



SEPARATION REPORT NO. 55


SEPARATION OF SACCHARIDES USING TSKgel AMIDE-80, A PACKING MATERIAL FOR HIGH-PERFORMANCE NORMAL PHASE PARTITION CHROMATOGRAPHY¹


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Contact us today for more information

EMEA  +49 6155 704 37 12

USA  +1-800-366-4875 option #3

 www.tosohbioscience.com

 techsupport.tbhg@tosoh.com

 techservice.tbl@tosoh.com

1. Introduction

Saccharides are fundamental substances that express various bioactivities and may exist independently or form complexes with proteins or lipids. The analysis of saccharides and sugar chains in complex carbohydrates provides valuable information for the medical, research and food industries. In the past, various methods have been used to analyze saccharides, including differing high-performance liquid chromatography (HPLC) modes.

Saccharides can be classified into *monosaccharides*, *disaccharides*, *oligosaccharides*, and *polysaccharides* based upon the degrees of polymerization and condensation. Methods of separating *monosaccharides* and *disaccharides* include anion exchange chromatography, in which an anionic saccharide-boric acid complex is formed in the presence of boric acid¹, normal phase chromatography²⁻⁶, ligand exchange chromatography⁷, and anion exchange chromatography under strong basic pH conditions⁸.

The methods of choice for the separation of *oligosaccharides* include gel filtration chromatography⁹, normal phase partition chromatography^{2,5,6,10} and reversed phase partition chromatography^{11,12}, while *polysaccharides* are most often separated by gel filtration chromatography.

It is critical that the features of these separation modes are understood and that the optimal separation mode for the target sample is selected.

Among these HPLC modes, normal phase chromatography, often also referred to as hydrophilic interaction chromatography (HILIC), selectively retains polyhydric alcohols such as saccharides, while most of the substances with low polarity, as well as monohydric alcohols, elute unretained or near the elution volume of an unretained compound. Normal phase chromatography, in tandem with a differential refractometer as a detector, has long been used for the analysis of saccharides, as it provides good selectivity with relatively short analysis times.

Traditional packing materials for saccharide separations often included ion-exchange resins^{2,10} and amino-bonded silica gel^{3,4,6}. These packing materials were not always satisfactory in terms of physical and chemical stability. TSKgel Amide-80 is a packing material in which the stationary phase consists of nonionic carbamoyl groups that are chemically bonded to the silica gel particles. Having a nonionic stationary phase, compared to so-called amino-bonded phases, affords TSKgel Amide-80 excellent chemical stability. The H-atom in the -NH group in the stationary phase can form a hydrogen bond with oxygen atoms in hydroxyl groups or with a carbonyl group; it also strongly retains water. This forms a water-rich partitioning 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 the 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/water solvent systems such as acetonitrile/water. To illustrate this point, the chromatogram in Figure 1 shows the separation of various sugar alcohols. Basically, the more hydroxyl groups in a compound, the more polar it will be and the longer it will be retained on TSKgel Amide-80 columns. In addition, comparison of retention between mannitol and inositol, each with 6 hydroxyl groups, shows that inositol, which has a cyclic form and lower solubility in the mobile phase (acetonitrile/water system), is retained longer. As usual, solute retention is greatly affected by the polarity of mobile phase, showing a tendency for stronger retention as the water content in the mobile phase decreases. This is illustrated in Figure 2. This trend is not limited to sugar alcohols. It is also observed with monosaccharides, disaccharides and oligosaccharides.

In addition, monosaccharides elute from the column in the order of increasing number of carbon atoms. For instance, a pentose elutes before a hexose. Within a certain class, for instance hexoses, selectivity varies due to slight differences in molecular structure even among similar hexoses, which enables separation of individual class members.

When analyzing oligosaccharides, water content needs to be increased by 20 to 30% since retention would be too long when using the mobile phase that is suitable for the separation of monosaccharides. Moreover, in the case of reducing sugars whose molecular structures are known to include α or β type structures, pyranose or furanose structures, etc., instead of being present in solution as a single structure, they generally are able to convert to their alternate structure until equilibrium is reached. The rate constant of this conversion process, called mutarotation, is often rather slow under neutral conditions at room temperature.

As a result, two or more peaks may be separated and detected even for a single saccharide under such conditions. Figure 3 shows the effect of temperature on the chromatogram when D-glucose is separated on a TSKgel Amide-80 column. It is evident that the chromatogram shows multiple peaks at temperatures of 60°C or lower, each peak representing a distinct molecular structure.

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 raised to 70 °C and higher. 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 kept constant, in general elution times become shorter at higher temperatures.

