CHROMSYMP. 765

USE OF REVERSED-PHASE CHROMATOGRAPHY IN CARBOHYDRATE ANALYSIS

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SUMMARY

   High-performance liquid chromatography of carbohydrates on different types of reversed-phase column with refractive index detection and water as the eluent is reported. Comparisons between the octadecylsilica packing materials of several suppliers have been made, and the influence of the column temperature, the length of the alkyl chain bonded to the silica, and the pore diameter of silica on the retention behaviour of starch hydrolysates and some other sugar samples has been investigated. The determination on an octadecylsilica column of di-D-fructose dianhydrides, formed in the glucose–fructose process, is described.

   It was concluded that decreasing the column temperature results in increased retention times and better resolution; decreasing the length of the alkyl chain or using a silica with larger pore diameters decreases the resolution and retention times. It was also shown that a combination of the chromatographic system described in the study and the modern thermospray quadrupole mass spectrometer works quite satisfactorily with the starch hydrolysate sample.

INTRODUCTION

   The analysis of carbohydrate syrups, obtained by different processes, is normally carried out by high-performance liquid chromatography (HPLC), which is rapid and convenient. Column packings used include polystyrene-based cation[1–8] and anion-exchange resins[9], an amino-bonded silica[8,10], or silica coated with an amino modifier in the eluent[11]. We have used these columns very successfully in our laboratories, but the limitation of the ion-exchangers is their poor resolution of oligosaccharides. The amino-bonded phase currently used is very useful and even quite durable for sugar analysis if handled correctly[8]. Recently many authors have published excellent papers reporting the separation of glucose oligomers on the octadecylsilica (ODS) column with water as the eluent[12–14]. The elution order is that of increasing molecular weight. Reversed-phase chromatography with pure water as the eluent offers two important advantages compared with ion-exchange or amino columns: a rigid stationary phase and a cheap, non-toxic eluent. In addition, it is a good alternative method of identifying sugars in complex mixtures, especially when connected to a mass spectrometer as reported here.

