No. 079

TOSOH

SEPARATION REPORT

Separation of Saccharides Using TSKgel Amide-80, a Packing Material for High-performance Normal Phase Partition Chromatography (2)

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TOSOH CORPORATION

1. Introduction

Saccharides are very important substances in the industrial fields as raw materials for food, paper, pulp, fiber, brewed or fermented products, and medical products. Furthermore, in recent years, saccharides and the sugar chain domain of complex carbohydrates have been found to be involved in biological functions, and saccharides are under attention as biochemically important substances. Therefore, an efficient method of analyzing saccharides or sugar chains is demanded in wide areas of engineering, agriculture, science, pharmacy, medicine, etc.

There are various methods of separating saccharides by high-performance liquid chromatography[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, anion exchange chromatography under strong alkaline, and affinity chromatography.

Among various methods, normal phase chromatography is a method in which saccharide separation is conducted by the difference in hydrophilicity of the saccharides. Hydrophilicity of a saccharide is determined by the number, directions, and positions of hydroxyl groups, and the binding positions among monosaccharides are also associated for disaccharides and oligo saccharides. Since molecular weight of the oligomer can be assumed from the retention capacity, it is also called the size fractionation chromatography[3].

Conventionally, silica gel packing materials with amino groups bonded[4 – 6] were used often as the packing material for normal phase chromatography on saccharides. However, these packing materials had weaknesses of having low chemical stability, low recovery of reducing sugars, etc. In order to overcome such weaknesses, a packing material in which carbamoyl groups are bonded to silica gel instead of aminopropyl groups (TSKgel Amide-80) was developed and commercialized to be applied widely in separation of unsaturated disaccharides[7], separation of glycosides[8], analysis of derivatized oligosaccharides[9 – 15], etc.

The fundamental properties of this packing material and applications (using acetonitrile/water as the mobile phase and detection by differential refractometer) have already been provided in Separation Report No.55 “Separation of Saccharides Using TSKgel Amide-80, a Packing Material for High-performance Normal Phase Partition Chromatography (1).” This report introduces a comparison between TSKgel Amide-80 and other silica-type packing materials, effect of organic solvents in the mobile phase, effect of addition of amine, and some applications of highly-sensitive analysis by derivatization of sacchrides.

2. Comparison of Three Types of Amino-Type Silica Packing Materials

2-1 Chemical Stability

Figure-1 shows the changes in capacity factor (k’) of trehalose for TSKgel Ammide-80 and other amino-type silica packing materials under the condition of continuous flow of acetonitrile/water = 75/25 as eluent. However, analysis with TSKgel Amide-80 has been conducted at 80°C to suppress anomer separation from reducing sugars. While capacity factor (k’) deteriorated drastically after 200 hours of continuous liquid flow for the three amino-type silica packing materials to (A) 66.0%, (C) 67.3% and (D) 78.5% of the initial values at the start of liquid running, it is evident that TSKgel Amide-80 shows chemical stability by the value of (B) 96.2%.