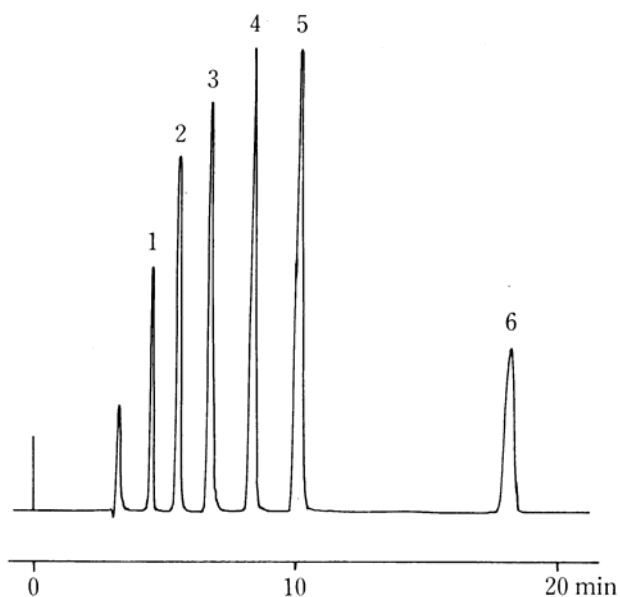


Figure 1 Separation of polyols

Column: T SKgel Amide-80
 4.6mm ID × 25cm
 Eluent: ACN/water = 75/25
 Flow rate: 1.0mL/min
 Temperature: 25 °C
 Detection: RI
 Sample: 1. ethylene glycol 2. glycerol 3. erythritol
 4. xylitol 5. mannitol 6. inositol

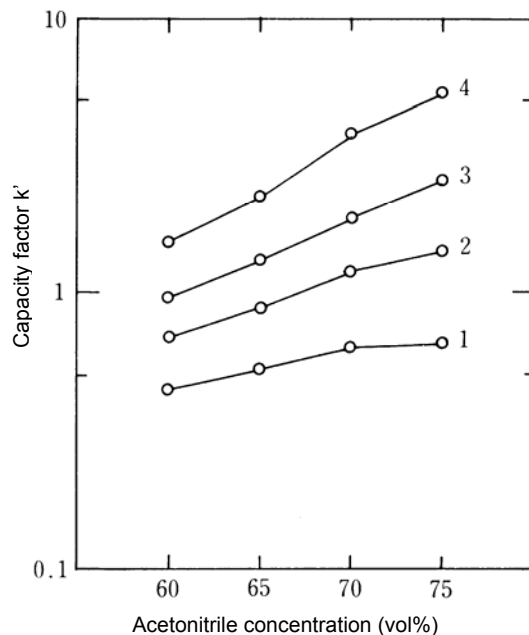


Figure 2 Effect of acetonitrile concentration in eluent on retention of polyols

Column: T SKgel Amide-80
 4.6mm ID × 25cm
 Eluent: ACN/ water
 Flow rate: 1.0mL/min
 Temperature: 25 °C
 Detection: RI
 Sample: 1. ethylene glycol 2. xylitol
 3. mannitol 4. inositol