A number of diketose dianhydrides are known that are formed by elimination of two molecules of water between two ketose units[15]. The D-fructose derivatives have been made by the action of strong acid on D-fructose. These components are also formed in fructose–glucose processes, causing losses and some other minor problems. There is a lack of information on how the analysis of difructose anhydrides can be performed. Krol has tried paper chromatography[16,17] and Tsang[18] has used columns of ion-exchange resin in the calcium form to separate these components. It seems that an effective ODS column, with water as the eluent, is probably the best analysis method for difructose dianhydrides.
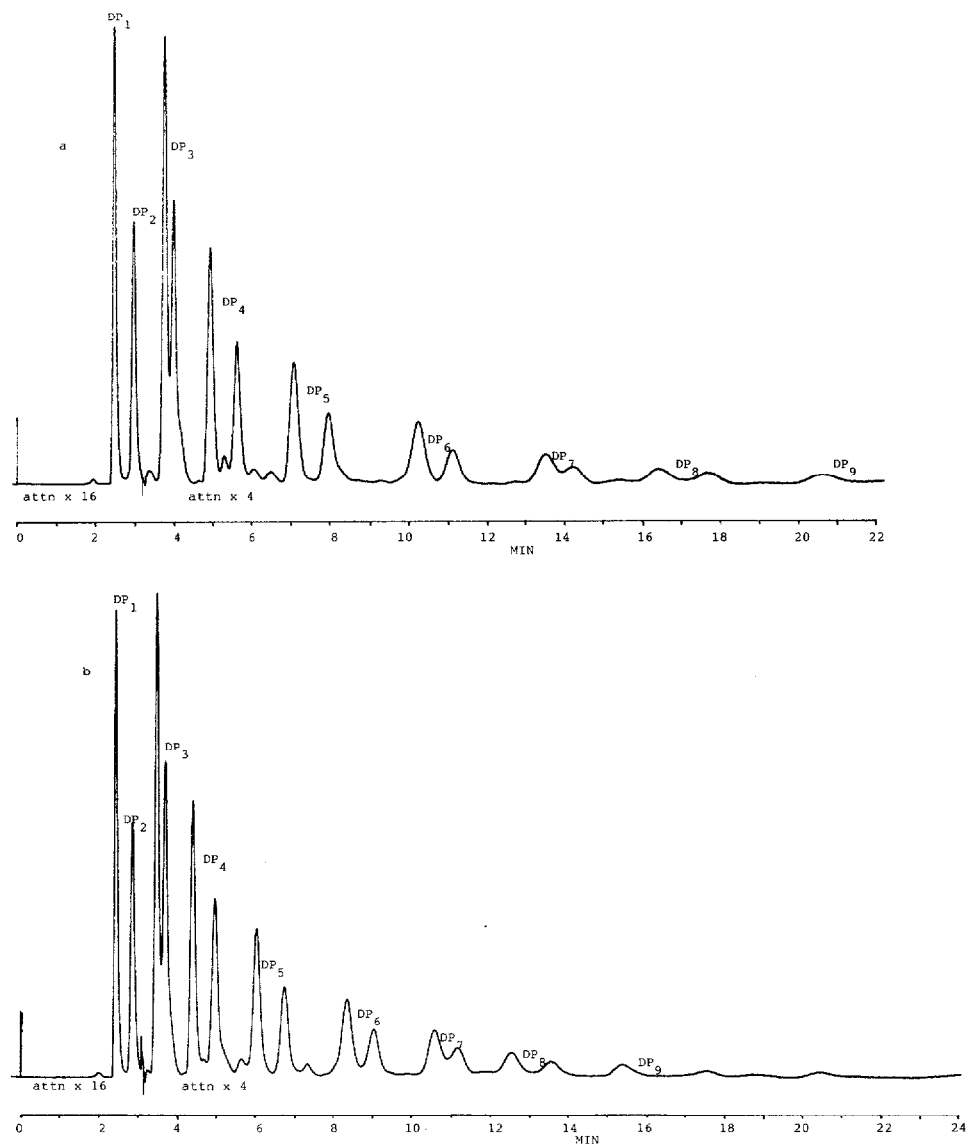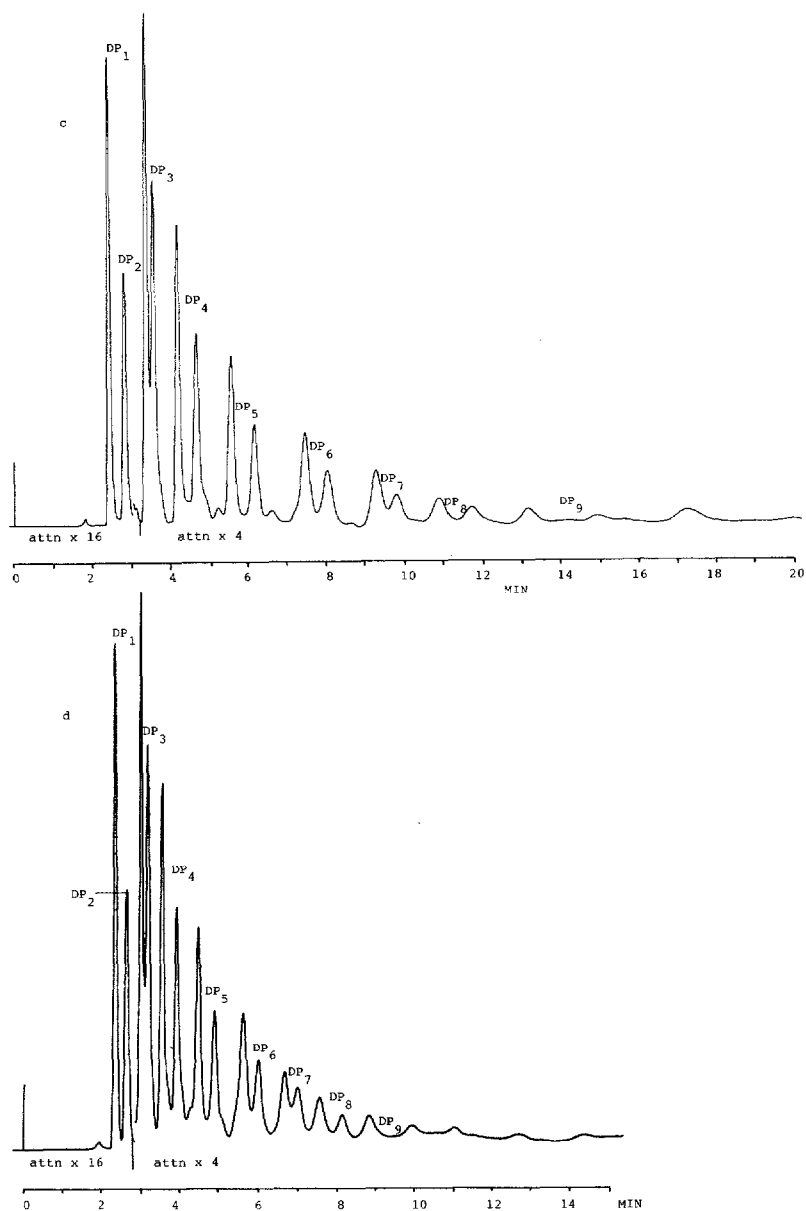

Fig. 1.

