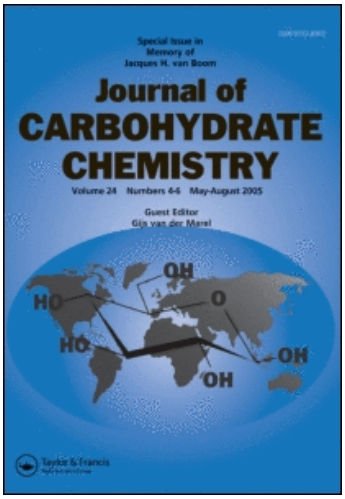

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THE USE OF REVERSE-PHASE COLUMNS FOR SEPARATION OF UNSUBSTITUTED CARBOHYDRATES

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ABSTRACT

Reverse-phase chromatography can be used to separate unsubstituted oligosaccharides using water as the eluent. The retention time of the individual oligosaccharides was found to be dependent on the molecular weight of the oligosaccharide and the type and the anomeric configuration of the linkage. This technique can be used for characterizing polysaccharides based on identification of the oligosaccharide fractions obtained by partial acid hydrolysis or for the isolation and purification of oligosaccharides.

INTRODUCTION

Unsubstituted carbohydrates can be separated by high performance liquid chromatography using amino-bonded silica gel or by ion-exchange columns. The amino-bonded columns were first introduced in 1975[1] and have been used to separate a wide range of mono- and oligosaccharides.[2-5] More recently, techniques have been developed to use silica gel columns and reverse-phase (RP) columns for carbohydrate separations by adding small amounts of amines[6-8] to the

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eluent. The major disadvantages of the amino-containing silica gel columns are their low efficiencies, their short lifetime, and the necessity of using solvents such as acetonitrile/water in which carbohydrates have limited solubility.

Ion-exchange columns have been used for many years to separate carbohydrates.[9] Recently, high performance cation exchange columns have been developed for the separation of unsubstituted carbohydrates.[10-13] These columns have many advantages. They are rugged and use aqueous solutions as the eluent. However, their capacity is fairly low and they give poor separations, especially for the higher molecular weight oligosaccharides.

In the last two years there have been several reports on the use of RP columns for the separation of unsubstituted carbohydrates including oligosaccharides produced by partial hydrolysis of chitin,[14] human milk oligosaccharides,[15] cello- and maltodextrins,[16,17] invert sugar, sucrose and raffinose,[18] ascorbic and isoascorbic acid,[19] cyclosophoraoses,[20] and unsaturated disaccharides produced by enzymatic hydrolysis of chondroitin sulfate.[21]

In this study, the separation of a series of oligosaccharides produced by acid hydrolysis were separated using RP chromatography. The effects of type of linkage, anomeric configuration and the molecular weight were determined. Five different types of RP columns that differed in carbon loading, end capping and the type of solid support were evaluated in this study.

RESULTS AND DISCUSSION

Reverse-phase columns have not been widely used with unsubstituted monosaccharides since there is very little interaction between the carbohydrate and the column. However, if the polarity of the carbohydrate is decreased by converting one of the hydroxyl groups into a less polar group such as methoxyl group, then a RP column can be used. The effect of reducing the carbohydrate's polarity on its chromatographic behavior is shown in Figure 1 for D-glucose and two monomethylated products. It should be noted that the retention time of the D-glucose on the column is the same as for other hexoses.

Another factor besides polarity that affects the interaction between a RP column and a carbohydrate is molecular weight. This is shown for the separation of a series of maltodextrins using water as the eluent on a RP-18 column (FIG. 2). The individual maltodextrins are well resolved on the analytical column; however, the peaks are broad and not very symmetrical. The shape of the peaks is not due to overlap of the individual oligomers but is caused by anomerization of the terminal reducing group. This was confirmed by determining the retention time of pure maltodextrins and by reduction of the oligosaccharides with sodium borohydride into their corresponding sugar alcohol (FIG. 3). With both the cellodextrin and maltodextrin systems, reduction of the terminal aldehyde group gave a symmetrical peak that had a shorter retention time than the starting material.