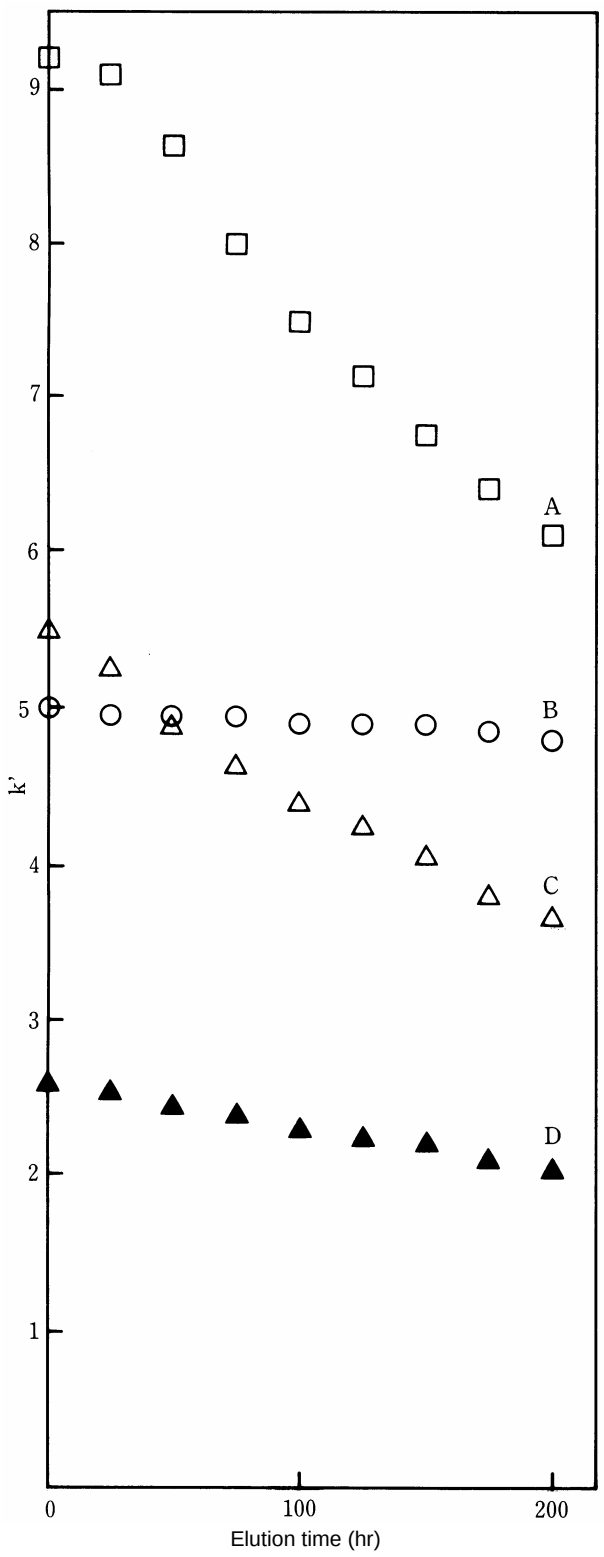


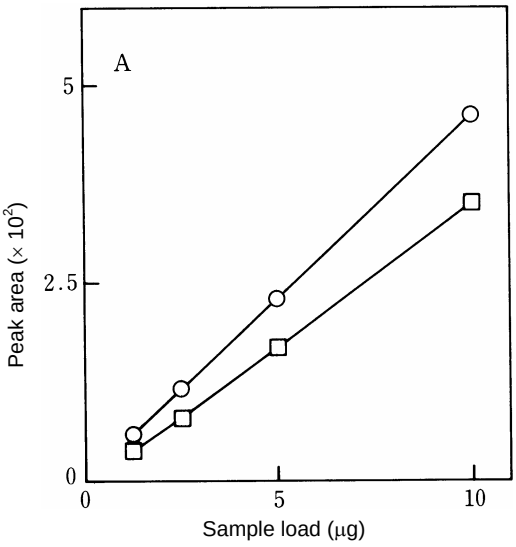
Figure-1 Chemical stability

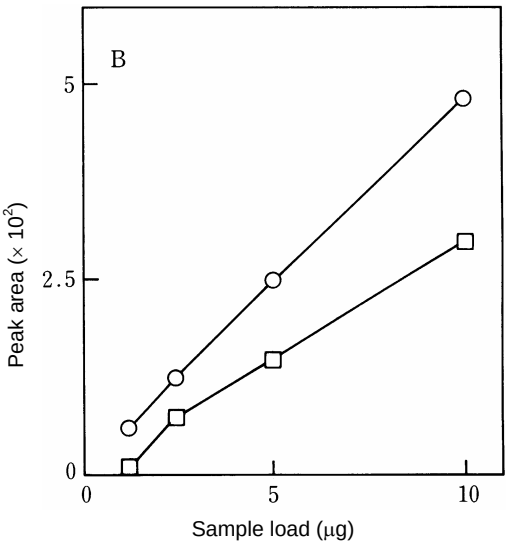
Column: A: Amino-type silica column by manufacturer A (4.6mmI.D. × 25cm)
B: TSKgel Amide-80 (4.6mmI.D. × 25cm)
C: Amino-type silica column by manufacturer B (4.6mmI.D. × 25cm)
D: Amino-type silica column by manufacturer C (4.6mmI.D. × 25cm)
Column temperature: A, C, D: 25°C, B: 80°C
Eluent: Acetonitrile/water = 75/25
Flow rate: 1.0 mL/min
Detection: RI
Sample: Trehalose (1g/L), 20μL