Fig. 1. Influence of temperature on the retention of carbohydrates on column 1; eluent, water; flow-rate, 1.0 ml/min; detector, refractive index, $\times 16$ and $\times 4$; sample, 1.0% (w/v) dried corn syrup. The chromatograms were obtained at (a) 5°C, (b) 15°C, (c) 25°C and (d) 35°C. DP = dextrose polymer, the subscript denotes the number of monomer units, *i.e.* DP_1 = glucose, DP_2 = maltose, DP_3 = maltotriose, etc.

EXPERIMENTAL

The experiments were carried out using a Varian 5000 liquid chromatograph (Varian Aerograph, Walnut Creek, CA, U.S.A.) with a Knauer Type 98.00 refractive

TABLE I

EFFECT OF TEMPERATURE ON THE RETENTION TIMES OF SUGARS ON COLUMN 1

Sugar	Retention time (min)		
	25°C	15°C	5°C
Fructose	2.50	2.55	2.53
Maltose	2.82	2.91	2.97
Sucrose	3.38	3.50	3.75
Panose	3.68	3.85	4.15
Stachyose	4.22	4.50	5.12
Raffinose	4.64	4.95	5.83
Diheterolevulosan I	3.25	3.36	3.46
Diheterolevulosan II	3.92	4.04	4.35
Difructose anhydride I	4.16	4.36	4.77
Difructose anhydride II	3.79	3.90	4.19
Difructose anhydride III	3.81	4.00	4.23

index detector (Knauer, Bad Homburg, F.R.G.). The stainless-steel columns, 250 × 4.6 mm I.D., were packed in 2-propanol slurry with methanol as the pressurising solvent, with the following modified 5-μm silicas: column 1, Spherisorb S 5 ODS 2 (Phase Separations, Queensferry, U.K.); column 2, Nucleosil 5 C_{18} (Macherey-Nagel, Düren, F.R.G.); column 3, Vydac 201 HSB 5 reverse phase (The Separations Group, Hesperia, CA, U.S.A.); column 4, Spherisorb S 5 C_8 (Phase Separations); column 5, Spherisorb S 5 C_6 (Phase Separations); column 6, Vydac 201 TPB 5 reverse phase (The Separation Group); column 7, Spherisorb 5 C_{18} (300 Å) (Phase Separations); and column 8, Shandon PB 17 B, WP 300, C_{18} (Shandon Southern, Cheshire, U.K.). The pure size in the first five is ca. 100 Å, in the rest 300 Å.
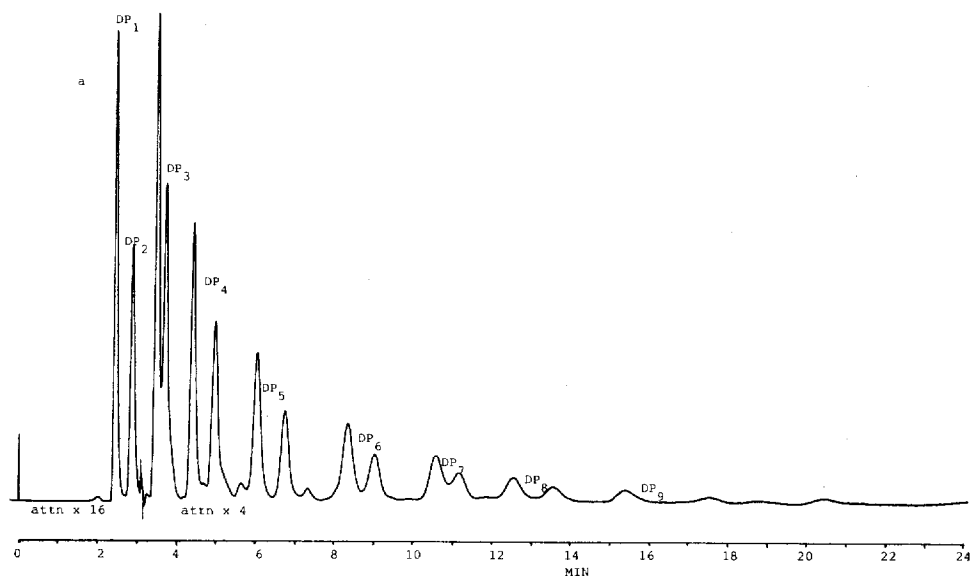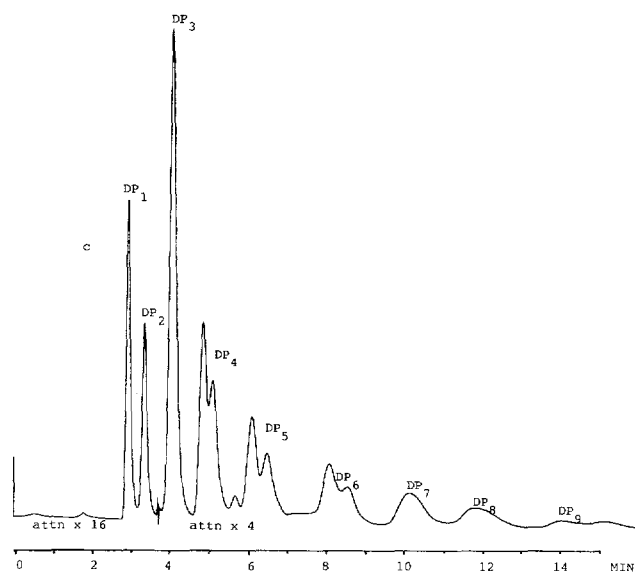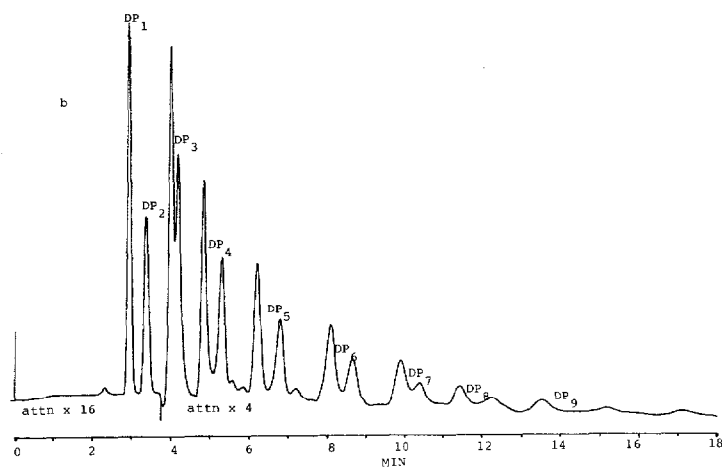