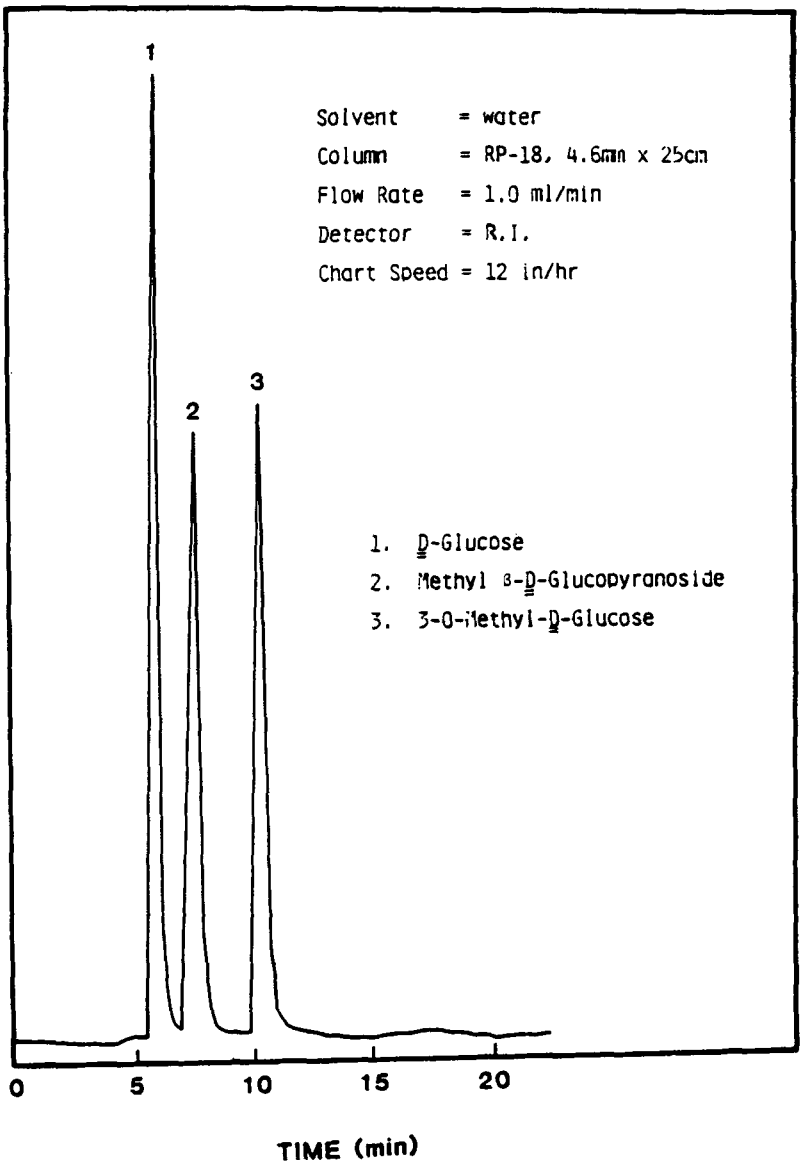

FIG. 1. The Separation of D-Glucose, Methyl β-D-Glucopyranoside and 3-O-Methyl-D-Glucose on a RP Column.