2-2 Quantitative Recovery of Monosaccharides

Figure-2 shows the relationship between injection volumes and the peak area on chromatogram when three different saccharides are separated. In TSKgel Amide-80 (○), all of one non-reducing sugar (mannitol) and two reducing sugars (glucose and xylose) yielded linear relationships from 1.25 to 10μg. On the other hand, in the three types of amino-type silica packing materials, linearity is not seen in the range below 1.25μg for glucose and below 10μg for xylose, indicating that recovery was deteriorated (only the data for the column by manufacturer A is shown). This is surmised to have been caused by the formation of 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, no formation of glycosyl-amine binding occurs. Hence, it is capable of quantitative recovery even for trace amounts of reducing sugars.

As shown above, TSKgel Amide-80 excels in chemical stability and recovery compared to conventional amino-type silica packing materials, and it is evident that it is useful especially in continuous analysis and trace amount analysis.





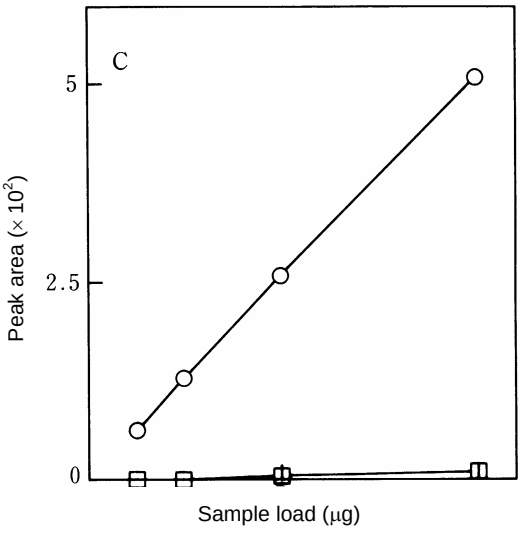


Figure-2 Quantitative recovery of monosaccharides

Column: ○ TSKgel Amide-80 (4.6mmI.D. × 25cm)
□ Amino-type silica column by manufacturer A (4.6mmI.D. × 25cm)
Eluent: Acetonitrile/water = 75/25
Flow rate: 1.0 mL/min
Detection: RI
Column 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 when four types of organic solvents are used as the mobile phase on TSKgel Amide-80 are shown in Figure-3. Under identical solvent concentration (75%), the smaller the proton acceptor dissolution parameter was used (A < B < C < D), the longer the saccharide retention time was. Ethanol had very small retention, and could not be used for separation of monosaccharides unless its concentration was increased to 95%. Among these solvents, acetonitrile and acetone are considered as being easy to handle.

3-2 Selectivity

Table-1 shows the effect of mobile phase composition on the separation factor (α) of anomers in TSKgel Amide-80. In addition, separation of β-cyclodextrin hydrolysate using acetonitrile or acetone as eluent is shown in Figure-4. Separation of cyclodextrins (three types) is shown in Figure-5. In Figure-6, separation of saccharide mixture in acetone system is shown. Based on Table-1, Figures-4 and -5, it is indicated that (1) acetone system has larger anomer splitting (Table-1 and Figure-4), that (2) acetonitrile system has better separation between α- and β-cyclodextrins (Figure-5), and that (3) acetone system has better separation between maltose and lactose (Figure-6), and thus there is a difference in selectivity between acetonitrile and acetone systems when the sample elution time is set nearly identical. Therefore, it is recommended that the solvent be selected depending on the target of analysis. Moreover, the acetone system with less toxicity is considered favorable when it is used in refinement of food, etc. upon consideration of the issue of remnant solvent in the refined product.

