## Note

# Rapid separation of linear and cyclic glucooligosaccharides on a cation-exchange resin using a calcium ethylenediaminetetraacetate solution as eluent

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Glucooligosaccharides are important constituents of the products of the potato starch industry. A variety of liquid chromatographic separations of such oligosaccharides has been described. Efficient separations of mono-, di- and even trisaccharides have been achieved with amine-modified silica columns eluted with acetonitrile–water mixtures[1-4]. For several reasons these systems are not preferred for the separation of oligosaccharides. Although the mono- and disaccharides are eluted rapidly as sharp peaks, the oligosaccharides with a higher degree of polymerization (DP 3–10) are eluted as broad peaks after a long time and polysaccharides such as starch are not eluted at all. Moreover, the low solubility of starch in the mobile phase may even cause serious problems.

Efficient separations of oligosaccharides with different DP are performed mostly on ion-exchange columns. On anion-exchange resins separations are achieved by partition chromatography using aqueous ethanol as mobile phase[5,6]. Anion-exchange resins are also used for the separation of carbohydrate–borate complexes[7,8]. The analysis of oligosaccharides produced from starch by enzymatic or chemical degradation is carried out mostly on cation-exchange resins at elevated temperature using water as eluent[9-13]. Often a strongly acidic cation-exchange resin in the calcium form with a cross-linking of about 4% is used. Due to the compressibility of the non-rigid stationary phase, low flow-rates have to be applied down to 0.3 ml min$^{-1}$ (ref. 12) and even 0.1 ml min$^{-1}$ (ref. 11), which require high precision high-performance liquid chromatographic (HPLC) pumps.

It is known that pure water at elevated temperatures is slightly corrosive. The use of electrochemical detectors in combination with HPLC has shown that the aqueous mobile phase often becomes contaminated with traces of metals leaching from the wetted (stainless) steel parts of the HPLC equipment[14,15]. The $Ca^{2+}$ in the cation-exchange resin will be replaced slowly by these metal ions thus changing the efficiency of the column. Owing to the formation of insoluble metal hydroxides and/or oxides, plugging of the column is also possible.

This paper describes an improved separation of glucooligosaccharides on a cation-exchange resin in the calcium form. By using a dilute calcium ethylenediaminetetraacetate (CaEDTA) solution as mobile phase the interference of traces of metals

leaching from the (stainless) steel equipment is eliminated. Column efficiency has been improved significantly by using a relatively short wide-bore column.

EXPERIMENTAL

A Hewlett-Packard 1084 B liquid chromatograph equipped with an automatic sample injection system (sample volume 50 $\mu$l) and an Hewlett-Packard 1033 differential refractometer detector was used.

Two different column systems were applied. System I consisted of two 6.2 mm bore columns of length 25 cm, coupled by a 7-cm stainless-steel capillary (0.25 mm I.D.). Column system II consisted of one 9.0 mm bore column of length 25 cm. Because of the dimensions of the column oven of the 1084 B liquid chromatographic system, columns longer than 25 cm could not be used.

All columns were home-packed with Aminex 50W-X4 purchased from Bio-Rad Labs. (particle diameter 20–30 $\mu$m). Before packing, the resin was purified and converted into the calcium form in a similar manner to that described by Ladisch *et al.*[10]. The resin in the calcium form was slurried in mobile phase in a ratio of 1:4, degassed in vacuum and poured into the filling column, the residual volume of which was filled with mobile phase. The resin was allowed to settle into the analytical column by sedimentation for 2 days. Then the top of the filling column was connected to the eluent pumps and the flow-rate was increased in 2 h to a value somewhat higher than that used during the actual analysis. The assembly was pumped at this flow-rate for 1 h. Then the flow-rate was stopped and the analytical column capped.

The temperatures of the columns and of the differential refractometer detector were 90°C and 37°C, respectively.

Column system I was eluted with only pure water at a flow-rate of 0.30 ml min$^{-1}$, whereas a solution of 50 ppm CaEDTA in water was used as mobile phase for column system II at a flow-rate of 0.50 ml min$^{-1}$. Before use, the mobile phases were filtered through 0.2-$\mu$m filters and deaerated at 90°C under reduced pressure.