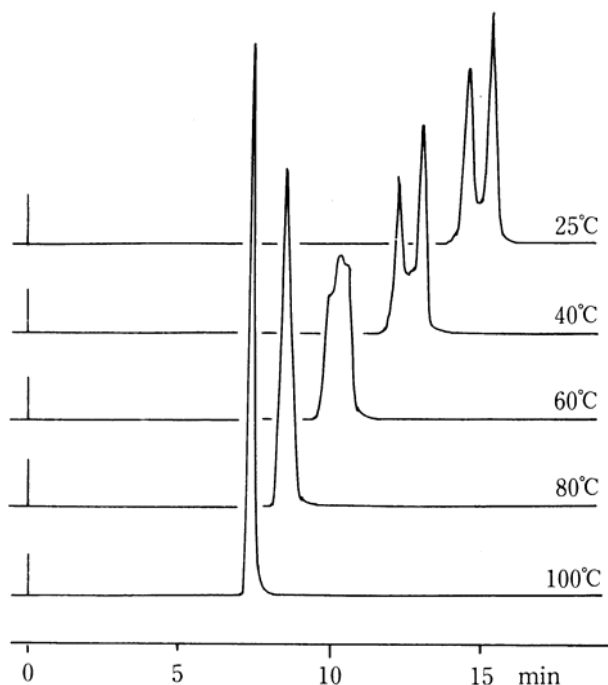


Figure 3 Effect of column temperature on D-glucose chromatogram

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

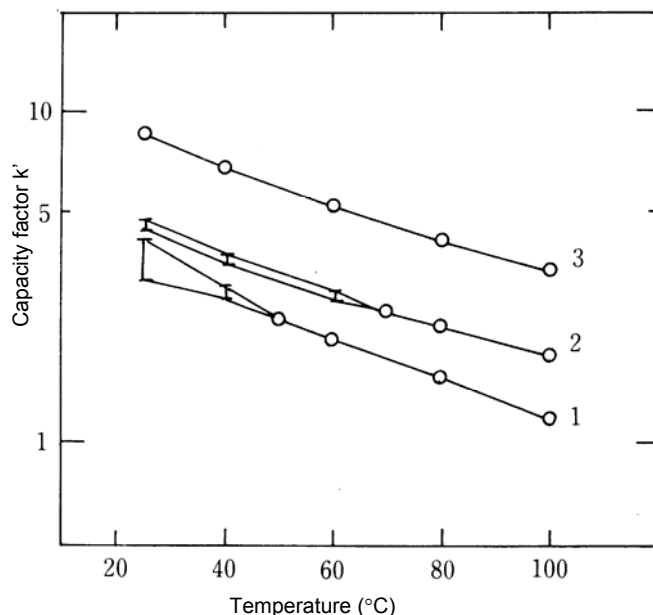


Figure 4 Effect of column temperature on retention

Column: T SKgel Amide-80
 4.6mm ID × 25cm
 Eluent: Acetonitrile/water = 80/20
 Flow rate: 1.0mL/min
 Detection: RI
 Sample: 1. fructose 2. glucose 3. sucrose

2-2 Height Equivalent to a Theoretical Plate

HETP, the height equivalent to a theoretical plate, usually abbreviated as H, is an expression of the efficiency of the column for a solute under a particular set of operating conditions. H is the ratio of the length of the column over the number of theoretical plates ($H=L/N$). H varies with flow rate, as is shown in Figure 5. The lower the value of H, the more efficient the column and the better the peaks are resolved.

When mannitol was injected at room temperature (25°C), N was calculated to be approximately 80,000 theoretical plates per meter at a flow rate between 0.15mL/min and 0.3mL/min. From a practical perspective, a flow rate in the range of 0.5 to 1.0mL/min is a good compromise between the efficiency of the separation and analysis time.

At 80°C (see Figure 6) the efficiency of *reducing sugars* such as D-glucose is optimal (lowest H-value) at 0.25mL/min, the lowest flow rate that was examined at this temperature. It is assumed that the optimum efficiency for *reducing sugars* is actually achieved at flow rates below 0.25mL/min.

Also at 80°C, minimum H values for *non-reducing sugars* such as mannitol and sucrose are obtained at flow rates between 0.5 and 1.5mL/min.

It is presumed that this phenomenon is caused by lower conversion rates between the anomeric forms of sugar compared to the solute's rate of partitioning between mobile and stationary phases of the column, which lead to band broadening.

Note that since TSKgel Amide-80 is a silica-based packing material, column lifetime will be adversely affected when the column is operated at high temperature in an acetonitrile/water solvent system.

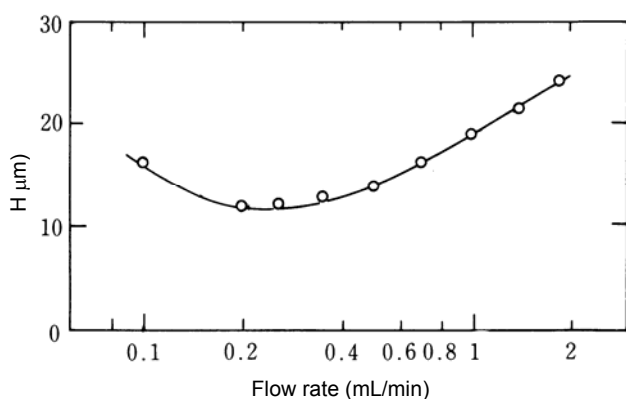


Figure 5 Effect of flow rate on H at 25°C

Column: T SKgel Amide-80
4.6mm ID × 25cm
Eluent: ACN/water = 75/25
Temperature: 25 °C
Detection: RI
Sample: mannitol