Table-1 Effect of mobile phase composition on separation 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

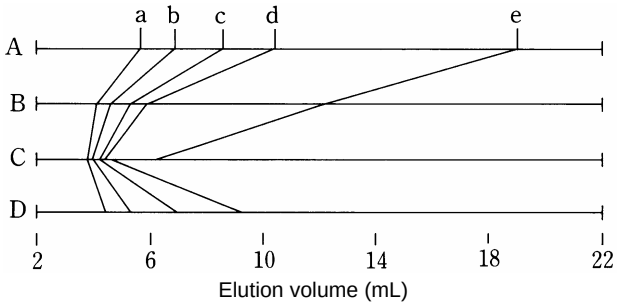


Figure-3 Changes in eluent composition and polyol retention volume on TSKgel Amide-80

Column: TSKgel Amide-80 (4.6mmI.D. × 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

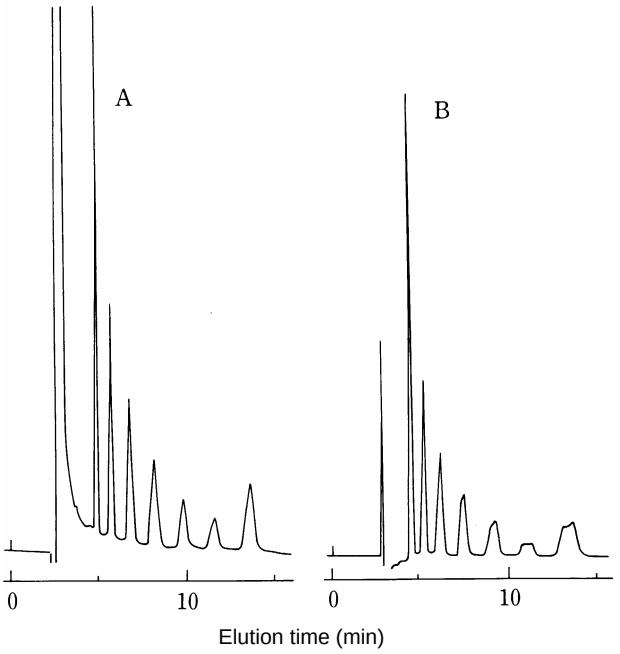


Figure-4 Separation of β-cyclodextrin hydrolysate

Column: TSKgel Amide-80 (4.6mmI.D. × 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

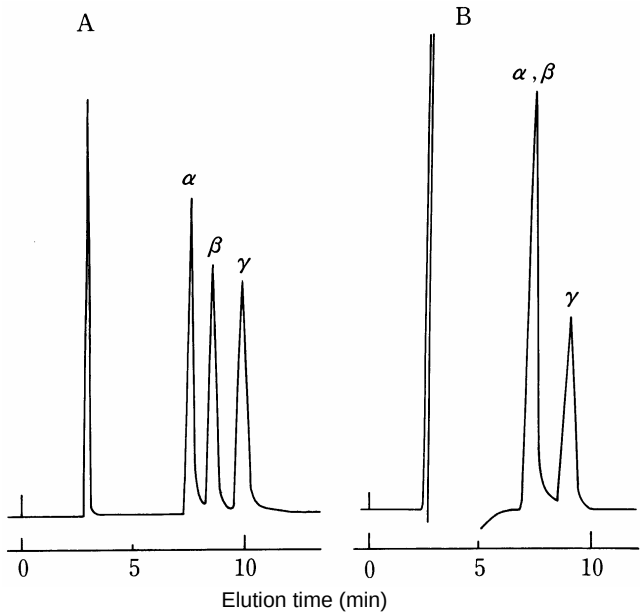


Figure-5 Separation of α, β, γ-cyclodextrins

Column:	TSKgel Amide-80 (4.6mmI.D. × 25cm)
Eluent:	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