CaEDTA was purchased from E. Merck (Darmstadt, G.F.R.) as Calcium-Titriplex and used without further purification.

RESULTS AND DISCUSSION

For the separation of oligosaccharides on Aminex 50W-X4 resin in the calcium form, columns of length 50 cm or more are commonly used[9–13]. Because of the dimensions of most commercially available ovens, the column system often consists of two or three separated columns connected to each other by stainless-steel capillary tubing coiled into curves of 180°. It is beyond question that this tubing will contribute to the dispersion and therefore its use should be minimized whenever possible.

According to the specifications[16], the flow regulation stability of the Hewlett-Packard 1084 B liquid chromatograph used is the summation of $\pm 1\%$ of the flow-rate setting and $\pm 0.02$ ml min$^{-1}$ per pump channel. Fig. 1 shows the calculated fluctuations in the flow-rate which one has to accept. As can be seen, fluctuations of more than 20% are not uncommon at the recommended minimum flow-rate of 0.10 ml min$^{-1}$. It is clear that this pump instability influences strongly the peak retention times. The flow-rate stability can be increased considerably by increasing the flow-
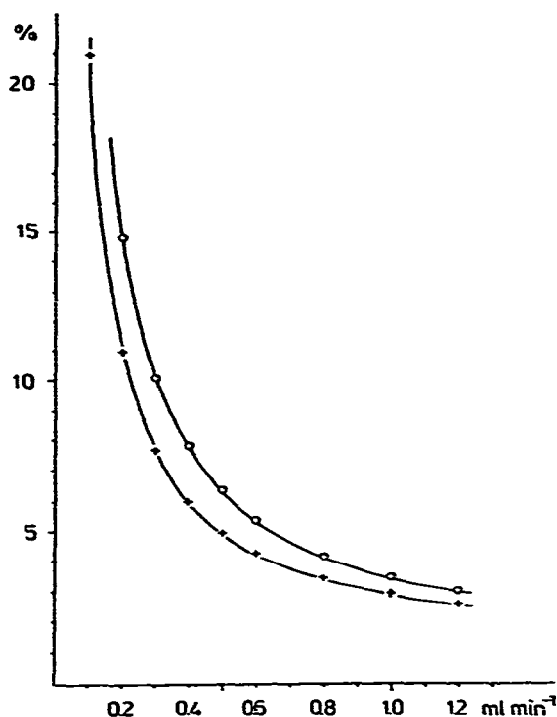
Fig. 1. Percentage flow fluctuation as a function of the flow-rate calculated according to the specifications of the Hewlett-Packard 1084 B liquid chromatograph. +, Flow fluctuations per pump channel; O, flow fluctuations when both pump channels are in use simultaneously.

rate. Unfortunately an increased flow-rate causes a higher column pressure and a decreasing efficiency[17]. However, by using a wider bore column a higher volume flow-rate can be applied without affecting the linear fluid velocity and thus the pressure gradient on the column. As predicted by the Van Deemter equation, it is the linear fluid velocity and not the volume flow-rate which affects the column efficiency. Therefore, by using a 9.0 mm instead of a 6.2 mm bore column, the flow-rate can be increased whereas at the same time the linear fluid velocity is even decreased. This improves the separation efficiency two-fold: (a) due to the higher flow-rate the pump stability increases, resulting in an increased reproducibility of the peak retention times; (b) the lower linear fluid velocity decreases the height equivalent to a theoretical plate (HETP).

A wider column diameter is also advantageous from the point of view of column packing. It is known that in the region of the column walls there is a badly packed region[18,19], which extends about 30 particle diameters inwards from the wall, and where the peak dispersion is much greater than in the core of the column. For a 6.2 mm bore column packed with Aminex 50W-X4 (20–30 $\mu$m particles) the badly packed region represents 35–50 % of the column cross-section. Using a 9.0 mm bore column this region is reduced to 25–36 % of the column cross-section. Thus the use of a wider bore column will improve the column performance.

