TOSOH

SEPARATION REPORT NO. 79

SEPARATION OF SACCHARIDES USING TSKgel AMIDE-80, A PACKING MATERIAL FOR HIGH PERFORMANCE NORMAL PHASE PARTITION CHROMATOGRAPHY(2)*

**Please refer to Separation Report 055 for Part 1 of this series.*

Table of Contents

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1. Introduction

Saccharides are very important substances in industrial applications. They are used as raw materials for food, paper, pulp, fiber, brewed or fermented products, and medical products. In recent years saccharides and the sugar chain domain of complex carbohydrates were discovered to play a role in biological functions, and as a result they are receiving increased attention as biochemically important substances. Therefore, an efficient method for analyzing saccharides or sugar chains is required in such areas as engineering, agriculture, science, pharmacy, medicine, etc.

There are various methods for separating saccharides using high performance liquid chromatography (HPLC)[1,2], including boric acid complex anion exchange chromatography, reversed phase chromatography, normal phase chromatography, ion exclusion chromatography, ion chromatography, gel filtration chromatography, ligand exchange chromatography, affinity chromatography and anion exchange chromatography under strong alkaline conditions.

Among the various methods, normal phase chromatography separates saccharides based on differences in hydrophilicity. Hydrophilicity of a saccharide is determined by the number and orientation of the hydroxyl groups. Since the molecular weight of an oligosaccharide can be derived from the retention or capacity factor, saccharide separations are also referred to as size fractionation chromatography[3].

Traditionally, amino-bonded silica gel [4–6] often was used as the packing material for normal phase chromatography of saccharides. However, these packing materials exhibited poor chemical stability and low recovery of reducing sugars. In order to overcome these shortcomings, a packing material in which carbamoyl instead of aminopropyl groups were bonded to silica gel was developed and commercialized (TSKgel Amide-80). This new bonded phase was designed for the analysis of unsaturated disaccharides[7], glycosides[8], and derivatized oligosaccharides[9–15], etc.

The fundamental properties of this packing material and several applications (using acetonitrile/water as the mobile phase and detection by differential refractometer) have already been provided in Separation Report No. 055 “Separation of Saccharides Using TSKgel Amide-80, a Packing Material for High Performance Normal Phase Partition Chromatography[1]”.

This report compares TSKgel Amide-80 with other silica-type packing materials and examines the effect of organic solvents and amines in the mobile phase.

2. Comparison to Three Different Amino-Type Silica-Based Packing Materials

2-1 Chemical Stability

The capacity factors (k’) of trehalose, a non-reducing sugar, on a TSKgel Amide-80 column and three amino-type silica-based packing materials are shown in Figure 1. This study was conducted under isocratic mobile phase conditions using 75% acetonitrile/25% water as an eluent. The analysis with the TSKgel Amide-80 column was performed at 80°C to suppress anomer formation (and separation) of reducing sugars.

The capacity factor (k’) for trehalose declined drastically after 200 hours of continuous eluent flow for the three amino-type silica-based packing materials. The capacity factor decreased 34% for column A, 32.7% for column C and 21.5% for column D from the initial measured values. The capacity factor decreased only slightly, or 3.8%, for the TSKgel Amide-80 column (B), which demonstrates the superior chemical stability of the amide stationary phase.

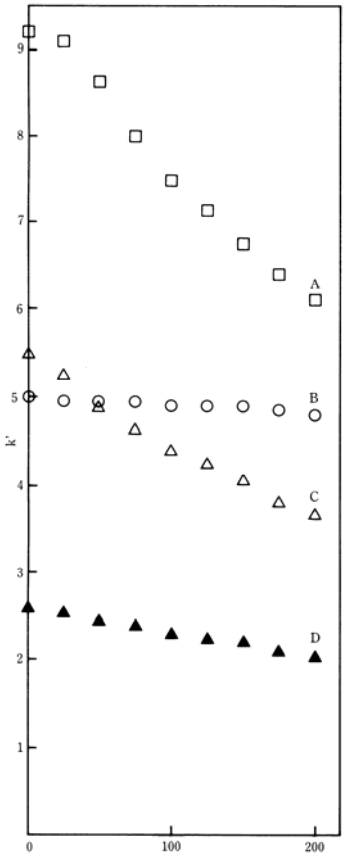


Figure 1 **Chemical stability**

Columns:	A: Amino-type silica column from manufacturer A (4.6mm ID × 25cm) B: TSKgel Amide-80 (4.6mm ID × 25cm) C: Amino-type silica column from manufacturer B (4.6mm ID × 25cm) D: Amino-type silica column from manufacturer C (4.6mm ID × 25cm)
Temperature:	A, C, D: 25°C, B: 80°C
Eluent:	Acetonitrile/water = 75/25
Flow rate:	1.0mL/min
Detection:	RI
Sample:	trehalose (1g/L), 20µL