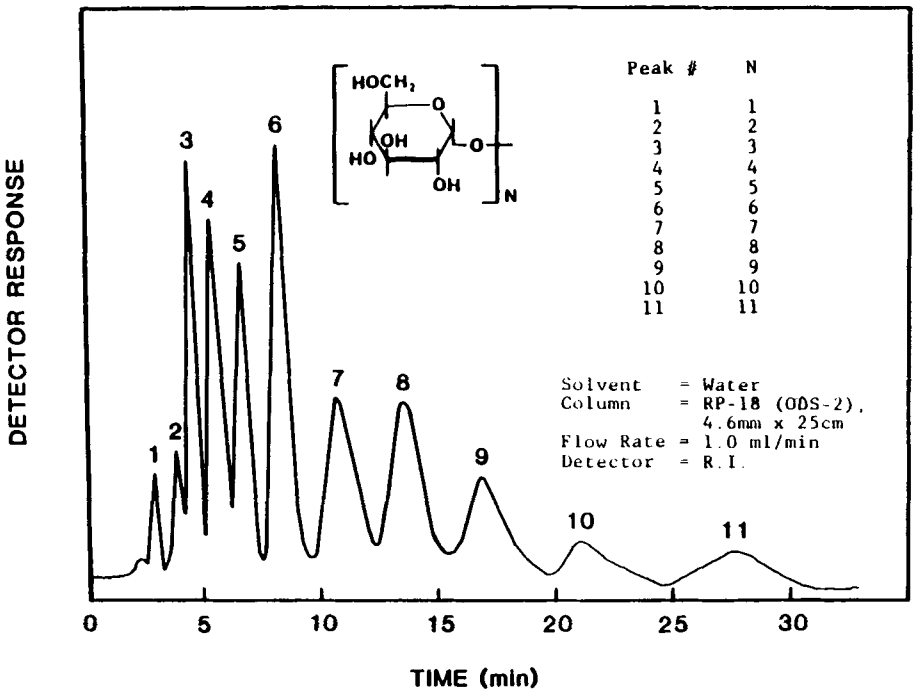

FIG. 2. Separation of a Series of Maltodextrins on a RP Column.

Some of the advantages of using RP columns for oligosaccharide separation are their wide availability, high capacity, and the use of water as an eluent. Isolation of pure oligosaccharide fractions can be done using a semi-preparative RP column. This is illustrated in Figure 4 which shows the base line separation of 25 mg of a maltodextrin mixture on this column.

There is a wide variety of RP columns commercially available. The silica gel based RP columns vary in the pore size of the silica gel, carbon loading, end capping, and the size of the alkyl chain attached to the silica gel. Recently, a new type of RP column has become available that

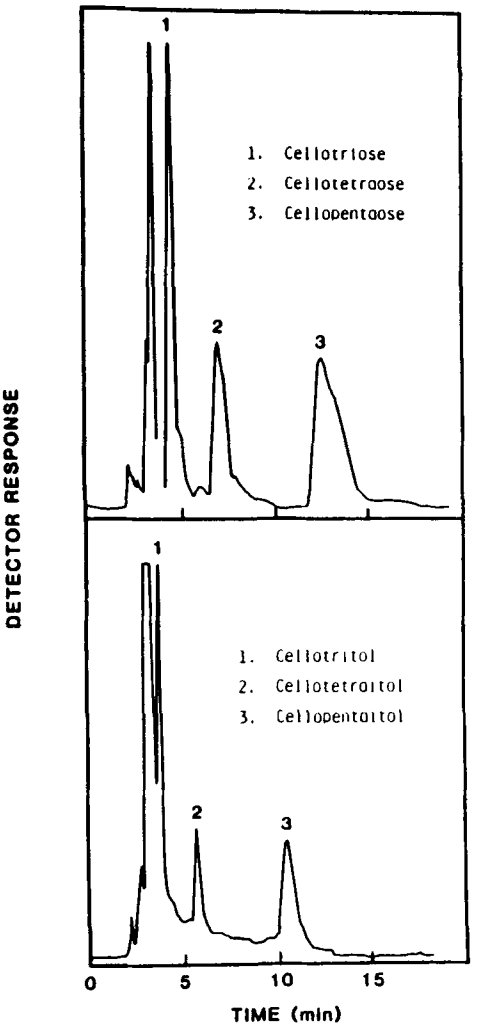

FIG. 3. The Separation of Reduced Cellodextrins on a RP Column Using Water as the Eluent. (Column: ODS-2, 25 x 4.6 mm (ID); Elution Solvent: Water, 1 mL/min; Detector: RI.

