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High-performance liquid chromatography of neutral oligosaccharides on a β-cyclodextrin bonded phase column

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ABSTRACT

A β-cyclodextrin bonded phase HPLC column was used for the separation of neutral oligosaccharides derived from starch, cellulose, pullulan, xylan, inulin, and mannan. Rapid, high-resolution separations resulted when acetonitrile–water mixtures were used as mobile phases. Retention times for oligosaccharides were dependent on both monosaccharide composition and linkage types. Compared to aminoalkyl silica gel phases, commonly used for carbohydrate analysis, the β-cyclodextrin bonded phase appeared to be similar in selectivity and superior in durability. It is anticipated that β-cyclodextrin bonded phase HPLC columns may be useful alternatives to commonly used aminoalkyl-modified silica gels for the separation and analysis of neutral oligosaccharides.

INTRODUCTION

Oligosaccharides, produced from the enzyme or acid catalyzed hydrolysis of polysaccharides, are important compounds both biologically and chemically. Determining the composition and relative quantity of each oligosaccharide present in such hydrolysates has traditionally been a difficult challenge. In recent years, many HPLC methods have been developed for this purpose. Separations of oligosaccharides up to about degree of polymerization (DP) 12 have been obtained on various ion exchange resins [1–5]. Oligosaccharides can also be separated on reversed-phase columns using a simple mobile phase, but the chromatograms are often difficult to interpret because anomers of each oligosaccharide are usually separated [6–8]. Chemically bonded amino-type columns give good resolution of oligosaccharides up to DP 30 [9–12], but these stationary phases can be unstable and degrade rapidly. The development of high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC–PAD) now allows for the separation of oligosaccharides up to DP 50 and beyond [13,14], as well as the separation of branched oligosaccharides [15,16]. However, specialized equipment is required for these analyses and quantification of oligosaccharides by pulsed amperometric detection is problematic.

In the present study, we examined the use of a β-cyclodextrin bonded phase HPLC column to separate oligosaccharides. Cyclodextrin bonded phase columns have been used primarily for separation of isomers [17–19]. Such separations were accomplished by the formation of transient inclusion complexes between the analytes and the cyclodextrin cavity. Recently [20,21] it has been shown that small molecular mass carbohydrates can be separated on a cyclodextrin

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bonded stationary phase column. We now report on the usefulness of this stationary phase for the separation of neutral oligosaccharides.

EXPERIMENTAL[a]

Materials

A malto-oligosaccharide sample (M250) was obtained from the Grain Processing Corp. (Muscatine, IA, USA). Pullulan and xylan were purchased from Pfanstiehl Laboratories (Waukegan, IL, USA). Mannan hydrolyzates were a gift of Dr. Allan G.W. Bradbury (Kraft General Foods, Tarrytown, NY, USA). Dextrans (mol. mass 70 000) were purchased from Pharmacia (Uppsala, Sweden). Amberlite IRA-94 resin, free-base form, was purchased from Sigma (St. Louis, MO, USA). Cello-oligosaccharides were prepared using a procedure to be published elsewhere. The Amberlite XAD-4 and MB-3 resins were purchased from Rohm and Haas (Philadelphia, PA, USA). All HPLC solvents were purchased from Baxter (Muskegan, MI, USA) and filtered through a 0.45-μm Nylon filter. Water used for HPLC analyses was purified through a Milli-Q filtration system from Millipore (Bedford, MA, USA).

Isolation of inulin oligosaccharides

Inulin oligosaccharides were isolated from Jerusalem artichokes as follows: Jerusalem artichokes (wet mass 500 g) were peeled and then juiced in an Acme Supreme Model 6001 Juicerator lined with Whatman No. 1 filter paper. The juice was collected in a beaker containing 500 ml of washed Amberlite MB-3 resin, and then passed through a glass column packed with an additional 100 ml of the same resin. The column was washed with water and the sample plus the wash were then decolorized by passing them through a glass column containing 200 ml of XAD-4 resin and 50 ml of MB-3 resin. The eluted sample was then evaporated to dryness under reduced pressure. Inulin oligosaccharide standards were purified by HPLC using a preparative-sized (300 × 22 mm I.D.) HPX-42A Ag^+ form column. The details of this purification will be published elsewhere.