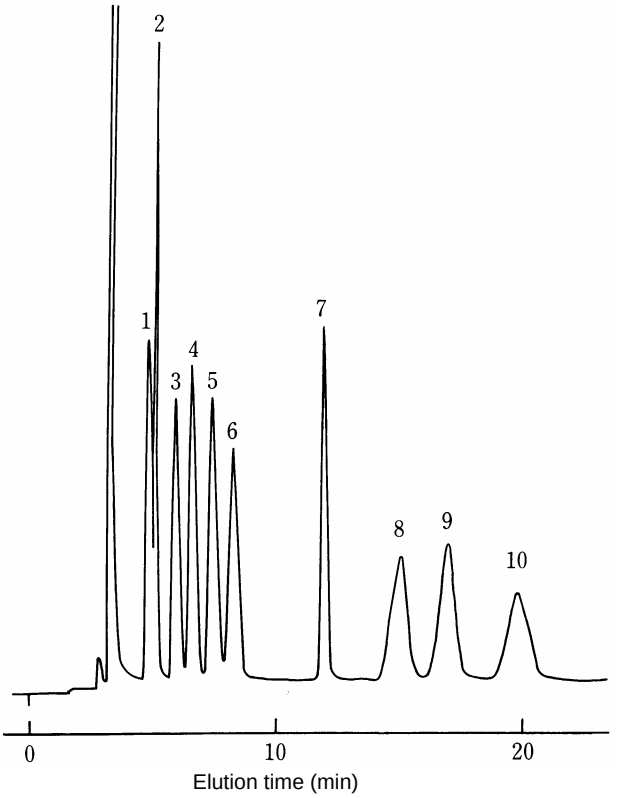


Figure-6 Separation of saccharide mixture

Column:	TSKgel Amide-80 (4.6mmI.D. × 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 Addition to the Mobile Phase

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

It has been described in Separation Report No.55 that the flow rate range of 0.5 to 1.5mL/min is needed for non-reducing sugars and 0.25mL/min or smaller for reducing sugars to obtain the minimum HETP in acetonitrile/distilled water mobile phase at 80°C. It is presumed that the reason why the flow rate that yields the minimum HETP for reducing sugars is fairly low compared to those of non-reducing sugars is that the anomer conversion rate is slower than the rate of partition to the column.

Table-2 summarizes the HETP values of four types of saccharides in TSKgel Amide-80 when five different types of 20mM organic amines are added to the acetonitrile/distilled water = 75/25 mobile phase. Considering the effect of HETP improvement and purity of commercial reagents, triethylamine and diethylaminoethanol seem to be practical.

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

An application of separating ten types of saccharides in a mobile phase containing 100mM triethylamine is shown in Figure-8. Regardless of the analysis being conducted at 25°C, anomer separation of reducing sugars was not observed. Therefore, separation of reducing sugars is possible under room temperature instead of increasing the measuring temperature to 80°C by using a mobile phase in which amines are added.

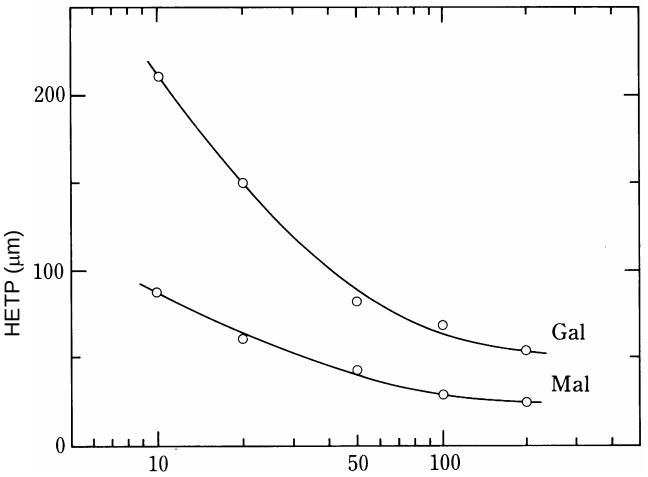


Figure-7 Effect of concentration of triethylamine added to mobile phase on HETP

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

Amine (20mM)	Saccharide 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 shouldered peaks