Fig. 2.

Fig. 2. Comparison of the retention of glucose oligomers under same chromatographic conditions (temperature, 15°C; eluent, water; flow-rate, 1.0 ml/min) on (a) column 1, (b) column 2 and (c) column 3.

The following conditions were used: column temperatures, 5–25°C; eluent, water; flow-rate, 1.0 ml/min; injection amount, 20 μl of 0.1% (w/v) solutions of samples.

Dried DE 42 corn syrup was obtained from Hayashibara Biochemical Labs. (Japan). The difructose dianhydrides were kindly donated by Dr. W. S. Charles Tsang (Sugar Processing Research, New Orleans, LA, U.S.A.) and all the other sugars were commercially available materials.

Liquid chromatography–mass spectrometry (LC–MS) was carried out at the application laboratory of S. N. Nermag (Paris, France).

TABLE II

COMPARISON OF CARBOHYDRATE RETENTION TIMES OF THREE DIFFERENT ODS COL-
UMNS

Sugar	Retention time (min)		
	Column 1	Column 2	Column 3
Fructose	2.55	2.97	3.09
Maltose	2.91	3.43	3.45
Sucrose	3.50	4.01	3.85
Panose	3.85	4.58	4.30
Stachyose	4.50	5.24	4.57
Raffinose	4.95	5.79	4.88
Diheterolevulosan I	3.36	4.38	3.85
Diheterolevulosan II	4.04	4.51	4.53
Difructose anhydride I	4.36	5.30	4.82
Difructose anhydride II	3.90	4.38	4.39
Difructose anhydride III	4.00	4.61	4.65