Partial acid hydrolyses

Partial acid hydrolyses of dextran, pullulan, and xylan were performed by dissolving the polysaccharide (2 g) in sulfuric acid (0.1 *M*, 25 ml) and refluxing the reaction mixture for one hour with stirring. The reaction was cooled in an ice water bath, and afterwards the sample was passed through a column that contained 30 ml of Amberlite IRA-94 resin. The effluent was diluted with ethanol so that the final concentration was 50:50. The mixture was then centrifuged (10 000 *g*, 20 min) to remove precipitates. The supernatant was then evaporated to dryness under reduced pressure. The resulting sample was used for chromatographic analyses.

Chromatography

β-Cyclodextrin bonded phase columns (Cyclobond I, 250 mm × 4.6 mm I.D.) were purchased from Rainin Instrument (Woburn, MA, USA). According to the manufacturer, Advanced Separation Technologies (Whippany, NJ, USA), the Cyclobond I phase is formed by coupling β-cyclodextrin molecules through a 10 atom spacer arm to 5-μm spherical silica gel particles. The resulting percentage of carbon is approximately 6.5%. The separation column was preceded by a cartridge-type precolumn (Alltech, Deerfield, IL, USA) packed with reversed-phase (C_{18}-bonded) silica gel. The HPLC system used was a Gilson Model 303 dual pump system equipped with a 811 dynamic mixer and a Rheodyne Model 7125 fixed loop (20-μl) injector. Samples were detected with a Waters 401 differential refractometer, and the data were recorded with a Dynamax HPLC Method Manager version 1.2. Oligosaccharide standards were made up at concentrations of approximately 1 to 3 mg/ml by first dissolving pure standards in water, then adding an equal amount of acetonitrile to produce an acetonitrile–water (50:50) solution. Samples were passed through a 0.45-μm Nylon filter prior to injection.

RESULTS AND DISCUSSION

β-Cyclodextrin bonded phase columns have

[a] Reference to a brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

been reported [20] to separate sugars in the same way as normal-phase silica gel columns that contain alkylamino, diol, or polyol groups. We have now found that this stationary phase is also useful for separation of larger oligosaccharides. Fig. 1 shows the HPLC separation of two types of gluco-oligosaccharides on this phase using acetonitrile–water eluents. Partial resolution, in ascending order of amylose-derived 1,4-linked (malto-) and dextran-derived 1,6-linked (isomalto-) oligosaccharides up to DP 25 and 15, respectively, was obtained in under 30 min using an acetonitrile–water (65:35) mobile phase (Fig. 1A and B). The resolution between adjacent pairs of oligosaccharides increased slightly up to about DP 12 (Fig. 2) and then gradually decreased (data not shown). Increasing the percentage of acetonitrile to 70% (Fig. 1C and D) increased the retention times of all oligosaccharides, allowed complete separation of early-eluting DP oligomers, and provided increased resolution for all DP pairs between 3 and 12