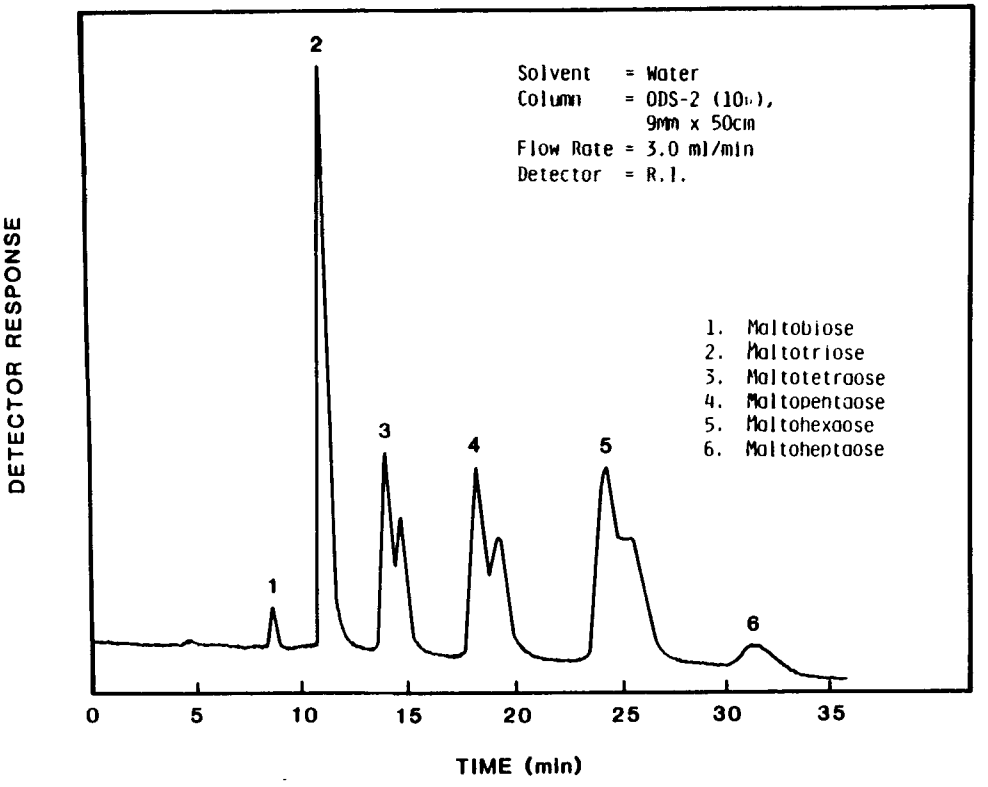

FIG. 4. Separation of Maltodextrins on a Semi-preparative RP Column.

is based on organic polymers (divinylbenzene-styrene). The major advantage of this column is its stability to a wide range of pH (i.e., from pH 1-13); in contrast, the silica gel based RP columns can be used only from pH 2-8.

Five types of RP columns were used in this study. The characteristics of each type are summarized in Table 1. The separation of a mixture of maltodextrins on all five columns is shown in Figure 5. The best column for separation of maltodextrins was the ODS-2 or the RP-18. They gave baseline separation of the maltodextrins based on their molecular weight. The ODS-2 column was used for the

TABLE 1. Characteristics of the RP Packing Material.

	Name	Solid support	Alkyl chain	Particle size	End capped	Carbon loading	Plates/ meter
1.	RP-18[a]	Silica gel	C_{18}	10	100%	15%	10,403
2.	RP-8[b]	Silica gel	C_8	10	100%	10%	10,725
3.	ODS-1[c]	Silica gel	C_{18}	10	50%	5%	14,865
4.	ODS-2[d]	Silica gel	C_{18}	10	70%	15%	33,575
5.	PRP-I[e]	Divinylbenzene-styrene	None	10	--	--	17,000

[a]Lichrosorb C-18.
[b]Lichrosorb C-8.
[c]Whatman C-18.
[d]Whatman C-18.
[e]Hamilton Divinylbenzene styrene.

