No. 055

TOSOH

SEPARATION REPORT

Separation of Saccharides Using TSKgel Amide-80, a Packing Material for High-Performance Normal Phase Partition Chromatography (1)

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TOSOH CORPORATION

1. Introduction

Saccharides are extremely fundamental substances. Saccharides express various bioactivities by not only existing singly but also forming complexes with proteins or lipids. Analysis of these saccharides and sugar chains in complex carbohydrates is considered with great importance in the fields of medicine and medical practice as well as food.

Analysis of saccharides has been conducted in various methods in the past. Various separation modes are used in high-performance liquid chromatography. Saccharides can be largely classified into monosaccharides, disaccharides, oligosaccharides, and polysaccharides by the degrees of polymerization and condensation.

Methods of separating monosaccharides and disaccharides include the method by anion exchange chromatography in which the nature to form anionic saccharide-boric acid complex under the boric acid existence[1)], the method by normal phase chromatography[2)-6)], the method by ligand exchange chromatography[7)], and the anion exchange chromatography under strong alkali[8)].

As methods to separate oligosaccharides, gel filtration chromatography[9)], normal phase partition chromatography[2), 5), 6), 10)], and reversed phase partition chromatography[11), 12)] are used in general. In addition, gel filtration chromatography is used in most cases as the method to separate polysaccharides.

To separate samples, it is critical that the features of these separation modes are understood and that the separation mode optimal for the target sample is selected.

Among these, separation method by normal phase chromatography selectively retains polyhydric alcohols such as saccharides while retaining little or eluting most of the substances with low polarity and monohydric alcohols to the area around V_0. Furthermore, since resolution among saccharides is relatively favorable and the analysis time is comparatively short in this case, the system combined with differential refractometer has been used since early times. However, the packing materials which have been used for this method were ion-exchange resins[2), 10)] and silica gel to which aminoalkyl groups are chemically bonded[3), 4), 6)] in most cases. These packing materials were not always satisfactory in terms of physical and chemical stability. TSKgel Amide-80 is a packing material in which the organic stationary phase containing nonionic carbamoyl groups is chemically bonded to silica gel. Having a nonionic stationary phase compared to the packing materials with chemical bonding to aminoalkyl groups, it has outstanding chemical stability. The NH bonding section in the stationary phase has a nature to form a hydrogen bond to the oxygen atom of a hydroxyl groups or a carbonyl group, and strongly retains water to form a polar partition phase in a mixed solution of water-soluble organic solvent and water, enabling separation in normal phase partition mode. It retains the saccharides and other polyols favorably and can be used under more practical elution conditions compared to nonionic diol-bonded silica gel.

This report introduces the fundamental properties of TSKgel Amide-80 and some applications in which it is used in separation of neutral monosaccharides, sugar alcohols, and oligosaccharides.

2. Elution Behaviors of Saccharides

2-1 Separation Mechanism and the Effect of Temperature

TSKgel Amide-80 retains polar compounds such as saccharides and polyols in organic solvent systems including water such as acetonitrile/water. A chromatogram of sugar alcohols is shown in Figure-1 as a separation example. Basically, the more hydroxyl groups the substance has, the better it is retained. In addition, comparison of retention between mannitol and inositol with the same number of hydroxyl groups, which is 6, shows that inositol which has a cyclic form and lower solubility to the mobile phase (acetonitrile/water system) is retained better. This retention strength is greatly affected by the polarity of mobile phase, showing tendency for retention to become stronger as the water content in the mobile phase becomes lower as shown in Fig.-2. Such tendency is not limited to sugar alcohols, but it is a phenomenon that is common to monosaccharides, disaccharides and oligosaccharides.

As to monosaccharides, tendency to elute increases in the order of pentose and hexose and selectivity varies by slight difference even among similar hexoses by the difference in molecular structure, which enables separation of each.

In oligosaccharide analysis, water content needs to be increased by 20 to 30% since retention is too large in the mobile phase for separation of monosaccharides. Moreover, considering the case of reducing sugars whose molecular structures are known to include α or β type structures, pyranose or furanose structures, etc. instead of simple singular structure, they generally repeat conversion in solutions until the equilibrium is reached. This is called mutarotation.