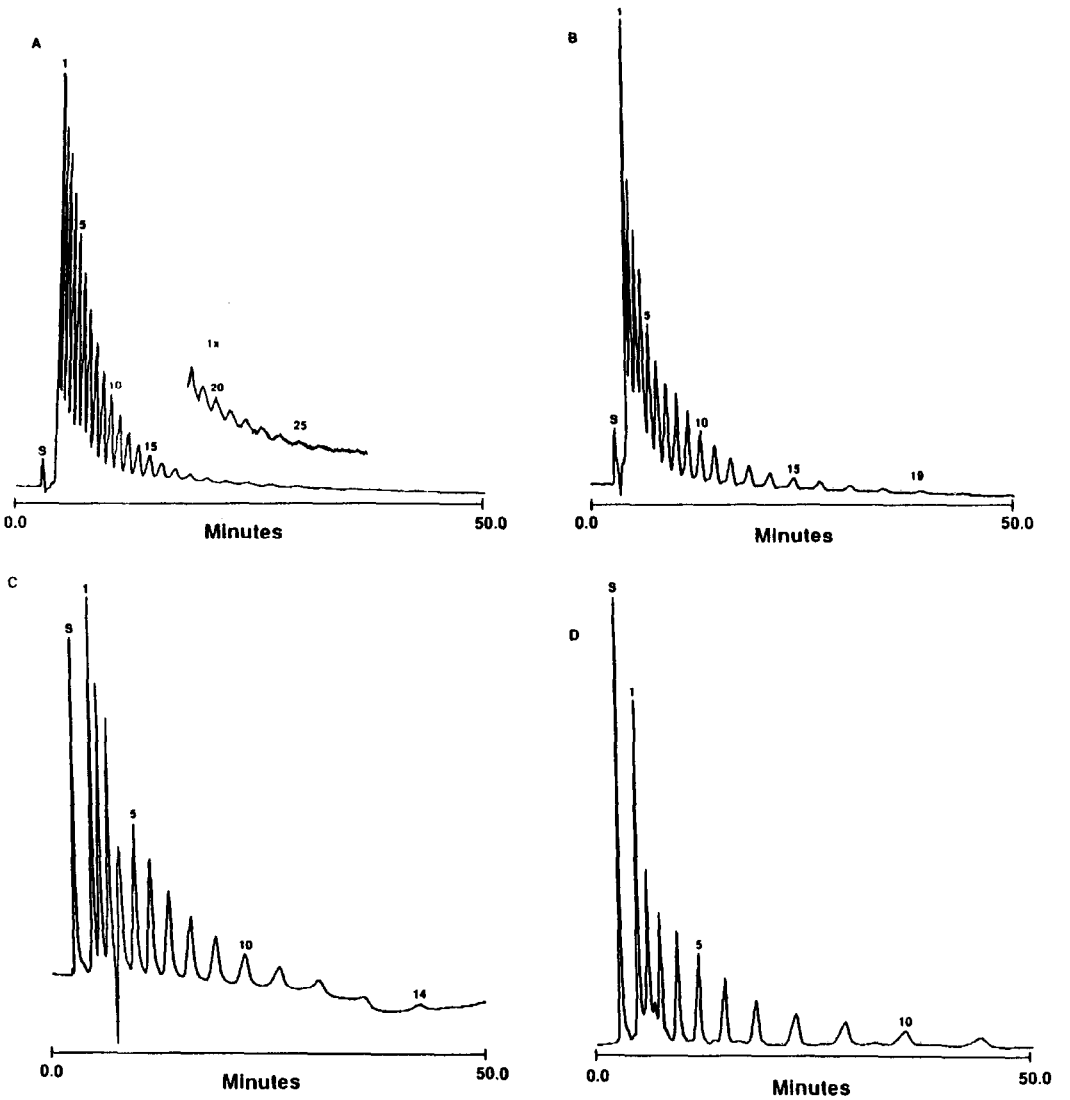

Fig. 1. The HPLC separation of (A) malto- and (B) isomalto-oligosaccharides using an acetonitrile–water (65:35) mobile phase and (C) malto- and (D) isomalto-oligosaccharides using an acetonitrile–water (70:30) mobile phase on a β-cyclodextrin bonded phase column (Cyclobond I, 250 mm × 4.6 mm I.D.). The column was operated at room temperature and 1 ml/min flow-rate with refractive index detection. The numbers above the peaks represent the DP values; S represents the solvent peak.

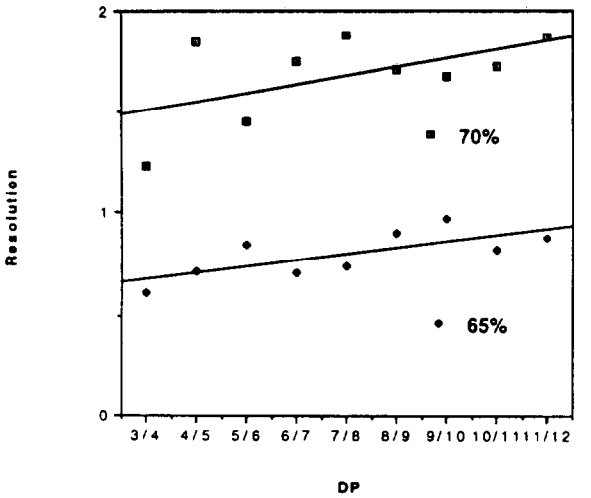

Fig. 2. Resolution values calculated for adjacent peaks of malto-oligosaccharides using mobile phases containing either 65% or 70% acetonitrile. Resolution values were calculated by the following equation, $R = \Delta t/t_w$, where Δt is the distance between adjacent peaks, and t_w is the average width of the peaks at the baseline.