2-2 Quantitative Recovery of Monosaccharides

Figure 2 shows the relationship between the sample load and peak area for three saccharides. For the TSKgel Amide-80 column, all but one non-reducing sugar (mannitol) and two reducing sugars (glucose and xylose) yielded linear relationships for sample loads ranging from 1.25μg to 10μg. Conversely, for the three different amino-type silica-based packing materials, linearity is not observed below 1.25μg for glucose and below 10μg for xylose, thus indicating poor sample recovery. Only the data for the column by manufacturer A is shown. The reason for the decline of recovery is thought to be caused by the formation of a glycosyl-amine bond between the aminopropyl groups of the packing materials and the reducing sugar. Since TSKgel Amide-80 employs carbamoyl groups instead of aminopropyl groups, formation of glycosyl-amine binding cannot take place. Hence, TSKgel Amide-80 provides quantitative recovery for trace amounts of reducing sugars.

As shown, TSKgel Amide-80 excels in terms of chemical stability and recovery compared to conventional amino-type silica-based packing materials, which makes this column ideal for continuous operation and trace analysis.

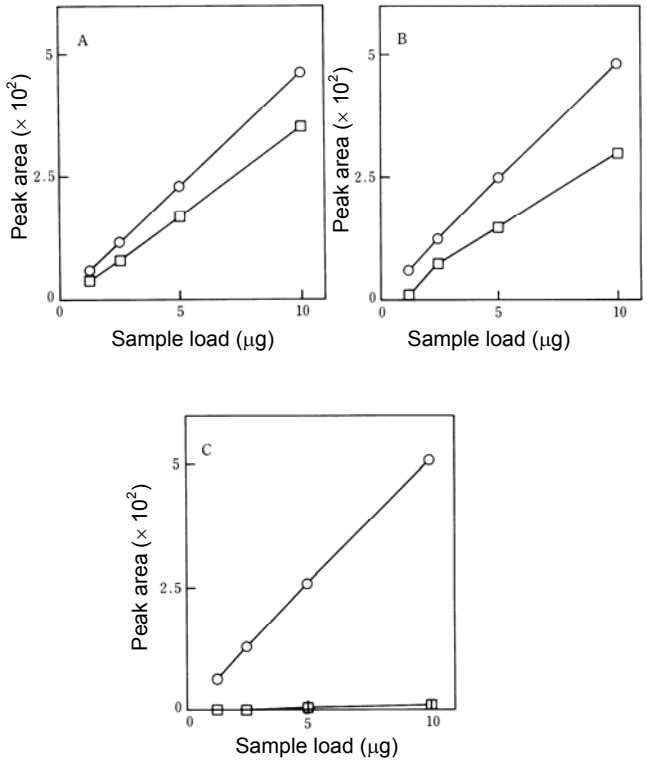


Figure 2 **Quantitative recovery of monosaccharides**

Columns: ○ TSKgel Amide-80 (4.6mm ID × 25cm)
□ Amino-type silica column from manufacturer A (4.6mm ID × 25cm)
Eluent: Acetonitrile/water = 75/25
Flow rate: 1.0mL/min
Detection: RI
Temperature: ○: 80°C □: 25°C
Sample: A: mannitol B: glucose C: xylose

3. Effect of Organic Solvents as Mobile Phase

3-1 Retention

The retention volumes of sugar alcohols on TSKgel Amide-80 in mobile phases containing various organic solvents are shown in Figure 3. Ethanol provided very short retention volumes and could not be used for the separation of monosaccharides unless its concentration was increased to 95%. Among these solvents, acetonitrile and acetone are considered the best solvents to use.