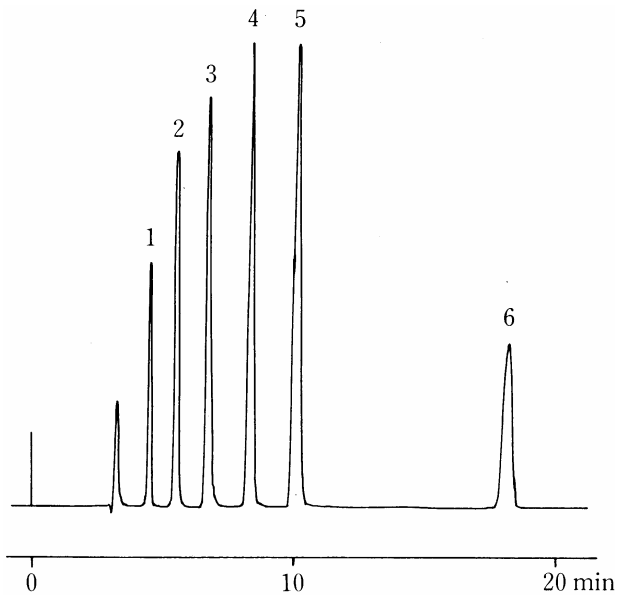

Figure-1 Separation of polyols

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 75/25
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: RI

1. Ethylene glycol
2. Glycerol
3. Erythritol
4. Xylitol
5. Mannitol
6. Inositol

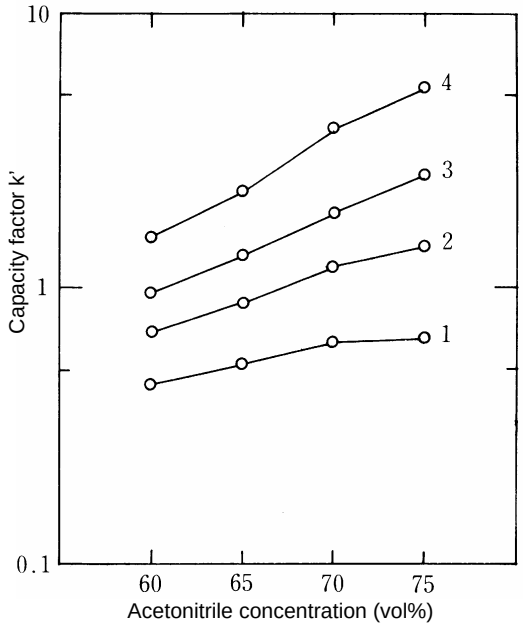

Figure-2 Effect of acetonitrile concentration in eluent on retention of polyols

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: RI

1. Ethylene glycol
2. Xylitol
3. Mannitol
4. Inositol

For example, when one saccharide molecule is focused, its molecular structure is supposed to convert continuously at a certain probability. Its conversion rate is generally slow in neutral environment at about room temperature, and two or more peaks may be separated and detected even for one type of saccharide under such conditions. Figure-3 shows the effect of temperature on the chromatogram when D-glucose is separated by TSKgel Amide-80. It is evident that the chromatogram is divided into two or more peaks by the difference in molecular structure under 60°C or lower.

In the case of D-glucose, it is detected as a single peak under the conditions shown in Figure-3 if the column temperature is increased to 70°C. Though the temperature that generates a single peak varies by the type of reducing sugar, most sugars can be detected as single peaks at temperatures of 80°C or higher.
Figure-4 shows the effect of temperature on separation. When elution conditions other than the temperature are stable, elution time becomes faster in general as the temperature is increased.