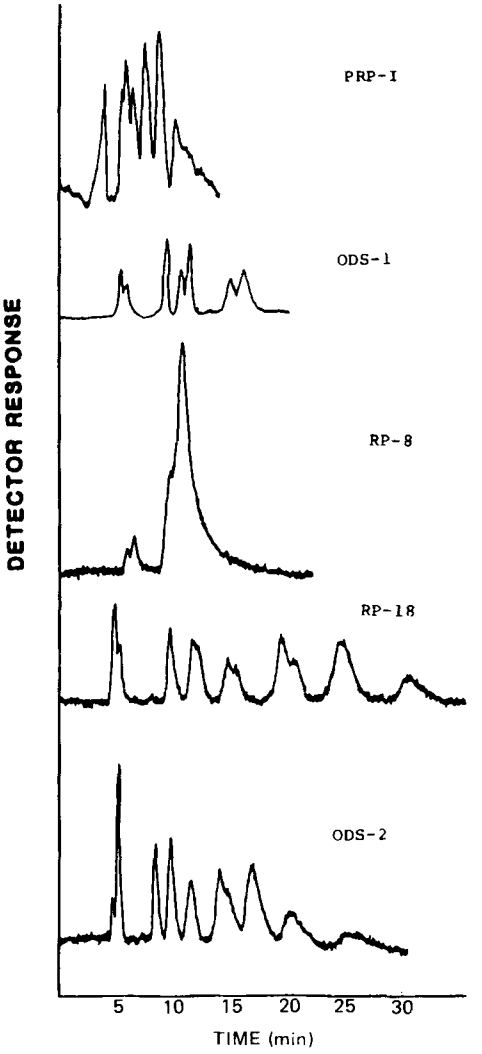

FIG. 5. Separation of the Maltodextrins on a Variety of RP Columns. (Column: 25 x 4.6 (ID); Elution Solvent: Water; Flow Rate: 1 mL/min; Detector: RI.)

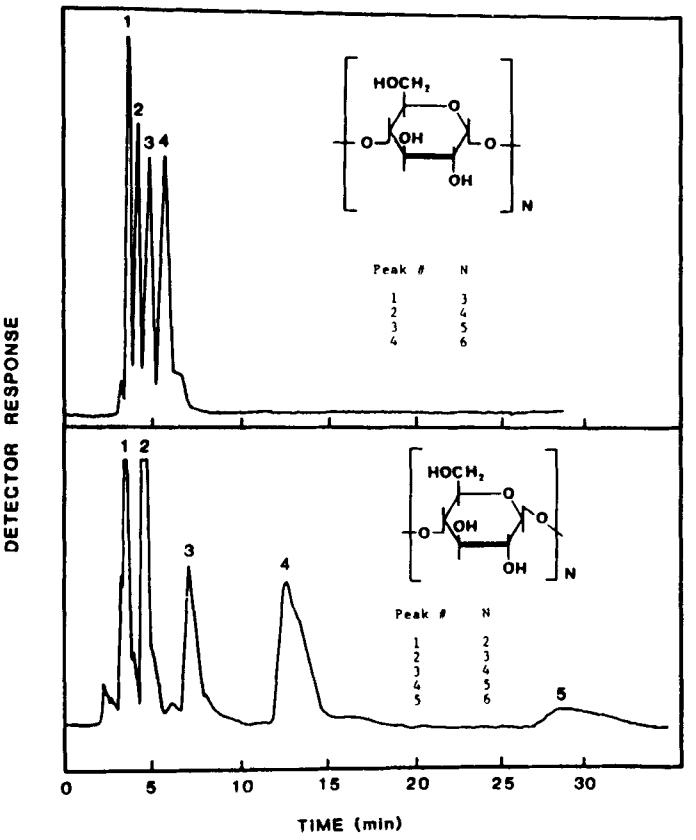

FIG. 6. Chromatographic Separation of Maltodextrins and Cellodextrins. (Column: ODS-2, 25 x 4.6 mm (ID); Elution Solvent: Water; Flow Rate: 1 mL/min; Detector: RI.)

remaining study because of its long-term stability for carbohydrates.