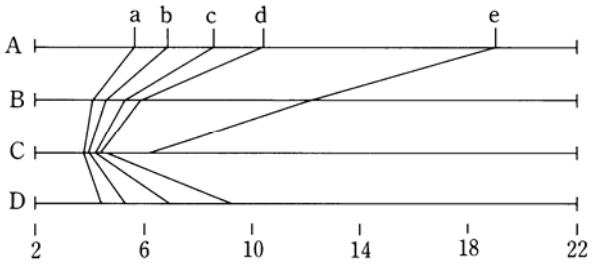


Figure 3 **Changes in eluent composition and polyol retention volume on a TSKgel Amide-80 column**

Column: TSKgel Amide-80 (4.6mm ID × 25cm)
Eluent: A: Acetonitrile/water = 75/25
B: Acetone/water = 75/25
C: 1, 4-dioxane/water = 75/25
D: Ethanol/water = 95/5
Flow rate: 0.3mL/min
Temperature: 25°C
Detection: RI
Samples: a. glycerin b. erythritol c. xylitol
d. mannitol e. inositol

3-2 Selectivity

Table 1 details the effect of mobile phase composition on the separation factor (α) of anomers using TSKgel Amide-80. In addition, the separation of β-cyclodextrin hydrolysate on a TSKgel Amide-80 column using acetonitrile or acetone as eluent is shown in Figure 4. The separation of three types of cyclodextrin and the separation of a saccharide mixture in an acetone solvent system, both using a TSKgel Amide-80 column, are shown in Figures 5 and 6, respectively. Based on this data, it can be concluded that the acetone solvent system provides enhanced anomer splitting (Table 1 and Figure 4), that the acetonitrile solvent system provides better separation between α- and β-cyclodextrins (Figure 5), and that the acetone solvent system provides better separation between maltose and lactose (Figure 6). Clearly, there is a difference in selectivity between the acetonitrile and acetone solvent systems when the analysis time is nearly identical. Therefore, it is recommended that the solvent be selected based on the goals of the analysis. Moreover, since the acetone solvent system is less toxic, it is favored when it is imperative to eliminate solvent residue in the refined product, such as when food components are purified.

Table 1 Effect of mobile phase composition on the selectivity factor (α) of anomers

Mobile phase	Separation factor (α)			
	Glucose	Xylose	Maltose	Lactose
Acetonitrile/water				
80/20	1.07	1.15	1.09	1.05
75/25	1.05	1.11	1.07	1.03
70/30	1.03	1.10	1.04	—
60/40	—	1.05	—	—
Acetone/water				
85/15	1.09	1.20	1.14	1.11
75/25	1.03	1.11	1.08	1.04

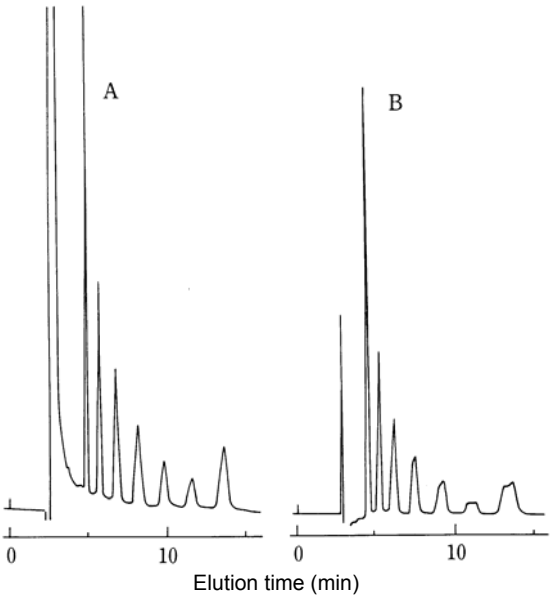


Figure 4 Separation of β-cyclodextrin hydrolysate

Column: TSKgel Amide-80 (4.6mm ID × 25cm)
Eluent: A: Acetonitrile/water = 60/40
B: Acetone/water = 65/35
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: RI
Sample: β-cyclodextrin hydrolysate