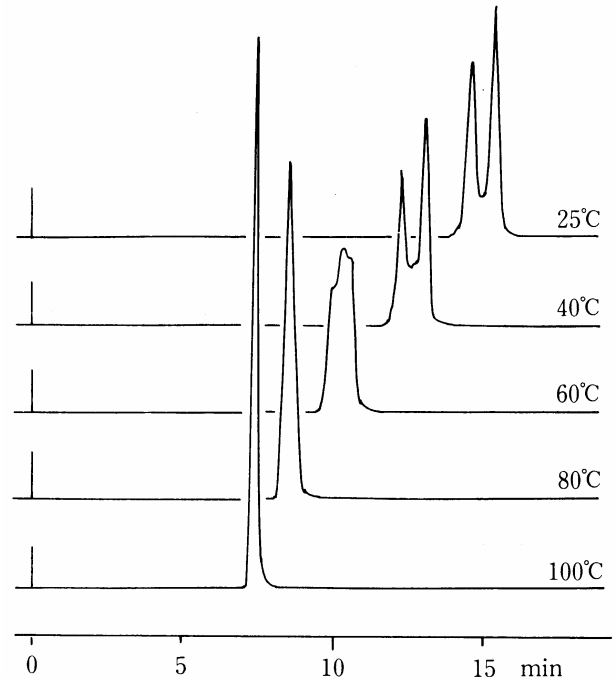

Figure-3 Effect of column temperature on D-glucose chromatogram

Column: TSKgel Amide-80
4.6mm ID × 25cm
Eluent: Acetonitrile/water = 80/20
Flow rate: 1.0mL/min
Detection: RI

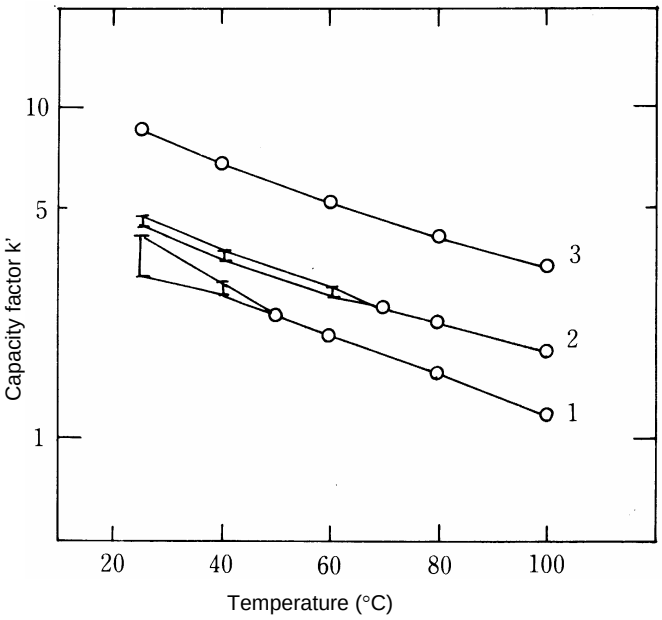

Figure-4 Effect of column temperature on retention

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 80/20
Flow rate: 1.0mL/min
Detection: RI

1. Fructose
2. Glucose
3. Sucrose

2-2 Height Equivalent to a Theoretical Plate (HETP)

Flow-rate dependency of height equivalent to a theoretical plate (HETP) is shown in Figure-5. When mannitol is used as sample under room temperature (25°C), HETP become minimum between 0.15mL/min and 0.3mL/min, yielding the theoretical plates of approximately 80,000TP/m. However, it is surmised that 0.5 to 1.0mL/min is appropriate as practical flow rate.
Furthermore, as shown in Figure-6, reducing sugars such as D-glucose show minimum HETP at 0.25mL/min within the flow rate range and the maximum value is surmised to be lower, while minimum HETP is shown at 0.5 to 1.5mL/min for non-reducing sugars such as mannitol and sucrose when the column temperature is 80°C.
It is presumed that this phenomenon is caused by lower structure conversion rates in sugars compared to the sample's partition and equilibration rate to the column which lead to band broadening. However, this TSKgel Amide-80 is a packing material that uses silica gel as the base material, it is not favorable in terms of column lifetime to use in a water-containing solvent system under higher temperatures.