RESULTS AND DISCUSSION

Influence of the temperature on the retention of carbohydrates

The DE 42 corn syrup and some separate sugar samples were run on column
1 at three different column temperatures. Table I and Fig. 1 show very clearly that
lowering the temperature results in longer retention times and better resolution. On
this particular column we could not obtain a single peak for each glucose oligomer,
with no separation of anomeric forms, at a temperature as high as 55°C, as has been
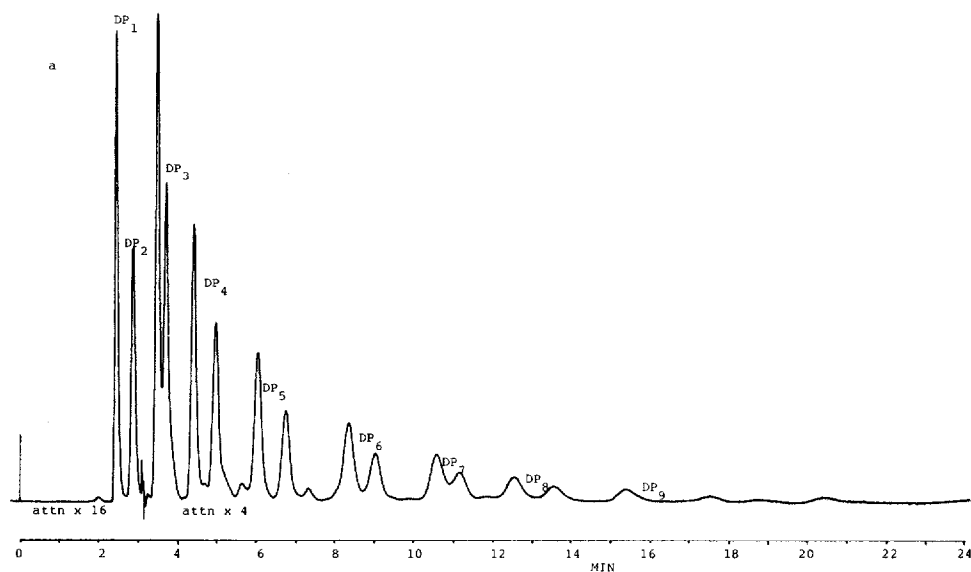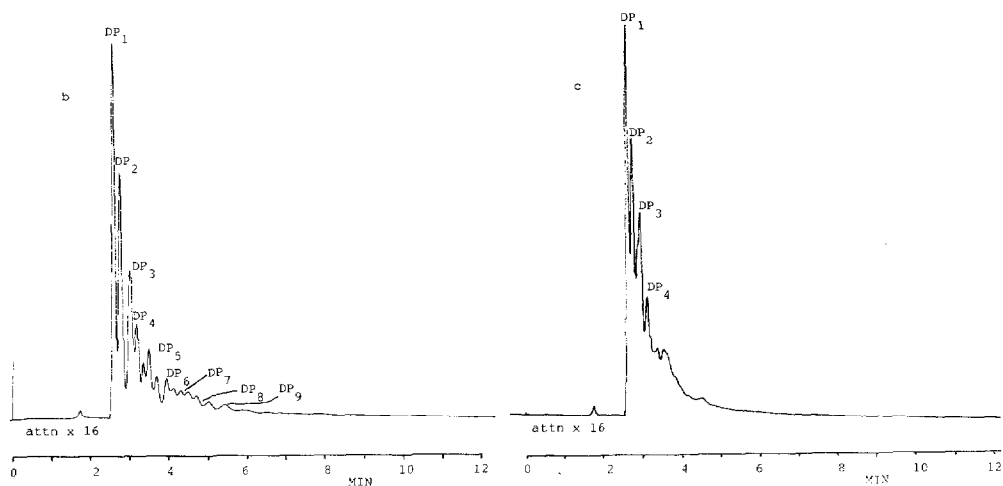reported previously[19].

Fig. 3.

Fig. 3. The effect of the alkyl chain length bonded to silica on the retention behaviour of glucose oligomers on (a) column 1, (b) column 4 and (c) column 5. The chromatographic conditions were as in Fig. 2.

TABLE III

THE EFFECT OF THE ALKYL CHAIN LENGTH ON RETENTION OF CARBOHYDRATES

Sugar	Retention time (min)		
	Column 1	Column 4	Column 5
Fructose	2.55	2.64	2.66
Maltose	2.91	2.75	2.77
Sucrose	3.50	3.02	2.96
Panose	3.85	3.04	2.94
Stachyose	4.50	2.96	2.83
Raffinose	4.95	3.21	3.02
Diheterolevulosan I	3.36	3.08	3.38
Diheterolevulosan II	4.04	3.49	3.37
Difructose anhydride I	4.36	3.59	3.48
Difructose anhydride II	3.90	3.45	3.34
Difructose anhydride III	4.00	3.58	3.50

Comparison of sugar retention on three 5-μm ODS columns

Chromatograms of the DE 42 corn syrup sample and the retention times of the sugars used in this work on columns 1, 2 and 3 at 15°C are shown in Fig. 2 and in Table II. It can be seen that column 1 is most suitable for these samples. Column 2 is also quite satisfactory, but the column 3 differs in selectivity from the other two. This may be due to the different types of silica produced by The Separations Group.