The retention time on the ODS-2 RP column depends on the type of sugar unit, linkage, the anomeric configuration and the molecular weight. The effect of some of these factors is illustrated in Figures 6 and 7 where a series of maltodextrins, cellodextrins and isomaltodextrins are

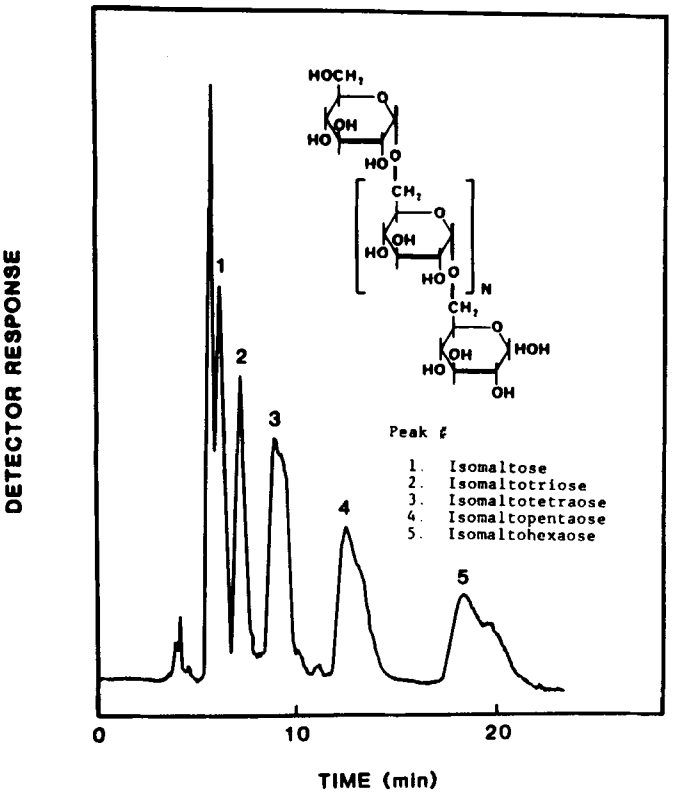

FIG. 7. Chromatographic Separation of the Isomaltodextrin Series. (Column: ODS-2, 25 x 4.6 mm (ID); Elution Solvent: Water; Flow Rate: 1 mL/min; Detector: RI.)

separated on the RP columns using the same chromatographic conditions.

The next series of figures shows the potential of using reverse-phase systems for characterizing oligosaccharide systems. Cyclodextrins are a series of cyclic dextrins produced by the action of Bacillus macerans on starch,[22] of which there are three major types: α, β, and γ, containing 6, 7, and 8 glucose units, respectively. The chromatographic

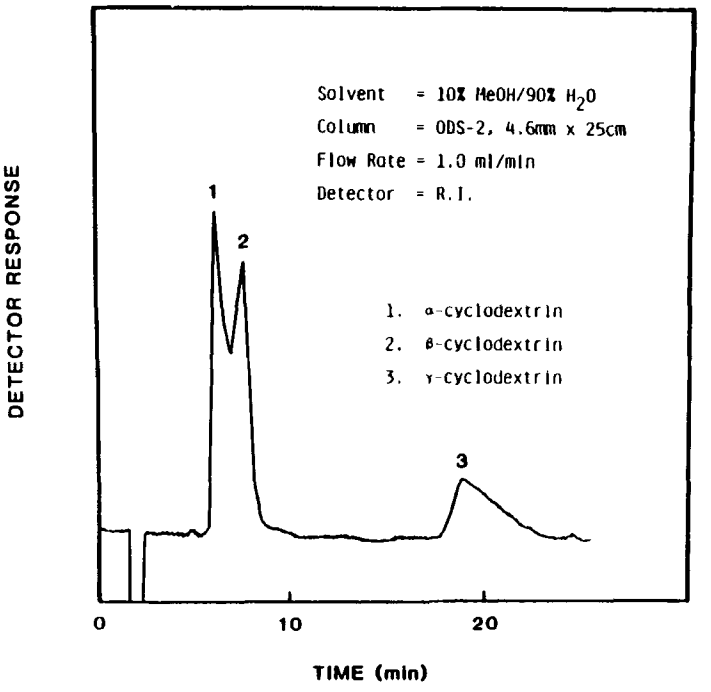

FIG. 8. Separation of α-, β- and γ-Cyclodextrins on a Reverse-phase Column.