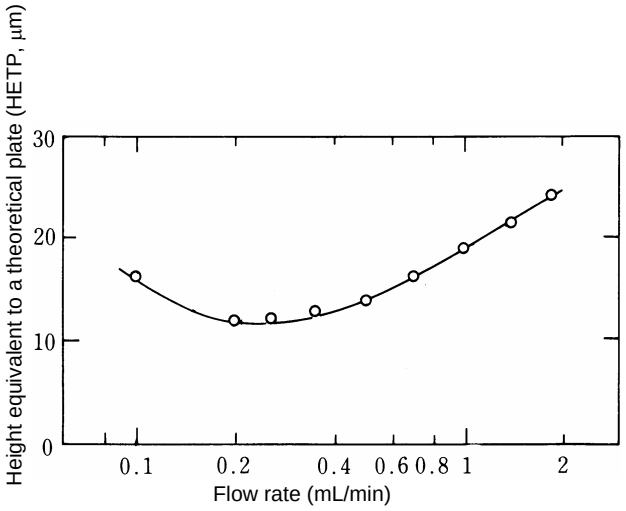

Figure-5 Effect of flow rate on HETP 1 (room temperature)

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 75/25
Temperature: 25°C
Detection: RI
Sample: Mannitol

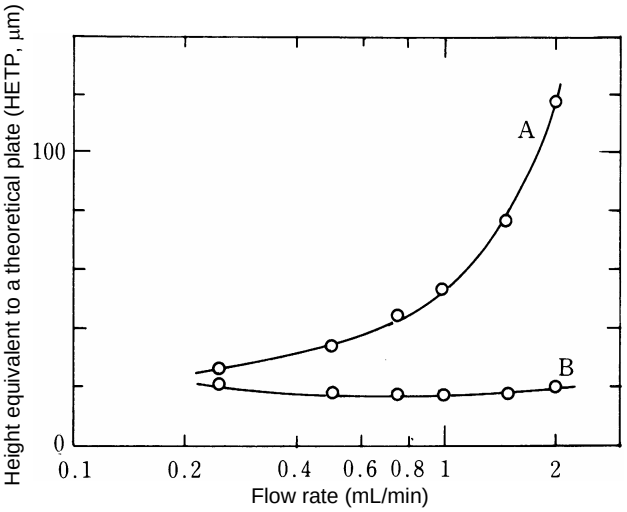

Figure-6 Effect of flow rate on HETP 2 (80°C)

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 80/20
Temperature: 80°C
Detection: RI
Sample: A: Glucose
B: Mannitol

2-3 Sample Load

Figure-7 shows the results of measuring the sample load by changing the composition of mobile phase. Based on this result, the maximum sample load* is shown to depend on the mobile phase composition. For example, 20μg or smaller per one component is the appropriate injection amount under the conditions to analyze monosaccharides and disaccharides, and 200μg or smaller per one component under the oligosaccharide conditions. This shows an tendency entirely reverse to the reverse-phase partition chromatography using the ODS column. Moreover, it is considered that 50μL or less is appropriate as one injection volume.

* Maximum sample load
It is defined as the maximum sample amount that can be loaded while maintaining the expansion of each component at the minimum level in one sample injection.
Although column will not be deteriorated even when the sample larger than the maximum sample load is injected at once, it is considered as a matter of course that resolution among components will be lower compared to appropriate levels.

2-4 Quantitativity

It is known that the reducing sugar and stationary phase react to form the Schiff base when aminoalkyl packing materials are used. A part of the reducing sugar may be lost during elution and may often lead to deterioration in recovery and large tailing in peaks.
On the other hand, TSKgel Amide-80 causes no such reaction and shows extremely good quantitativity even with trace samples. Figure-8 shows the relationship between the injection amount and peak area by differential refractometer, and it shows very favorable linearity.