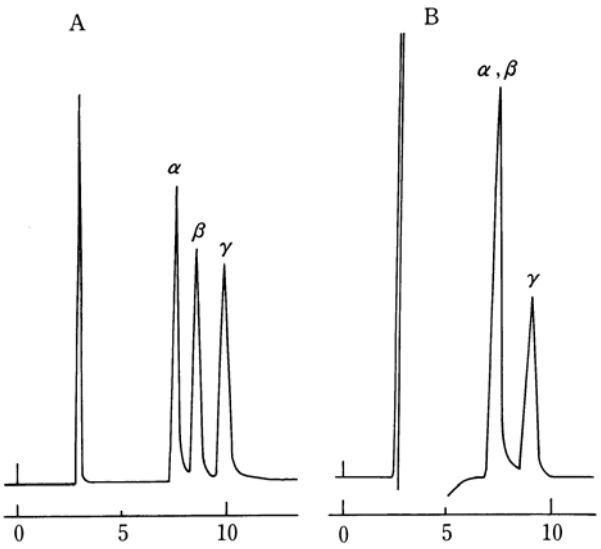


Figure 5: Separation of α, β, γ-cyclodextrins

Column: TSKgel Amide-80 (4.6mm ID × 25cm)
A: Acetonitrile/water = 60/40
B: Acetone/water = 65/35
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: RI
Samples: α, β, γ-cyclodextrins

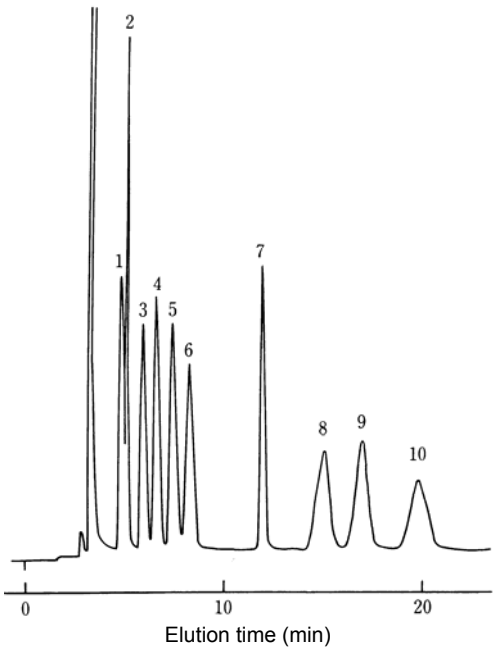


Figure 6 Separation of saccharide mixture

Column: TSKgel Amide-80 (4.6mm ID × 25cm)
Eluent: Acetone/water = 82/18
Flow rate: 1.0mL/min
Temperature: 80°C
Detection: RI
Samples: 10mmol/L monosaccharides, 5mmol/L disaccharides, 20μL

1. rhamnose 2. ribose
3. xylose 4. fructose
5. mannose 6. glucose
7. sucrose 8. maltose
9. lactose 10. isomaltose

4. Amine-Containing Additives in the Mobile Phase

4-1 Effect on Height Equivalent to a Theoretical Plate (HETP)

It was described in Separation Report No.55 that when using an acetonitrile/distilled water mobile phase at 80°C, the flow rate range of 0.5 to 1.5mL/min provides the highest efficiency for non-reducing sugars and 0.25mL/min or lower is required to attain the best efficiency for reducing sugars. It is believed that the reason why the optimum flow rate for reducing sugars is fairly low compared to that of non-reducing sugars is that the anomer conversion rate is slower than the rate of solute distribution between the mobile and stationary phase in the column.

Table 2 summarizes the HETP values of four types of saccharides on a TSKgel Amide-80 column when five different organic amines at a concentration of 20mmol/L are added to the acetonitrile/water (75/25) mobile phase. Balancing the extent of HETP improvement with the purity of commercial reagents, triethylamine and diethylaminoethanol appear to be most practical amine modifiers.

Figure 7 shows the effect on the HETP at various concentrations of triethylamine added to the mobile phase. As shown in the figure, the HETP of the saccharide clearly decreases as the concentration of triethylamine added to the mobile phase increases. This decrease in HETP is caused by the acceleration of the anomer conversion rate in reducing sugars due to the added organic amine.

An application showing the separation of ten saccharides in a mobile phase containing 100mmol/L triethylamine is shown in Figure 8. Despite conducting the analysis at 25°C, anomer separation of reducing sugars was not observed. Therefore, separation of reducing sugars in the presence of amine additives is possible at room temperature as an alternative to running at 80°C.