The addition of a small amount of CaEDTA to the mobile phase is advan-

tageous for several reasons. The main reason is the elimination of traces of heavy metals leaching from the stainless-steel parts of the equipment. These metals tend to form either insoluble (hydr)oxides and plug the column or replace the calcium ions in the resins causing a loss in separation efficiency. The added CaEDTA in the mobile phase chelates rapidly and effectively the leached metal ions by exchanging them for the calcium ions. Another advantage is that the CaEDTA and the traces of free calcium ions in the mobile phase continuously regenerate the resin. keeping the column in optimal condition. Moreover, it is not inconceivable that, analogous to the use of traces of triethylamine in the mobile phase[20], the EDTA catalyzes the mutarotation of the reducing sugars which results in reduced peak widths.

In Table I some column and separation parameters for column systems I and II are compared using a 1 % sucrose solution as sample. It is beyond doubt that the performance of system II is superior to that of system I. The average values and standard deviations in Table I have been calculated from eight chromatographic runs. The reported 50-fold increase in column pressure by Ladisch and Tsao[11] due the increase in column diameter was not observed. The packing in the 9.0 mm bore column is almost ideal as can be concluded from the extremely low effective plate height ($H_{eff} = 78 \pm 6$ $\mu$m) of about three resin particle diameters. The improved asymmetry factor indicates a considerable decrease in column wall effects.

TABLE I

COMPARISON BETWEEN SOME COLUMN AND SEPARATION PARAMETERS OF SYSTEMS I AND II. USING A 1 % SUCROSE SOLUTION AS SAMPLE

| Parameter | Column system I | Column system II |
|---|---|---|
| Column dimensions (mm) | $2 \times (250 \times 6.2)$ | $250 \times 9.0$ |
| Column volume (ml) | 15.1 | 15.9 |
| Eluent composition | Pure water | Aqueous 50 ppm CaEDTA |
| Volume flow-rate (ml min$^{-1}$) | 0.30 | 0.50 |
| Linear fluid velocity (mm sec$^{-1}$) | 0.17 | 0.13 |
| Retention time (min) of sucrose | $30.5 \pm 0.4$ | $16.09 \pm 0.08$ |
| Asymmetry factor* | $2.3 \pm 0.4$ | $1.5 \pm 0.3$ |
| $N_{eff}$** | $(3.0 \pm 0.3) \cdot 10^3$ | $(3.24 \pm 0.25) \cdot 10^3$ |
| $H_{eff}$*** ($\mu$m) | $167 \pm 16$ | $78 \pm 6$ |

* At 10 % peak height.
** $N_{eff} = 5.54 (t_R / W_{1/2})^2$, where $t_R$ = retention time and $W_{1/2}$ = peak width at half height.
*** $H_{eff} = L \cdot N_{eff}$ where $L$ = column length.

