedy and C.A. White, *Bioactive Carbohydrates In Chemistry, Biochemistry and Biology*, Ellis Horwood, Chichester, 1983, pp. 15–42.