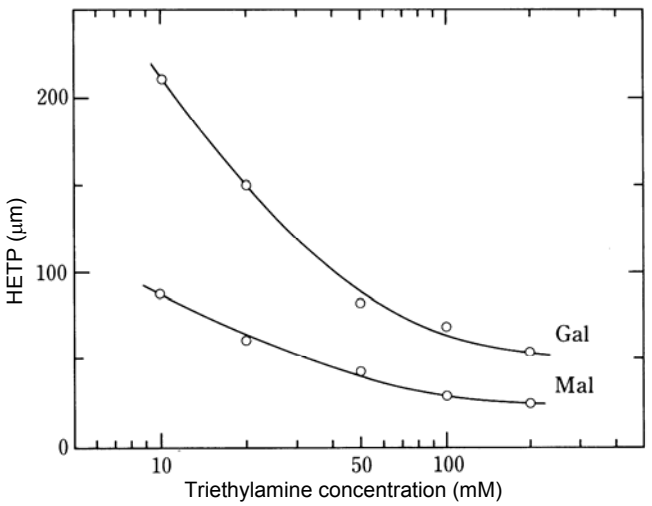


Figure 7 Effect of concentration of triethylamine added to mobile phase on HETP

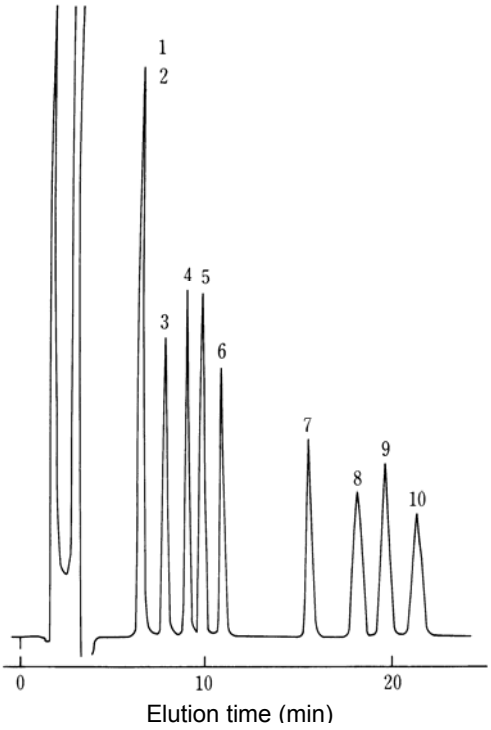


Figure 8 Separation of saccharides

Eluent: Acetonitrile/water = 75/25, containing 100mmol/L triethylamine
Temperature: 25°C
Other conditions: Same as Figure 6.

Table 2 Effect of the addition of amine on the separation of saccharides on TSKgel Amide-80

Amine (20mmol/L)	HETP at 25°C (μm)			
	Glucose	Galactose	Maltose	Lactose
Tris*	88	532	142	n.d.**
Ethanolamine	n.d.**	n.d.**	42	27
Triethylamine	n.d.**	150	59	28
Tributylamine	69	252	103	39
Diethylaminoethanol	51	283	72	41

*: Trishydroxymethylaminomethane
**: Calculation impossible due to the formation of a shoulder on the peak

5. High Sensitivity Analysis

5-1 Prelabeled High Sensitivity Analysis

High sensitivity is required for the analysis of trace components. For example, the pyridylamination method of derivatization has the following advantages:

(1) High sensitivity
(2) Various treatments are possible because pyridylamination derivatives are relatively stable against chemical reactions
(3) Separation by reversed phase chromatography is possible

An example of separating pyridylaminated saccharides following the reaction of dextran hydrolysate with 2-aminopyridine is provided in Figure 9. Saccharide hydrolysate was nearly baseline separated from pentamer to 25mer as a function of increasing molecular weight.

Fluorescence detection provided high sensitivity even when the amount of the pyridylaminated saccharide derivative was 1pmol or less. It is reasonable to expect that the application range for saccharide analysis can be extended to high sensitivity trace analysis.

A structural study of the sugar backbone of a glycoprotein is shown next. This application, which is often referred to as two-dimensional mapping or sugar chain mapping[9–15], is used to determine the sugar chain structure of an unknown oligosaccharide.