The effect of the length of the alkyl chain on the retention behaviour of carbohydrates

All the samples were run at 15°C column temperature on column 1, column 4 and column 5 phases manufactured by Phase Separations based on exactly the same silica. When the length of the alkyl chain is decreased the retention times of the
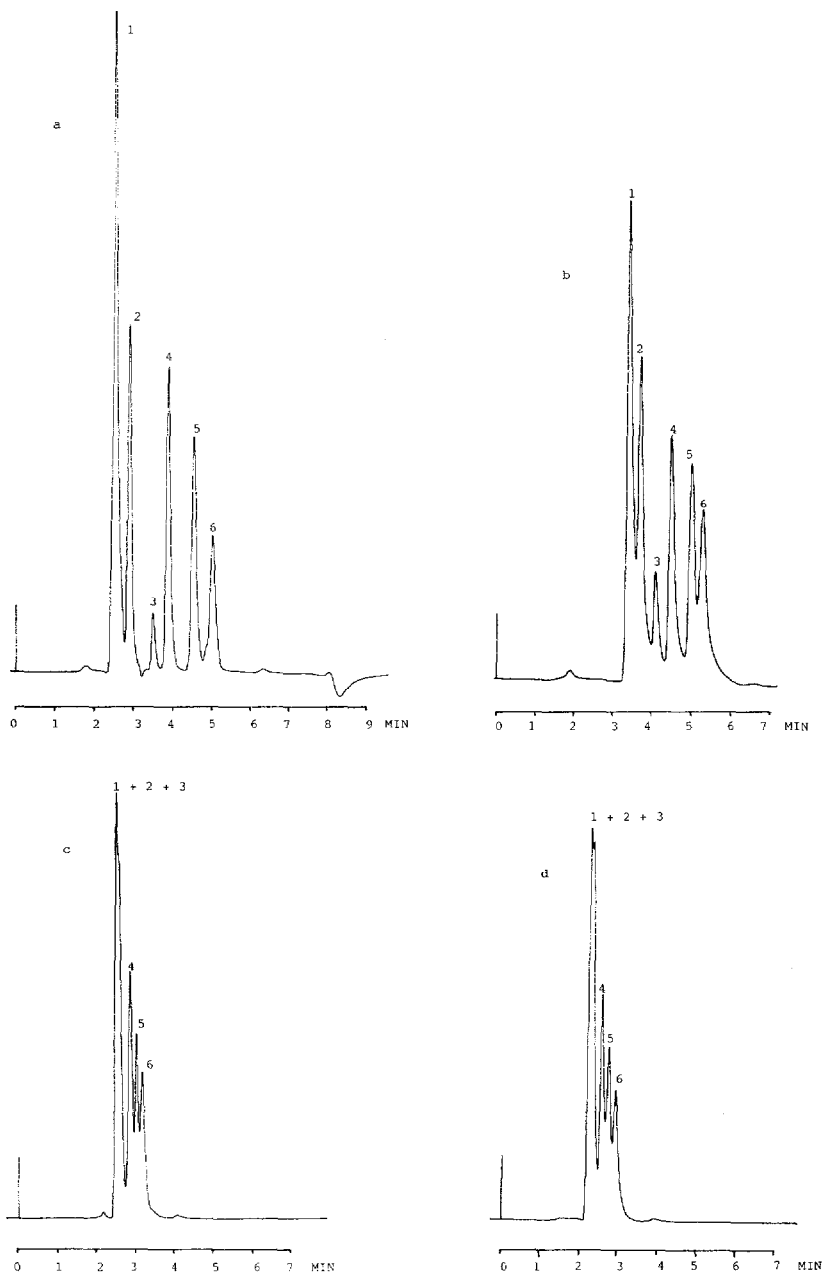
Fig. 4. The influence of pore diameter of silica on retention times of saccharides at 15°C column temper-
ature. The chromatograms were obtained on (a) column 1, (b) column 7, (c) column 6 and (d) column 8.
Peaks: 1 = fructose; 2 = maltose; 3 = sucrose; 4 = panose; 5 = stachyose; 6 = raffinose.

carbohydrates decrease, except in the cases of fructose, glucose and diheterolevulosan
I (Fig. 3 and Table III). The separation mechanism is probably due to hydrophobic

interactions, and when the hydrophobicity of the column material is lower (C_{18} > C_8 > C_6), the degree of interaction with the saccharides decreases, which means lower retention times.

The influence of pore diameter of silica on retention behaviour of carbohydrates

When packings based on 300-Å silica were used, it was found that the retention times and the resolution decreased significantly compared with the results obtained with 100-Å C_{18}-silica. This is illustrated in Fig. 4, which shows the chromatograms of a sugar mixture containing fructose, maltose, sucrose, panose, stachyose and raffinose, run under the same conditions on columns 1, 6, 7 and 8.