(Fig. 2). At the higher percentage of acetonitrile, however, oligosaccharides above approximately DP 11–14 did not elute within 50 min. At this acetonitrile concentration, it is expected that these larger oligosaccharides would be relatively insoluble and could precipitate onto the stationary phase, eventually leading to decreased column performance.

The difference in retention times between different linkage-classes of gluco-oligosaccharides is readily apparent in Fig. 3. Malto-oligosaccharides generally cluted at shorter retention times than the corresponding isomalto-oligosaccharides. It was possible to resolve similar DP values of isomalto- and malto-oligosaccharides starting at DP 8 with a 70% acetonitrile mobile phase (Fig. 3A). Increasing the percentage of acetonitrile to 75% allowed resolution of oligosaccharides above DP 3 (Fig. 3B).

Pullulan, a mixed-linkage homopolysaccharide composed of α-1,6 linked maltotriose units, was hydrolyzed and the resulting oligosaccharides were analyzed on this stationary phase. The chromatogram (Fig. 4A) was complex and appeared to contain peaks grouped in a unique triplet pattern. The chromatogram obtained with a higher concentration of acetonitrile in the

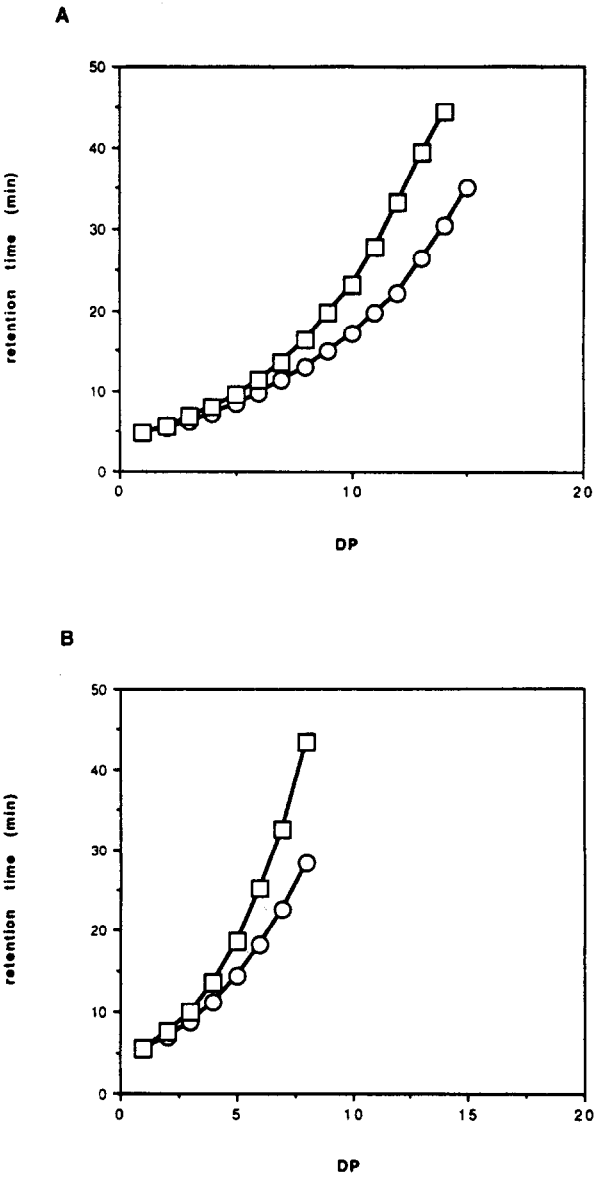

Fig. 3. Graph of retention time *versus* DP for two different classes of gluco-oligosaccharides [(○) malto- and (□) isomalto-series)] on a β-cyclodextrin column with (A) acetonitrile–water (70:30) and (B) acetonitrile–water (75:25) mobile phase. Other conditions as in Fig. 1.

mobile phase resolved some of the former peaks into multiplets (Fig. 4B). These additional, closely eluting peaks are thought to represent oligosaccharides with the same DP value, but with varying combinations of α-1,4 and α-1,6 linkages.