In this method, a standard sample of dextran hydrolysate is pyridylaminated (PA) before analysis by reversed phase and normal phase chromatography as shown in Figure 9. Then, a known oligosaccharide is pyridylaminated to compare its elution position with the elution position of the standard sample and an estimation of the number of glucose oligomer units is performed. By plotting the estimated glucose oligomer units (elution position) on a two-dimensional plot, points unique to the sample can be obtained. An unknown sample is pyridylaminated and both RPC and NPC chromatographic methods are compared to the plot to determine the structure of the unknown sample.

As described here, two-dimensional mapping is a valuable method which enables high-sensitivity analysis of the size and structure of sugar chains using PA-oligosaccharides. This method is considered useful in structural analysis of trace sugar chains. Furthermore, it is possible to determine the exact structure of a sugar chain by using structural methods such as NMR after an analytical technique such as HPLC.

Figure 10 shows an application of PA-oligosaccharide separation by reversed phase and normal phase chromatography. Moreover, glucose oligomer units estimated by the elution position for 6 types of PA-oligosaccharides are provided in Table 3.

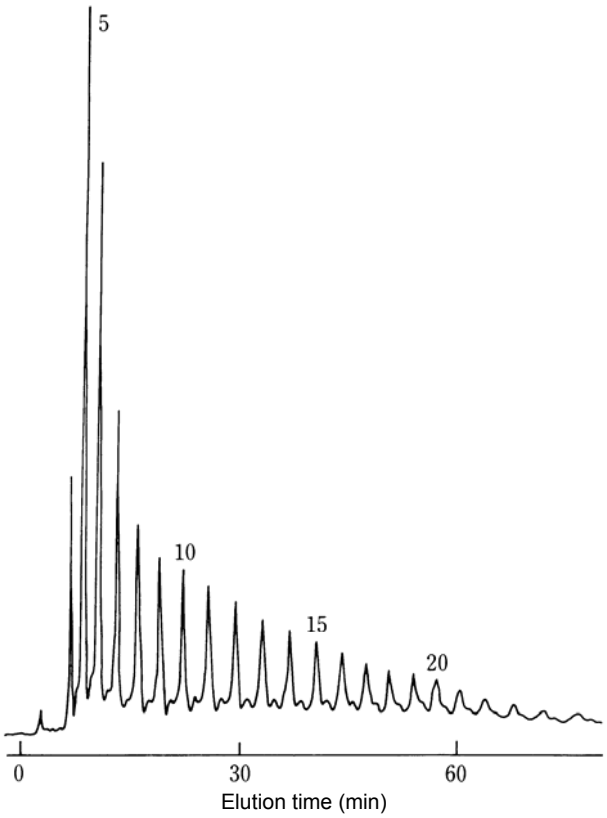
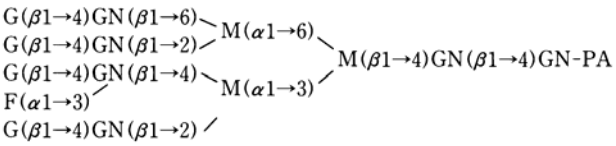
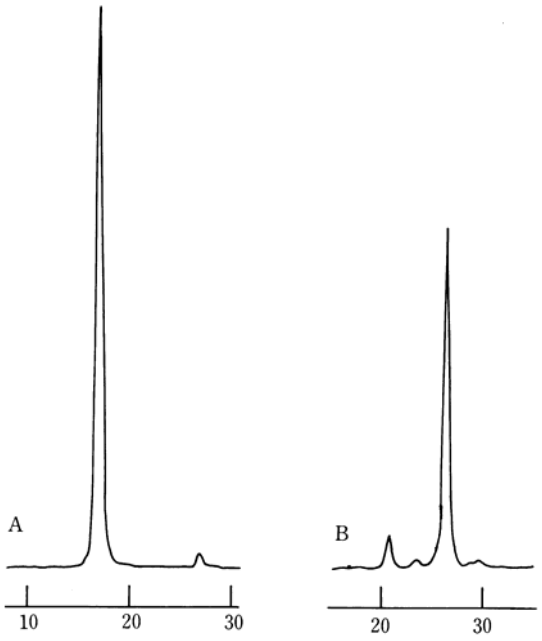