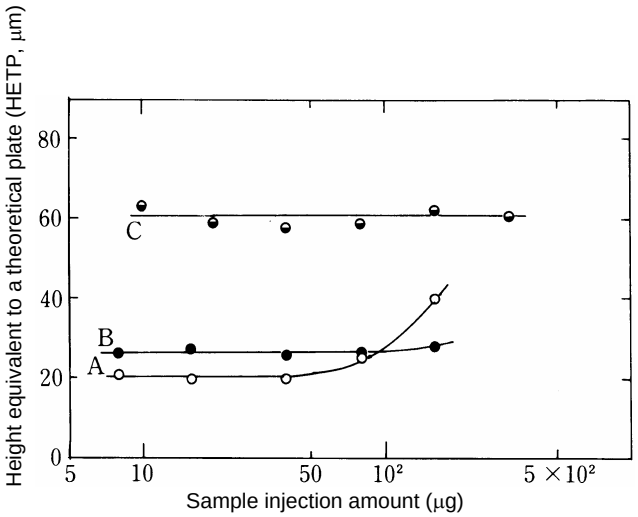

Figure-7 Sample load

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: A: Acetonitrile/water = 75/25
B: Acetonitrile/water = 65/35
C: Acetonitrile/water = 55/45
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: RI
Sample: A, B: ○, ● Mannitol
C: ◒ Maltopentaose

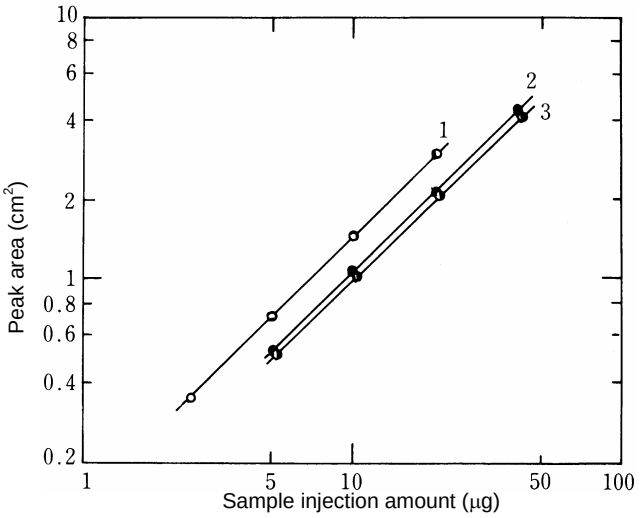

Figure-8 Relationship between sample injection amount and peak area

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 80/20
Flow rate: 1.0mL/min
Temperature: 80°C
Detection: RI

1. ○ Erythritol
2. ● Glucose
3. ◑ Xylose

3. Selection of Separation Conditions

As described in the previous section, it is necessary that various conditions be selected appropriately in order to obtain favorable resolution with good reproducibility. The items to be selected and a guideline for selection are provided as follows:

3-1 Factors Affecting Elution Volume and Elution Order

(1) Ratio of organic solvent and water in mobile phase
The fundamental concept is provided in Section 2-1. Please see Figure-2 and applications in Figures-9 and -10.

(2) Type of organic solvent in mobile phase
In general, acetonitrile and acetone are valid. Lower alcohols can be also applied for oligosaccharide separation.