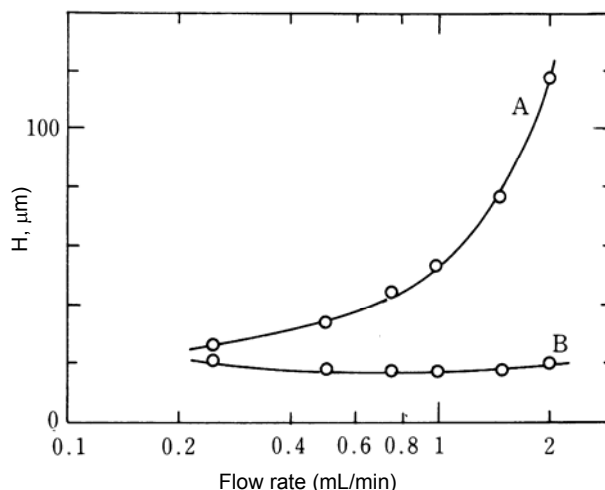


Figure 6 Effect of flow rate on H at 80°C

Column: T SKgel Amide-80
4.6mm ID × 25cm
Eluent: ACN/water = 80/20
Temperature: 80 °C
Detection: RI
Sample: A. D-glucose
B. mannitol

2-3 Sample Load

Figure 7 shows that maximum sample load* is affected by mobile phase composition. For example, a load of 20 µg or smaller per component is the appropriate injection amount to analyze monosaccharides and disaccharides under the mobile phase conditions listed in the figure, while 200 µg or smaller per component is the appropriate injection amount for oligosaccharides under the listed conditions. Moreover, it is considered that an injection volume of 50 µL or less is appropriate to prevent volume overloading of the column. Figure 7 also shows that more sample can be injected before overloading the column by increasing the percentage of water in the mobile phase.

- Maximum sample load is defined as the amount of sample that can be loaded onto the column without decreasing the width of the peak. Although higher sample loads may be injected onto the column, this inevitably will result in a loss of column efficiency.

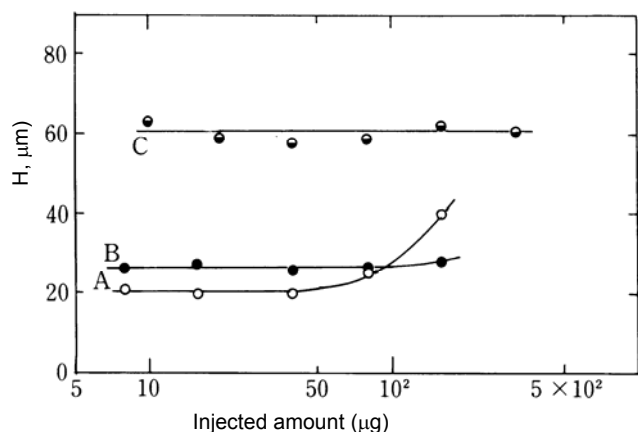


Figure 7 Sample load

Column: T SKgel Amide-80
4.6mm ID × 25cm
Eluent: A: ACN/water = 75/25
B: ACN/water = 65/35
C: ACN/water = 55/45
Flow rate: 1.0mL/min
Temperature: 25 °C
Detection: RI
Sample: A, B. mannitol
C. maltop entose

2-4 Quantification

It is known that reducing sugars react with an aminoalkyl stationary phase to form a Schiff's base. This results in an irreversible change to the stationary phase, thus making the amino column unsuitable for further analysis. On the other hand, reducing sugars do not react with a TSKgel Amide-80 column; allowing good quantitation even when injecting trace sample amounts. Figure 8 shows excellent linearity when studying the relationship between injection amount and peak area using a differential refractometer

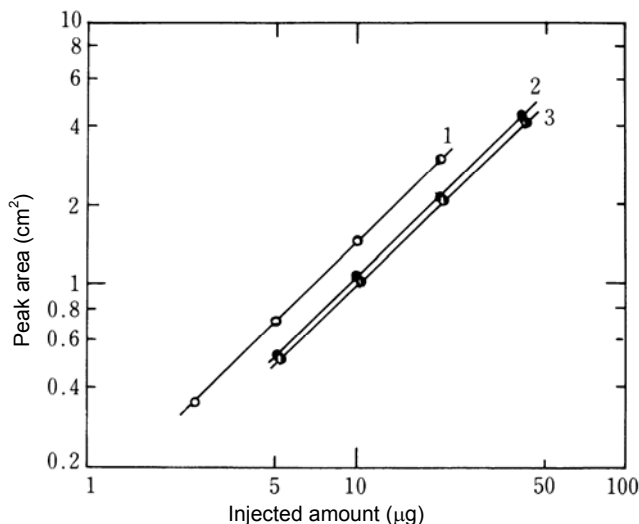


Figure 8 Relationship between sample injection amount and peak area

Column: T SKgel Amide-80
4.6mm ID × 25cm
Eluent: ACN/water = 80/20
Flow rate: 1.0mL/min
Temperature: 80 °C
Detection: RI
Sample: 1. erythritol
2. glucose
3. xylose

3. Method Development

As described in the previous section, it is necessary that various mobile phase conditions are investigated in order to optimize retention and selectivity. The following comments serve as a guideline for method development on TSKgel Amide-80 columns.