The analysis of di-D-fructose dianhydrides by reversed-phase chromatography with water as the eluent

We have tried to analyse di-D-fructose dianhydrides formed in the glucose-fructose process on several different HPLC columns, without any particular success until we used reversed-phase chromatography. Comparison of the retention times with those of authentic standards permitted identification of these components (Fig. 5), which is typical of the chromatograms given by fructose mother solutions. Identification by this method is not reliable and should be confirmed, *e.g.* by MS.
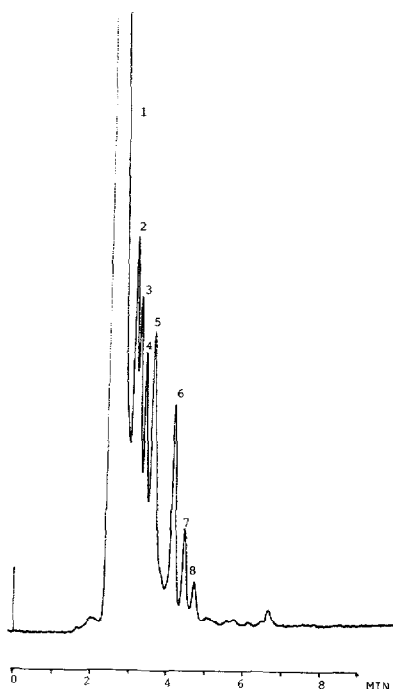

Fig. 5. The determination of di-D-fructose dianhydrides in fructose mother liquid on column 1 at 15°C. Peaks: 1 = fructose; 2 = unknown; 3 = unknown; 4 = diheterolevulosan I; 5 = sucrose; 6 = diheterolevulosan II; 7 = di-D-fructose dianhydride I; 8 = unknown.

Application of LC–MS with a thermospray interface

In the past few years more and more reports of the successful combination of LC and MS have been published. This is probably indicative of the improved performance of the instruments available today and the solution of problems that are not overcome by use of GC–MS. The application of the thermospray instrument in analysis of starch hydrolysate samples has been tested by coupling this to an ODS column, with water as the eluent at a flow-rate of 0.5 ml/min, and post-column addition of a chemical ionization agent, 0.05 M ammonium acetate, at a flow-rate of 0.5 ml/min. Total ion current (TIC) chromatograms of positive and negative ions are presented in Fig. 6. The LC–MS negative ion spectra of glucose and maltose are shown in Fig. 7. The spectra have not been completely interpreted, but the following fragment ions can be assigned: m/z 179 (glu $-$ H$^+$), m/z 161 (glu $-$ H$^+$ $-$ H$_2$O), m/z 143 (glu $-$ H$^+$ $-$ 2H$_2$O), m/z 215 (glu $-$ H$^+$ $+$ 2NH$_4^+$), m/z 221 (glu $-$ 2H$^+$ $+$ CH$_3$COO$^-$)?, m/z 341 (DP2 $-$ H$^+$), m/z 377 (DP2 $-$ H$^+$ $+$ 2H$_2$O).

CONCLUSION

Reversed-phase chromatography is a very good alternative to either ion-exchange or amino columns for analysis of sugars. Reversed-phase packings have the great advantage of separating oligosaccharides when pure water is used as the eluent. The separation can be carried out at room temperature, but by lowering the temperature it is possible to increase the resolution and retention of carbohydrates. By making the alkyl chain bonded to silica shorter, or by using silica with a larger pore diameter, it is possible to change the retention behaviour of oligosaccharides significantly.
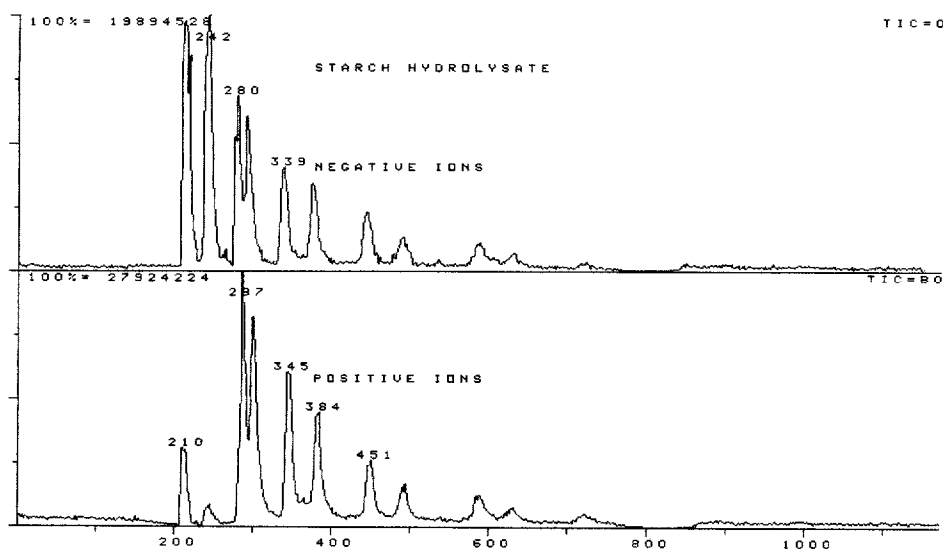