β-1,4 Linked manno- and cello-oligosaccha-

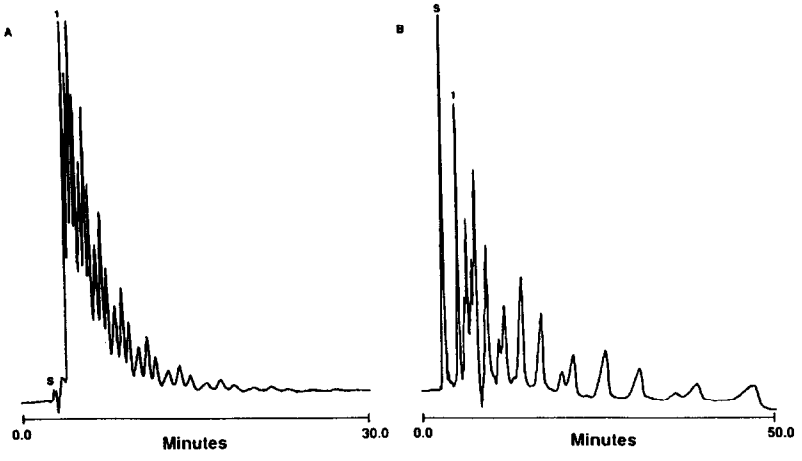

Fig. 4. The separation of pullulan-derived oligosaccharides on the β-cyclodextrin column with an (A) acetonitrile–water (65:35) and (B) acetonitrile–water (70:30) mobile phase. Other conditions as in Fig. 1.

rides from DP 2 to 8 were also readily separated (chromatograms not shown) under similar conditions used for malto-oligosaccharides. Because of the close structural similarity, it was not possible to resolve similar DP isomers of all three classes in the same chromatogram under DP levels of about 7 (Fig. 5).

Inulin-derived oligosaccharides, up to DP 13 ($GlcFru_{12}$), were separated in approximately 20 min using an acetonitrile–water (70:30) mobile phase (Fig. 6A). These oligosaccharides are composed of a sucrose molecule with additional β-1,2 linked fructofuranose units. Better resolution of the early-eluting fructo-oligosaccharides could be obtained with an acetonitrile–water (75:25) mobile phase (Fig. 6B). Under most conditions, the inulin-derived fructo-oligosaccharides eluted earlier than the corresponding manno- and isomalto-oligosaccharides (Fig. 7).

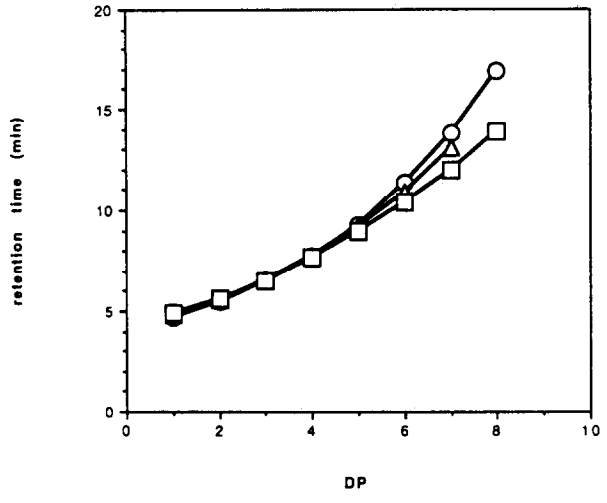

Fig. 5. Dependence of DP on retention time of (○) manno-, (□) malto-, and (△) cello-oligosaccharides on a Cyclobond I column eluted with acetonitrile–water (70:30). Other chromatographic conditions as in Fig. 1.

β-1,4 Linked xylo-oligosaccharides, DP 2–8, were well separated using an acetonitrile–water (80:20) mobile phase (chromatogram not shown). At acetonitrile concentrations lower than 80%, however, DP 2–5 were weakly retained and poorly resolved. The shorter retention times for xylo- *versus* gluco-oligosaccharides would be expected on normal-phase chromatographic systems because the C-5 methylene carbon of the xylopyranose residues results in oligosaccharides that are less polar than the corresponding gluco-oligosaccharides.