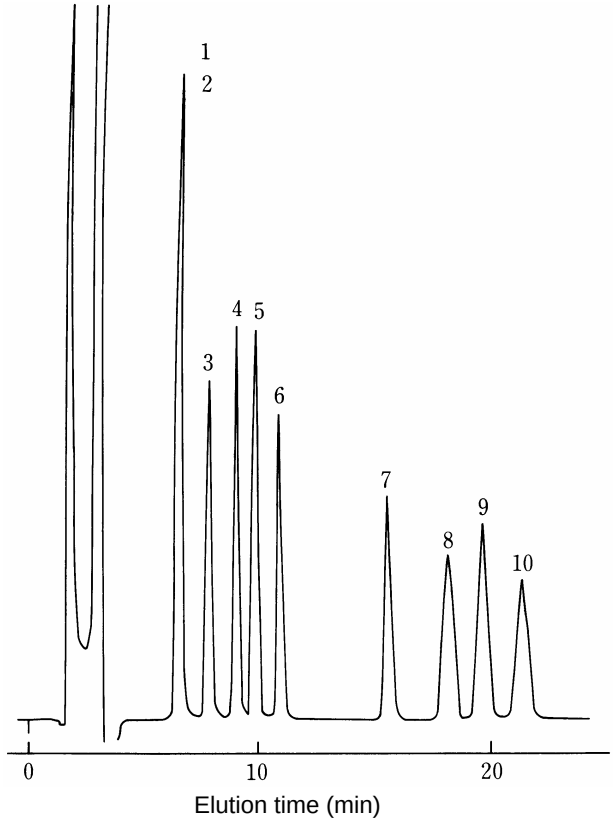


Figure-8 Separation of saccharides

Eluent:	Acetonitrile/water (75/25) containing 100mM triethylamine
Column temperature:	25°C
Other conditions:	Same as Figure-6.

5. Highly-Sensitive Analysis

5-1 Prelabeled Highly-Sensitive Analysis

An advantage of highly-sensitive analysis is that it can analyze trace components. Especially, the pyridylamination method 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 becomes possible.

An example of actually pyridylaminating and separating saccharides is provided. Figure-9 shows an application in which analysis of dextran hydrolysate was conducted after pyridylaminating it with 2-aminopyridine. Saccharide hydrolysate was separated nearly completely from pentamer to 25mer depending on the molecular weight, and fluorescence detection was possible with high sensitivity even when the amount of the pyridylaminated derivative of saccharide is 1pmol or less. It is hence expected that the application range of saccharide analysis may be extended by high-sensitivity analysis.

Next, an application to the sugar chain structure study of glycoprotein is provided. This application which is called the two-dimensional mapping (sugar chain mapping)[9 – 15] is useful for estimation of the sugar chain structure of an unknown oligosaccharide.

In this method, pyridylaminated (PA) dextran hydrolysate (standard sample) is separated by reversed phase chromatography 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 estimate as the glucose oligomer unit. By plotting the estimated glucose oligomer unit (elution position) on a two-dimensional coordinate axis, coordinate points unique to the sample can be obtained. Then an unknown sample is pyridylaminated to perform both chromatographic methods to obtain the coordinate points in a similar fashion so that the structure of the unknown sample can be estimated by comparing with the coordinate points of the known oligosaccharide.

As described here, two-dimensional mapping is a method which enables highly-sensitive analysis of the size and structure of sugar chains using PA-oligosaccharides, and it is considered as useful in structure analysis of trace sugar chains in organisms. Furthermore, it is possible to determine the exact structure of sugar chains by subsequently using analytical methods such as NMR after separation by HPLC.

Figure-10 shows an application in 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.