Fig. 6. TIC chromatograms of positive and negative ions of the glucose oligomers; conditions as mentioned in text.
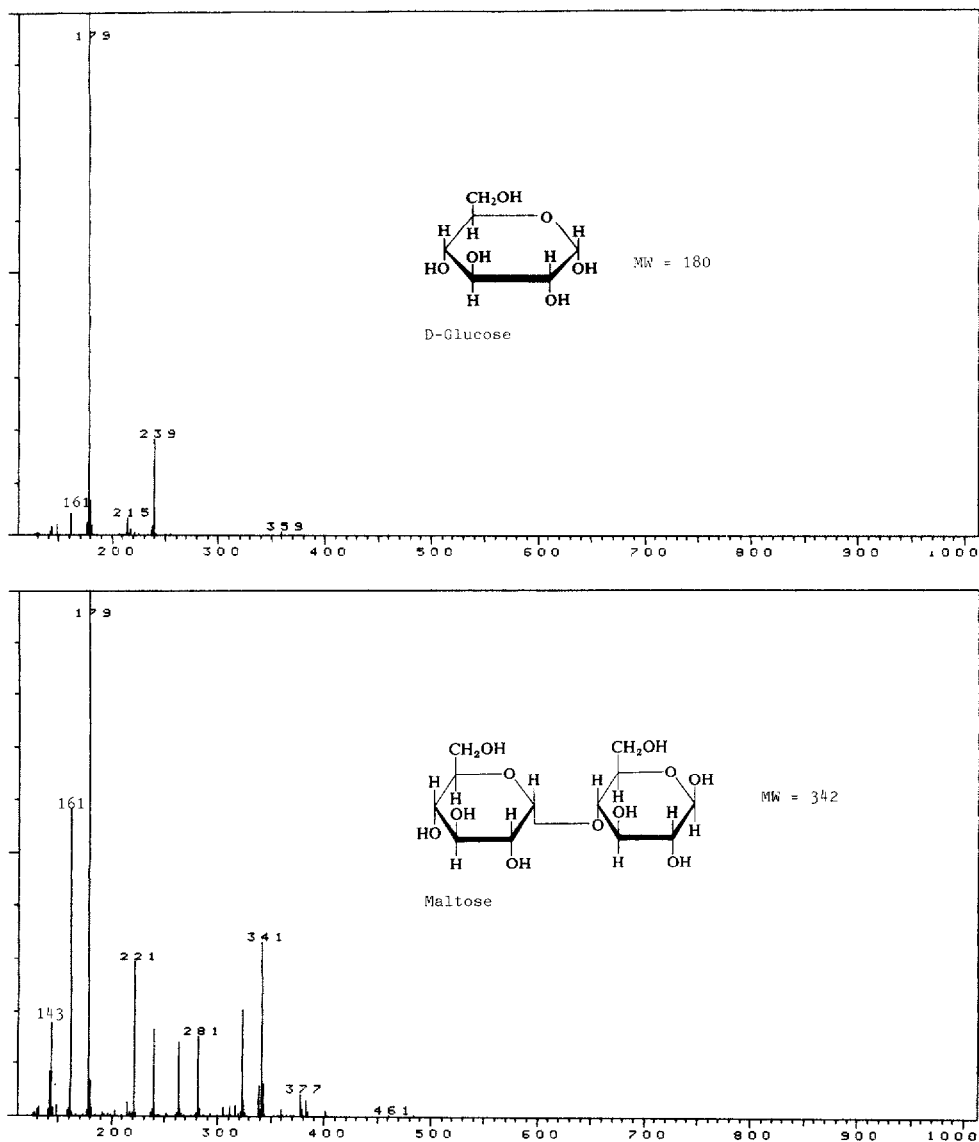
Fig. 7. The LC–MS negative ion spectra of glucose and maltose.

ACKNOWLEDGEMENTS

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