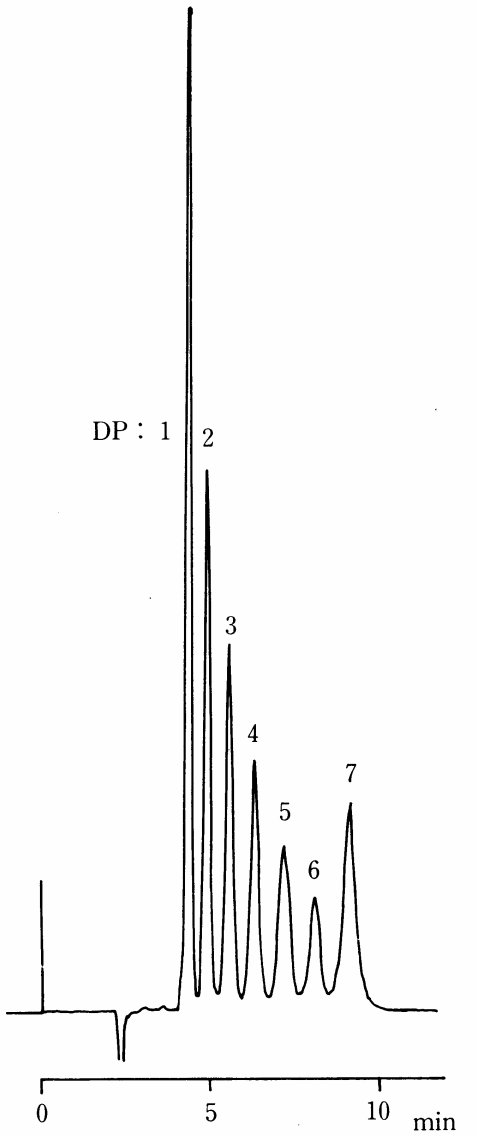

Figure-9 Separation of β-cyclodextrin acid hydrolysate

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 55/45
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: RI

(3) pH and salt concentration in mobile phase
For elution of basic samples, the pH needs to be lowered to about 3.0 or addition of approximately 10 to 20mM salt is required.

(4) Column temperature
The fundamental concept is provided in Section 2-1. Figures-3 and -4 show the results of investigating the effects on chromatogram and capacity factors.

3-2 Factors Affecting Band broadening

(1) Flow rate
Effect of flow rate has already been described in Section 2-2 in relation to the height equivalent to a theoretical plate. Please see Figures-3, -5 and -6.

(2) Column temperature
Sections 2-1 and 2-2 describe the effect of temperature on chromatogram and height equivalent to a theoretical plate. Please see Figures-3, -5 and -6.

(3) Particle size of packing materials
TSKgel Amide-80 uses 5μm particles and yields high column efficiency.

(4) Composition of sample solution
It will be described in Section 5-1.

(5) Sample load and injection volume
It has been shown in Section 2-3. Please see Figure-7.

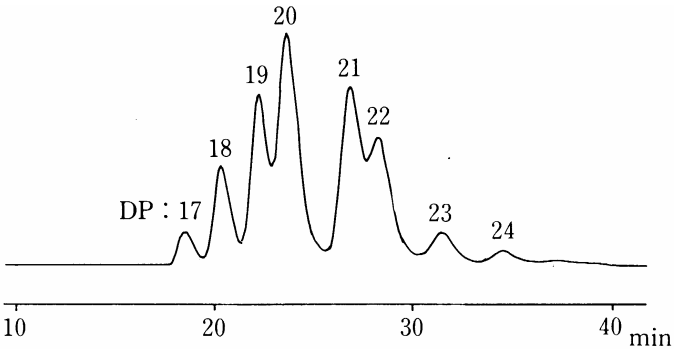

Figure-10 Separation of cyclosophoran

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 57/43
Flow rate: 1.0mL/min
Temperature: Room temperature
Detection: RI

(This chromatogram is included by the courtesy of Dr. Hisamatsu at Department of Agricultural Science, Mie University.)

4. Applications

4-1 Separation of Oligosaccharides (β-cyclodextrin acid hydrolysate)

As shown in Figure-9, glucose to heptamer (open-form β-cyclodextrin) are nearly completely separated within 10 minutes. Furthermore, if there is any intact β-cyclodextrin, it is eluted at the position of tetramer under these conditions.