The retention times of the fructo- and xylo-oligosaccharides were consistently shorter than the corresponding gluco- and manno-oligosaccharides. These trends are similar to that noticed by Armstrong and Jin [20] for the separation of monosaccharides on this stationary phase. The order of retention for monosaccharides was shown to be pentoses < ketohexoses < aldohexoses. In this report we show that this trend continues for homo-oligosaccharides composed of these monosaccharide units. Thus, the relative

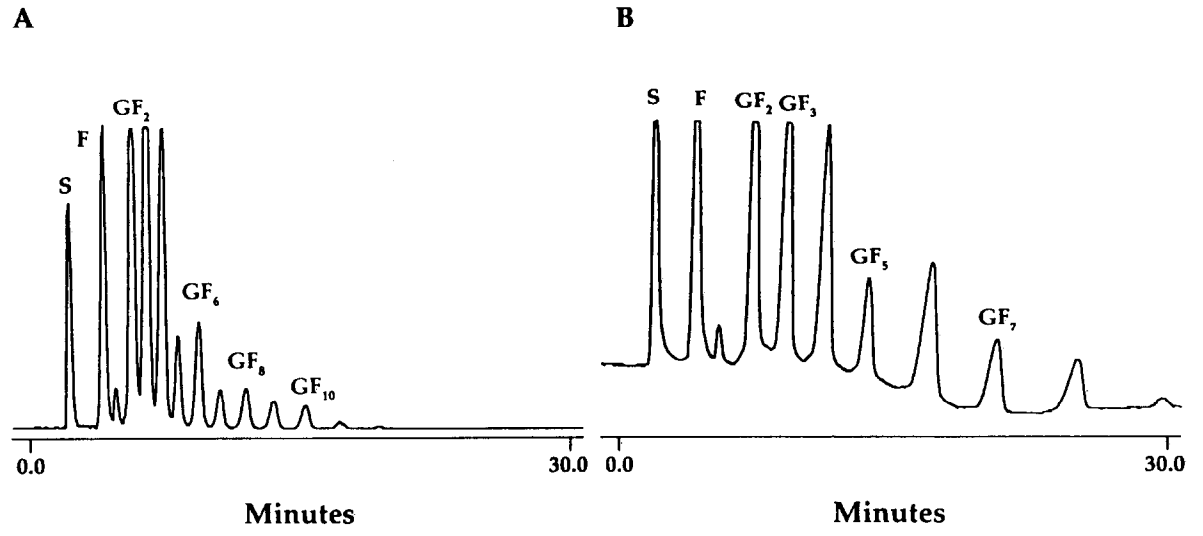

Fig. 6. Separation of inulin-derived oligosaccharide standards using (A) acetonitrile–water (70:30) and (B) acetonitrile–water (75:25). F = fructose, GF_2 = 1-kestose (DP 3), GF_4 = DP 5, etc. Other conditions as in Fig. 1.

retention is: xylose-containing homo-oligosaccharides < those composed of fructose < those composed of glucose or mannose. It is noteworthy that this same order of elution was found when these oligosaccharides were separated on polar alkylamino silica gel stationary phases [21], indicating that the selectivity of these two phases is similar. Alkylamino-type silica gel phases provide excellent selectivity and capacity for carbohydrates, but they are known to have a comparatively short "life" [22]. Diol-modified silica gels [22] and polyamine-bonded vinyl alcohol co-polymer gel columns [15] have been suggested as more durable alternatives. It is not presently known if the Cyclobond I column would be more durable than improved amino-type organic polymer gel columns [15] since they have not been directly compared. The Cyclobond I column is a much more versatile column, however, since it may be also used with different mobile phases, to separate isomeric chiral molecules using an inclusion complexation mode [17–19]. A disadvantage of the diol phases, is that they resolve anomeric forms of reducing sugars, leading to complex chromatographic peaks that are difficult to integrate and quantify. The cyclodextrin stationary phases can be used to resolve anomers, but only under conditions of low temperature (0°C) and with special mobile phases [23]. Under the conditions used in our study, all oligosaccharides eluted with relatively narrow and symmetrical peaks, indicating the absence of anomer resolution. It is not clear why sugar anomers are not separated under these conditions. Perhaps the interaction between the cyclodextrin functionality and the reducing sugar leads to a significant increase in

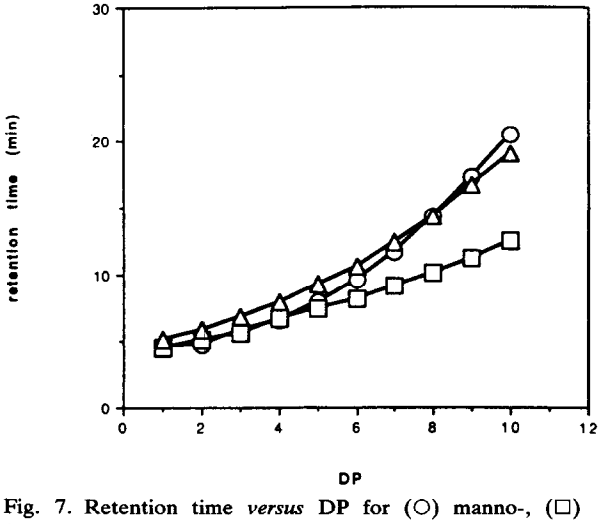

Fig. 7. Retention time *versus* DP for (○) manno-, (□) fructo-, and (△) isomalto-oligosaccharides on the β-cyclodextrin bonded phase column. The analyses were obtained using an acetonitrile–water (70:30) mobile phase. Other conditions as in Fig. 1.