In Fig. 2 a comparison is shown between the separation of an acid-hydrolyzed potato starch syrup (5 %) on both column systems. Due to the faster separation and the occurrence of less peak dispersion on the 9.0 mm bore column, the resolution of column system II is better than that of system I. The addition of CaEDTA to the mobile phase has no influence on the separation mechanism of the glucooligosaccharides on the cation-exchange resin. Whether or not CaEDTA is added to the mobile phase. there exists a linear relationship between the retention time and the logarithm of the molecular weight of the eluted glucooligomers (Fig. 3). This in-
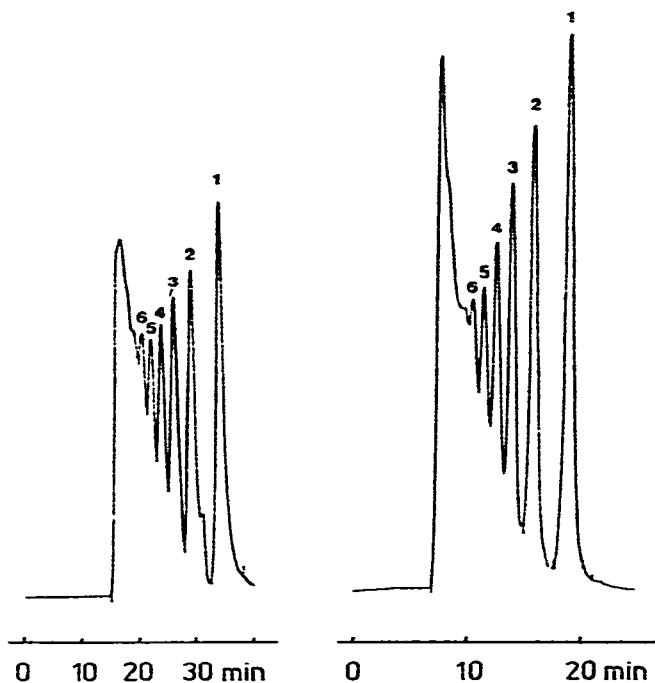
Fig. 2. Comparison between the separation performance of column system I (left) and column system II (right) using an acid-hydrolyzed potato starch syrup (5%) as sample. Peaks: 1 = glucose; 2 = maltose; 3 = maltotriose; 4 = maltotetraose; 5 = maltopentaose; 6 = maltohexaose.
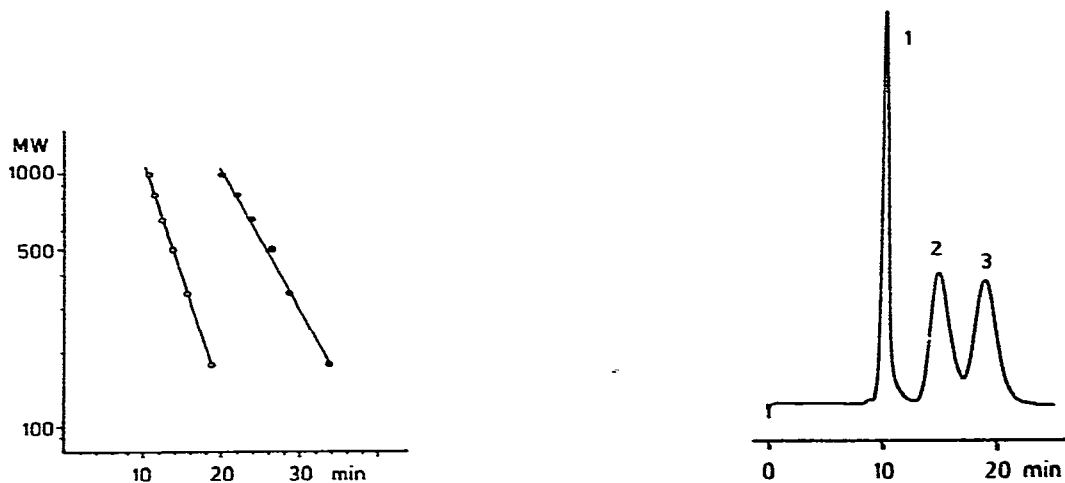


Fig. 3. Plot of the logarithm of the molecular weight (MW) *versus* the retention time of the eluted glucooligomers on column system I (●) and column system II (○).

Fig. 4. Chromatogram of a mixture of cyclodextrins using column system II. eluted with an aqueous 50 ppm CaEDTA solution. Peaks: 1 = $\alpha$-cyclodextrin (1%, w/v); 2 = $\gamma$-cyclodextrin (1%, w/v); 3 = $\beta$-cyclodextrin (1%, w/v).

dicates that in both systems the separation of the glucooligosaccharides is based amongst others on size exclusion effects.

Just as in the ca -: of linear glucooligomers, the analysis time of mixtures of cyclodextrins is shortened considerably by using column system II. Fig. 4 shows the chromatographic separation of a mixture of $\alpha$, $\beta$ and $\gamma$-cyclodextrins. The separation is complete within 23 min on column system II. The separation of the same mixture on column system I with a comparable resolution has been described by Hokse[12] and took about 36 min.