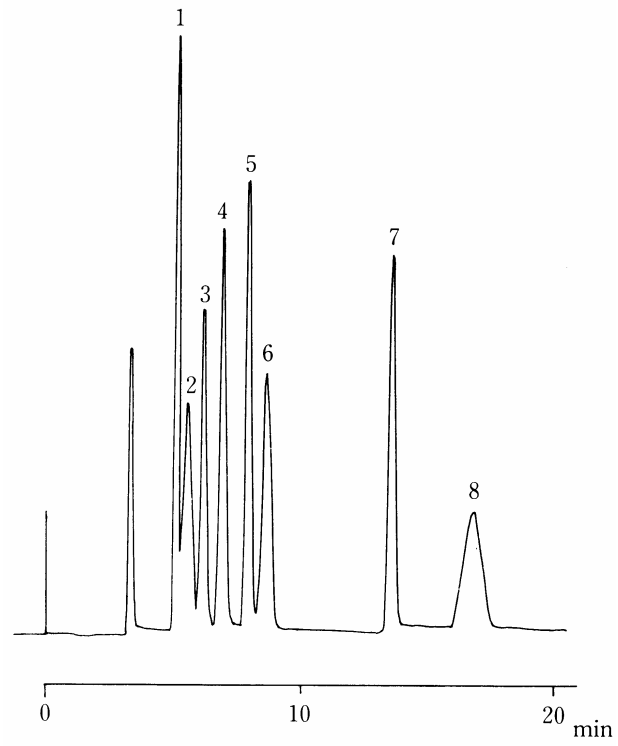

Figure-11 Separation of monosaccharides and disaccharides

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 80/20
Flow rate: 1.0mL/min
Temperature: 80°C
Detection: RI

1. Rhamnose
2. Fucose
3. Xylose
4. Fructose
5. Mannose
6. Glucose
7. Sucrose
8. Maltose

4-2 Separation of Oligosaccharides (cyclosophoran: cyclic β-1, 2-glucan)

As shown in Figure-10, degrees of polymerization from 17 to 24 are separated.

4-3 Separation of a Mixture of Monosaccharides and Disaccharides

Figure-11 shows the chromatogram. It is recommended that separation of saccharides containing reducing sugars be conducted at column temperature of 80°C as in the conditions of this chromatogram.

4-4 Separation of Glycosides (crude stevioside)

Figure-12 shows the chromatogram. Evidently, it is considered that using TSKgel Amide-80 will simplify the measurement of polar impurities which are usually difficult to measure in reversed-phase partition chromatography.

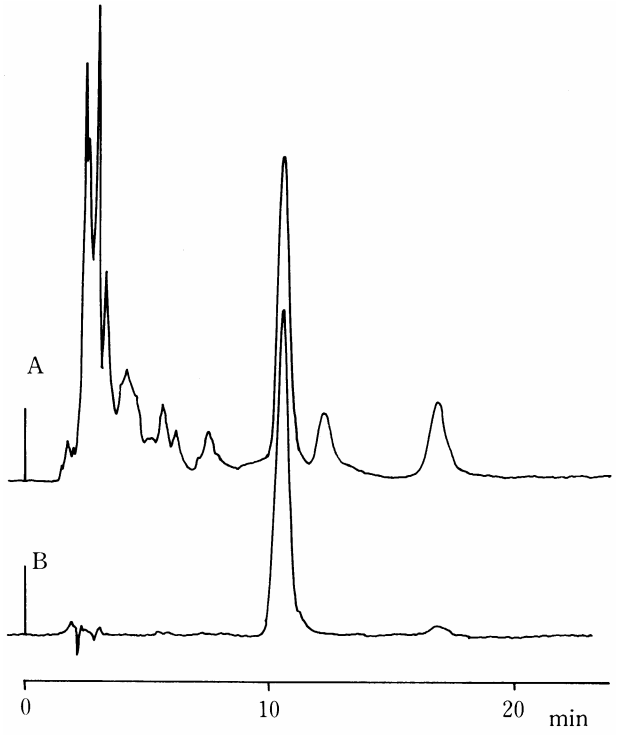

Figure-12 Chromatogram of crude and purified stevioside

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 80/20
Flow rate: 1.0mL/min
Temperature: 25°C
Detection: UV (210nm)

5. Notes on Measurement

5-1 Solvent in a sample solution

Generally in normal phase partition chromatography, polarity of the sample solvent has a great effect on band broadening. Table-1 shows the results of one experiment. Hence, band broadening is increased when the polarity of the sample solvent is higher (that is, elution is faster). Therefore, it is considered that favorable injection should be made by dissolving the sample in mobile phase if a dry sample is used, and by diluting the sample approximately to the mobile phase composition with the organic solvent used in the mobile phase if an aqueous solution sample is used. If the composition of the solvent in a sample solution differs from that of the mobile phase, it is assumed that its effect will be naturally larger depending on the injection volume.