the sugar's mutarotation rate. This action [23] would prevent the separation of the two anomeric forms. It was previously reported [20] that cyclodextrin bonded phases are quite durable for separations of sugars. This was confirmed in this study for larger oligosaccharides; we noted negligible changes in retention times during approximately eight months of heavy use.

CONCLUSION

The β-cyclodextrin bonded phase column was found to be quite useful for the separation of a variety of different neutral oligosaccharides. Retention of oligosaccharides was dependent on both sugar composition and linkage. A normal-phase separation mechanism appeared to be operating since increasing the percentage of acetonitrile, in all cases, led to an increase in oligosaccharide retention times. Because of its selectivity and durability, it is likely that this phase could replace alkylamino-type normal phases for some oligosaccharide applications.

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REFERENCES

1 H.D. Scobell and K.M. Brobst, *J. Chromatogr.*, 212 (1981) 51–64.
2 K. Brunt, *J. Chromatogr.*, 246 (1982) 145–151.
3 J. Havlicek and O. Samuelson, *Anal. Chem.*, 47 (1975) 1854–1857.
4 K.B. Hicks and A.T. Hotchkiss, Jr., *J. Chromatogr.*, 441 (1988) 382–386.
5 A.G.J. Voragen, H.A. Schols, M.F. Searle-van Leeuwen, G. Beldman and F.M. Rombouts, *J. Chromatogr.*, 370 (1986) 113–120.
6 L.A.Th. Verhaar, B.F.M. Kuster and H.A. Claessens, *J. Chromatogr.*, 284 (1984) 1–11.
7 G.D. McGinnis, S. Prince and J. Lowrimore, *J. Carbohydr. Chem.*, 5 (1986) 83–97.
8 N.W.H. Cheetham, P. Sirimanne and W.R. Day, *J. Chromatogr.*, 207 (1981) 439–444.
9 F.M. Rabel, A.G. Caputo and E.T. Butts, *J. Chromatogr.*, 126 (1976) 731–740.
10 V. Kahle and K. Tesarik, *J. Chromatogr.*, 191 (1980) 121–128.
11 W.R. Praznik, R.H.F. Beck and E. Nitsch, *J. Chromatogr.*, 303 (1984) 417–421.
12 T. Akiyama, *J. Chromatogr.*, 588 (1991) 53–59.
13 K. Koizumi, Y. Kubota, T. Tanimoto and Y. Okada, *J. Chromatogr.*, 464 (1989) 365–373.
14 A.T. Hotchkiss, Jr. and K.B. Hicks, *Anal. Biochem.*, 184 (1990) 200–206.
15 K. Koizumi, M. Fukuda and S. Hizukuri, *J. Chromatogr.*, 585 (1991) 233–238.
16 R.N. Ammeraal, G.A. Delgado, F.L. Tenbarge and R.B. Friedman, *Carbohydr. Res.*, 215 (1991) 179–192.
17 J.I. Seeman, H.V. Secor, D.W. Armstrong, K.D. Ward and T.J. Ward, *J. Chromatogr.*, 483 (1989) 169–177.
18 D.W. Armstrong and W. Li, *Chromatography*, 2 (1987) 43–48.
19 M. Krause and R. Galensa, *J. Chromatogr.*, 588 (1991) 41–45.
20 D.W. Armstrong and H.L. Jin, *J. Chromatogr.*, 462 (1989) 219–232.
21 Z.L. Nikolov, M.M. Meagher and P.J. Reilly, *J. Chromatogr.*, 321 (1985) 393–399.
22 C. Brons and C. Olieman, *J. Chromatogr.*, 259 (1983) 79–86.
23 D.W. Armstrong and H.L. Jin, *Chirality*, 1 (1989) 27–37.