The lifetime of the 9.0-mm column, packed with the Aminex 50W-X4 in the calcium form and eluted with an aqueous 50 ppm CaEDTA solution, is long. The column described in this paper and used for daily routine analysis of both linear glucooligosaccharides and cyclodextrins has served continuously for more than nine months, without significant loss in separation efficiency. Such columns can be packed with good reproducibility, as indicated in Table II.

TABLE II

COMPARISON OF THE PACKING REPRODUCIBILITY OF TWO 9.0 mm BORE COLUMNS

Average values and standard deviations are calculated from eight chromatographic runs using a 1 % sucrose solution as sample.

| Separation parameter | Column packed April 1980 | Column packed February 1982 |
|---|---|---|
| Asymmetry factor | $1.5 \pm 0.3$ | $1.1 \pm 0.1$ |
| $N_{eff}$ | $(3.24 \pm 0.25) \cdot 10^3$ | $(3.28 \pm 0.15) \cdot 10^3$ |
| $H_{eff}$ ($\mu$m) | $78 \pm 6$ | $76 \pm 4$ |

CONCLUSIONS

The use of a wide-bore 9.0-mm column of length 25 cm, packed with Aminex 50W-X4 in the calcium form and eluted with an aqueous 50 ppm CaEDTA solution, shortens significantly the separation time of mixtures of oligosaccharides, improves the peak shape and increases slightly the sensitivity of the system, compared with a narrower bore column system of 6.2 mm I.D.

The addition of CaEDTA to the mobile phase eliminates the interfering effects of metals and prolongs considerably the lifetime of the column.

REFERENCES

1 *Analysis of Carbohydrates in Food by HPLC, Application Note AN 232-14*, Hewlett-Packard, G.F.R., December 1979.
2 *Carbohydrates, Waters Technical Bulletin*, Waters Assoc., Milford, MA, February 1981.

3   M. T. Yang, L. P. Milligan and G. W. Mathison, *J. Chromatogr.*, 209 (1981) 316.
4   D. L. Hendrix, R. E. Lee, Jr., J. G. Baust and H. James, *J. Chromatogr.*, 210 (1981) 45.
5   J. Havlicek and O. Samuelson, *Carbohyd. Res.*, 22 (1972) 307.
6   J. Havlicek and O. Samuelson, *Anal. Chem.*, 47 (1975) 1854.
7   L. A. Verhaar and J. M. H. Dirkx, *Carbohyd. Res.*, 53 (1977) 247.
8   M. Sinner and J. Puls, *J. Chromatogr.*, 156 (1978) 197.
9   H. D. Scobell, K. M. Brobst and E. M. Steele, *Cereal Chem.*, 54 (1977) 905.
10  M. R. Ladisch, A. L. Huebner and G. T. Tsao, *J. Chromatogr.*, 147 (1978) 185.
11  M. R. Ladisch and G. T. Tsao, *J. Chromatogr.*, 166 (1978) 85.
12  H. Hokse, *J. Chromatogr.*, 189 (1980) 98.
13  J. Schmidt, M. John and C. Wandrey, *J. Chromatogr.*, 213 (1981) 151.
14  L. J. Felice, W. P. King, L. A. Pachla, R. M. Riggin, R. E. Shoup and P. T. Kissinger, *J. Chem. Educ.*, 54 (1977) 50.
15  K. Brunt, C. H. P. Bruins and D. A. Doornbos, *Anal. Chim. Acta*, 125 (1981) 85.
16  *Hewlett-Packard Liquid Chromatograph Operating Manual of the 1080 Series.* Hewlett-Packard, Böblingen, 1978, pp. 1–4.
17  H. D. Scobell and K. M. Brobst, *J. Chromatogr.*, 212 (1981) 51.
18  J. H. Knox (Editor), *High Performance Liquid Chromatography*, Edinburgh University Press, Edinburgh, 1978, p. 82.
19  L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979, p. 230.
20  L. A. Th. Verhaar and B. M. F. Kuster, *J. Chromatogr.*, 210 (1981) 279.