5-2 Eluent Composition and Pressure Drops

Due to the properties of TSKgel Amide-80 columns, pressure drops inflicted under a certain flow rate depends on the viscosity and composition (polarity) of the mobile phase. Please note that the pressure thus increases in accordance with the increase in water content of the mobile phase. Normally with acetonitrile/water system, it is desired that the water is not increased to more than 60%. Please use with a low flow rate under the conditions containing high water content.

5-3 Column Oven and Injection Valve

When analyzing a reducing sugar using TSKgel Amide-80, it is conducted by maintaining the column oven at about 80°C. It is surmised that in many cases an organic solvent with a boiling point of 100°C or lower is used as the sample solvent. If a column oven with built-in injection valve is used in this case, quantitativity may become deteriorated by solvent expansion or vaporization. It is recommended that the injection valve be fixed at a section under room temperature and that the valve and column be connected by a stainless tube with inner diameter of 0.2mm.

5-4 Adsorption of sample components and Cleaning Method

(1) Removal of polar substances (neutral polysaccharides, etc.)

In the case of TSKgel Amide-80, adsorption of hydrophobic substances without charge rarely occurs. The substances that are easily bound include polysaccharides.

Table-1 Effect of Sample Solvent Composition

Sample solvent	Mannitol		Sucrose	
Acetonitrile/water (v/v)	Elution volume (mL)	Theoretical plates (TP/column)	Elution volume (mL)	Theoretical plates (TP/column)
75/25	10.34	14,807	15.00	13,850
60/40	10.32	12,190	14.96	11,738
45/55	10.25	5,510	14.89	5,331
0/100	10.22 *	—	14.86 *	—

* The peak had shoulders. The highest point of the peak is considered as the elution volume.

Separation conditions

Column: TSKgel Amide-80
4.6mm I.D. × 25cm
Eluent: Acetonitrile/water = 75/25 (v/v)
Flow rate: 1.0mL/min
Detection: RI
Temperature: 25°C
Sample: Mannitol, sucrose, 2.0mg/mL, 20μL each

To remove such substances, for example, running an acetonitrile/water system (45/55, v/v) at flow rate of 0.5mL/min for about 3 hours will be sufficient normally. In addition, baseline becomes stable shortly after cleaning the column with solvent containing water content of about 5% higher than the mobile phase for measurement prior to the measurement.

(2) Removal of ionic binding substances (cationic substances)

Since TSKgel Amide-80 uses silica gel as the base material, trace amount of silanol group remains on the packing material surface with negative charge and binds to cationic substances under neutral environment. Cationic substances can be eluted and removed by adding a trace amount of salt in the mobile phase. For example, running the acetonitrile/50mM phosphate buffer pH6.0 (50/50) at a flow rate of 0.5mL/min for about 3 hours as a cleaning liquid will remove them in most cases.

6. Conclusion

TSKgel Amide-80 is a packing material for high-performance normal phase partition chromatography which has been developed to simplify and speed up the analysis of polyols such as saccharides by high-performance liquid chromatography method. It has overcome the weaknesses of conventional normal phase partition chromatography packing materials and achieved high precision as well as favorable reproducibility. We have confidence that it will be useful if it is used with good understanding of the characteristics and procedure for the packing material.

Furthermore, TSKgel lineup includes the TSKgel Sugar AX series (anion exchange method – with use of saccharide-boric acid complex formation), TSKgel SCX (H^+ type), TSKgel PW type for aqueous solution system gel filtration chromatography (containing PW_{XL} series for oligosaccharide/polysaccharide separation), and aminopropyl chemical bonding type silica gel TSKgel NH_2-60 for normal phase partition chromatography as the packing materials and packing columns for saccharide analysis. Please make use of these products as well.

Literature

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