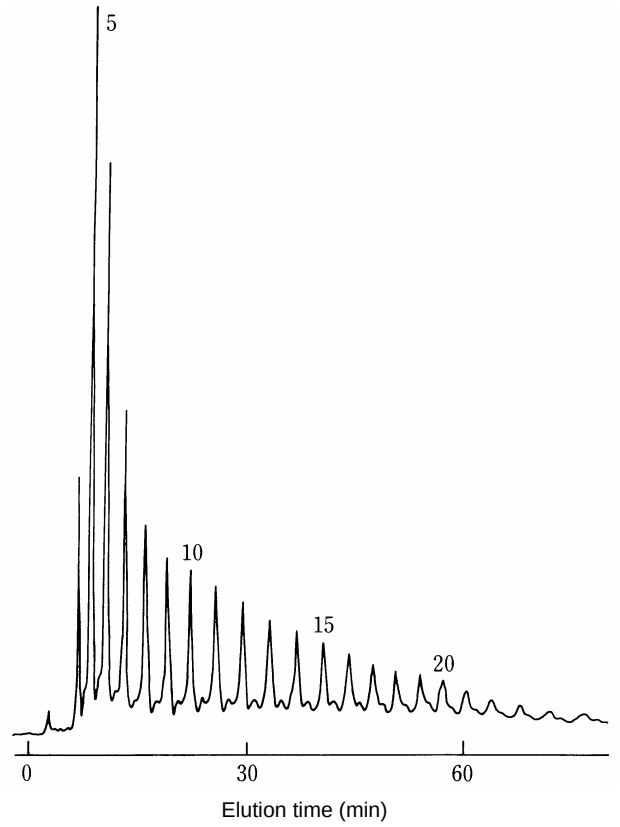


Figure-9 Separation of pyridylaminated derivative of dextran hydrolysate

Column:	TSKgel Amide-80 (4.6mmI.D. × 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 derivative of dextran hydrolysate 0.5g/1.1μL

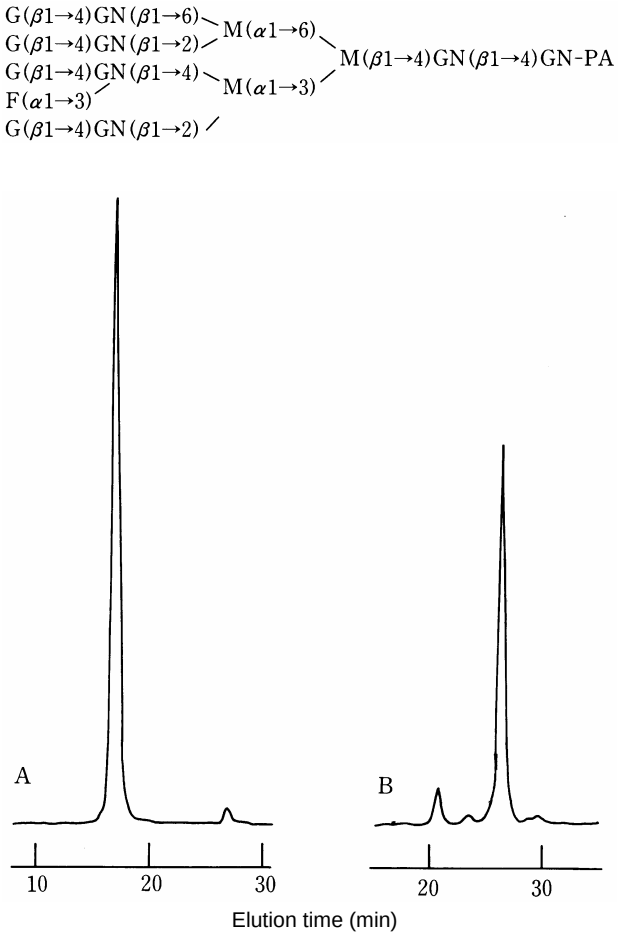


Figure-10 Analysis of pyridylaminated derivative of oligosaccharide on TSKgel ODS-80TM and TSKgel Amide-80

Column:	A: TSKgel ODS-80T_M (4.6mmI.D. × 15cm)
	B: TSKgel Amide-80 (4.6mmI.D. × 25cm)
Eluent:	A: a: 10mM 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 time of pyridylaminated oligosaccharides in TSKgel Amide-80 and TSKgel ODS-80TM

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 calculated
3): Glucose unit referred from the literature [9]

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

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

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

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

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

6. Conclusion

TSKgel Amide-80 is a packing materials for normal phase partition chromatography which has overcome the weaknesses of conventional amino-type silica columns. TSKgel Amide-80 delivers excellent separation not only of monosaccharides and disaccharides, but also of oligosaccharides.
Furthermore, TSKgel includes other packing materials and packing columns for saccharide analysis such as the TSKgel SugarAX series (boric acid complex anion exchange method), TSKgel SCX (H^+ type) (ion exclusion method), TSKgel PW, PW_{XL} series (gel filtration method), and TSKgel NH_2-60 (amino-type normal phase partition method). Please make use of these products as well.

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