elution patterns of the three cyclodextrins are shown in Figure 8. These compounds interact very strongly with the reverse-phase columns. When water is used as the eluent, the cyclodextrins are completely held on the column. Only when the methanol concentration reaches approximately 10% will the cyclodextrins move on the column. The strong attraction between the cyclodextrins and the column may relate to the ability of the cyclodextrins to form inclusion compounds with organic compounds. On very mild acid hydrolysis, the cyclic structure is opened up and a series of maltodextrins are formed (FIG. 9). The number of individual oligomer fragments can be related to the number

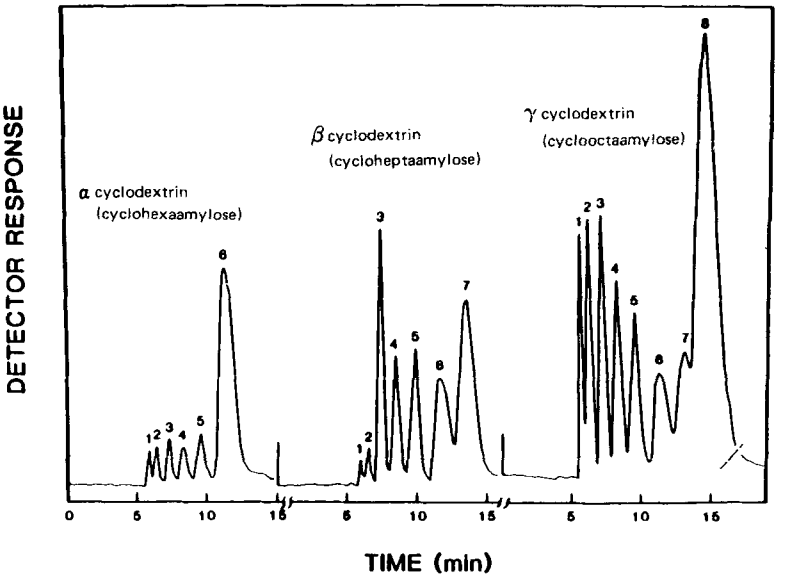

FIG. 9. Separation of Partially Hydrolyzed α-, β-, and γ-Cyclodextrins on a RP Column. (Column: ODS-2, 25 x 4.6 mm (ID); Elution Solvent: Water; Flow Rate: 1 mL/min; Detector: RI.)

of units in the initial starting product; for example, α-cyclodextrin contains six glucose units tied up in a cyclic structure on hydrolysis. It gives non-cyclic fragments containing one to six anhydro glucose units per molecule. Secondly, it should be noted that the cyclic structure can be completely separated from the corresponding linear oligosaccharide by simply adjusting the methanol/water ratio.

In conclusion, RP columns can be used to separate a wide range of oligosaccharides. The exact relationship between retention times and the structure is still not clear and will need to be clarified by further research.

EXPERIMENTAL

The high performance liquid chromatography used in this study was a Perkin-Elmer Model 601 liquid chromatograph equipped with a 3000 psi pumping system and a refractive index detector (Waters Associates Model 202) and a Perkin-Elmer Series 3 liquid chromatograph equipped with a refractive index detector (Waters Associates Model 202).

The methylated sugars, glycosides, maltodextrins, cyclodextrins, isomaltodextrins, and the cellodextrins were obtained commercially and used without further purification. The maltodextrins from cyclodextrins were prepared by hydrolyzing the corresponding cyclodextrin with 1% H_2SO_4 for 60 minutes.

The analytical RP columns were obtained commercially (Alltech Associates, Inc., Deerfield, IL) and were 4.6-mm ID and 25-cm in length. The type of packing material is given in Table 1. The semi-preparative column was a 9.4-mm ID and 50-cm long filled with ODS-2 RP packing (Whatman #6526-41).

All separations were done using water as the eluent, with the exception of the cyclodextrins where 10% methanol/ 90% water was used as the eluent.

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