Figure 9 Separation of pyridylaminated derivatives of dextran hydrolysate

Column: TSKgel Amide-80 (4.6mm ID × 25cm)
Eluent: A: 3% acetic acid-triethylamine (pH7.3)/ acetonitrile = 35/65
B: 3% acetic acid-triethylamine (pH7.3)/ acetonitrile = 50/50
A → B (for 50 min linear gradient)
Flow rate: 1.0mL/min
Temperature: 40°C
Detection: FS (Ex. 320nm, Em. 400nm)
Sample: pyridylaminated derivatives of dextran hydrolysate 0.5g/1.1μL





PA-oligosaccharide structures

1 G(β1-4)GN(β1-2)—M(α1-6)
G(β1-4)GN(β1-2)—M(α1-3)
M(β1-4)GN(β1-4)GN-PA

2 G(β1-4)GN(β1-2)—M(α1-6)
G(β1-4)GN(β1-4)
G(β1-4)GN(β1-2)
M(α1-3)
M(β1-4)GN(β1-4)GN-PA

3 G(β1-4)GN(β1-2)—M(α1-6)
G(β1-3)GN(β1-4)
G(β1-4)GN(β1-2)
M(α1-3)
M(β1-4)GN(β1-4)GN-PA

4 G(β1-4)GN(β1-6)
G(β1-4)GN(β1-2)
M(α1-6)
G(β1-4)GN(β1-4)
G(β1-4)GN(β1-2)
M(α1-3)
M(β1-4)GN(β1-4)GN-PA

5 G(β1-4)GN(β1-2)—M(α1-6)
G(β1-4)GN(β1-4)
F(α1-3)
G(β1-4)GN(β1-2)
M(α1-3)
M(β1-4)GN(β1-4)GN-PA

6 G(β1-4)GN(β1-6)
G(β1-4)GN(β1-2)
M(α1-6)
G(β1-4)GN(β1-4)
F(α1-3)
G(β1-4)GN(β1-2)
M(α1-3)
M(β1-4)GN(β1-4)GN-PA

Figure10 Analysis of pyridylaminated derivative of oligosaccharide on TSKgel ODS-80T$_M$ and TSKgel Amide-80

Column: A: TSKgel ODS-80T$_M$ (4.6mm ID × 15cm)
B: TSKgel Amide-80 (4.6mm ID × 25cm)
Eluent: A: a: 10mmol/L phosphate buffer (pH3.8)
b: a + 0.5% n-butanol
a/b (80/20) → (40/60) linear gradient (80 minutes)
B: Conditions identical to Figure 9
Flow rate: 1.0mL/min
Temperature: A: 55°C, B: 40°C
Detection: FS (Ex. 320nm, Em. 400nm)
Sample: PA-oligosaccharide

Table 3 Retention times of pyridylaminated oligosaccharides on TSKgel Amide-80 and TSKgel ODS-80T$_M$ Columns

Sugar chain	TSKgel Amide-80			TSKgel ODS-80T$_M$	
	Retention time (mL) [1]	Converted glucose unit Measured value [2)]	Literature value [3]	Retention time (mL) [1]	Converted glucose unit Measured value [2]
1	13.7	6.9	7.0	15.3	9.6
2	17.9	8.3	8.3	25.8	12.3
3	17.5	8.2	8.2	26.7	12.6
4	23.5	9.9	9.9	17.7	10.3
5	20.6	9.1	9.0	24.4	11.9
6	26.2	10.6	10.5	16.9	10.1

1) Elution volume
2) Glucose unit values, calculated
3) Glucose unit values reported in the literature[9]

6. Conclusion

TSKgel Amide-80 is a packing material for normal phase partition chromatography which overcomes the weaknesses of conventional amino-type silica-based columns. TSKgel Amide-80 provides excellent separations for mono-, di- and oligosaccharides.

In addition to the TSKgel Amide-80 columns, there are many other TSKgel HPLC products utilized in saccharide analysis, such as the TSKgel SugarAX series (anion exchange method using boric acid as a counter ion), TSKgel SCX (H^+ type) (ion exclusion method), TSKgel PW and PW_{XL} series (gel filtration method), and TSKgel NH_2-60 (amino-type normal phase partition method). See the Tosoh Bioscience Chromatography Catalog for more details on these columns.

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