3-1 Factors Affecting Retention 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) **Organic solvent in mobile phase**
Acetonitrile and acetone have been used successfully as mobile phase components when working with TSKgel Amide-80 columns. Lower alcohols can be also applied for oligosaccharide separation.
- (3) **Mobile phase pH and salt concentration**
For elution of basic samples, the pH needs to be lowered to about 3.0. In addition, approximately 10 to 20mM salt is required.
- (4) **Column temperature**
The fundamental concept is provided in Section 2-1. Figures 3 and 4 show that efficiency and retention vary with column temperature.

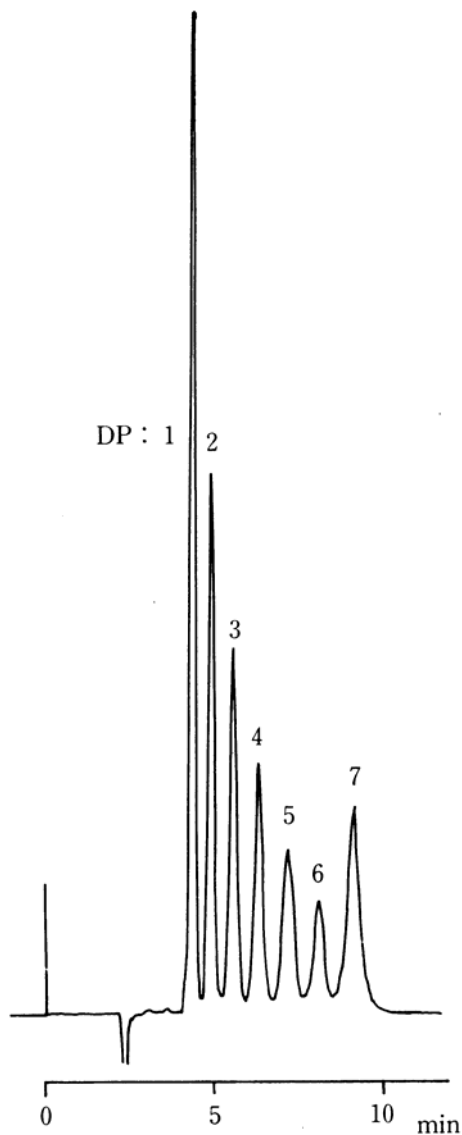


Figure 9 Separation of β -cyclodextrin acid hydrolysate

Column: T SKgel Amide-80
 4.6mm ID \times 25cm
 Eluent: ACN/water = 55/45
 Flow rate: 1.0mL/min
 Temperature: 25 °C
 Detection: RI

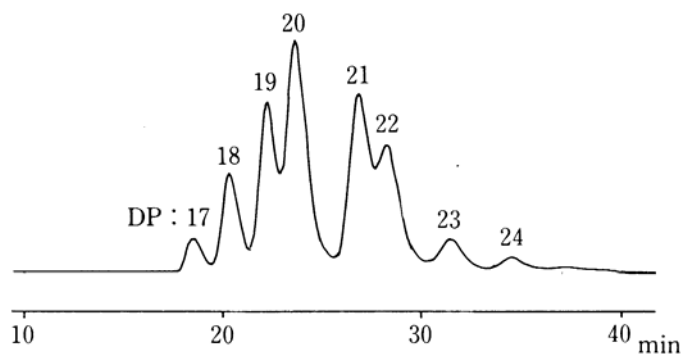


Figure 10 Separation of cyclodextran

Column: T SKgel Amide-80
 4.6mm ID \times 25cm
 Eluent: ACN/water = 57/43
 Flow rate: 1.0mL/min
 Temperature: 25 °C
 Detection: RI

3-2 Factors Affecting Band Broadening

- (1) **Flow rate**
 The effect of flow rate was 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 retention, peak shape and columns efficiency. Please see Figures 3, 5 and 6.
- (3) **Particle size**
 Conventional TSKgel Amide-80 columns contain 5 μ m particles. Recently more efficient 3 micron columns have become available.
- (4) **Sample solution**
 See Section 5-1.
- (5) **Sample load and injection volume**
 See Section 2-3 and Figure 7.

4. Applications

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

As shown in Figure 9 with the analysis of open-form β -cyclodextrin, glucose and all oligomers up the heptamer are completely separated within 10 minutes. Any intact β -cyclodextrin that may exist is eluted at the position of the tetramer under these conditions.

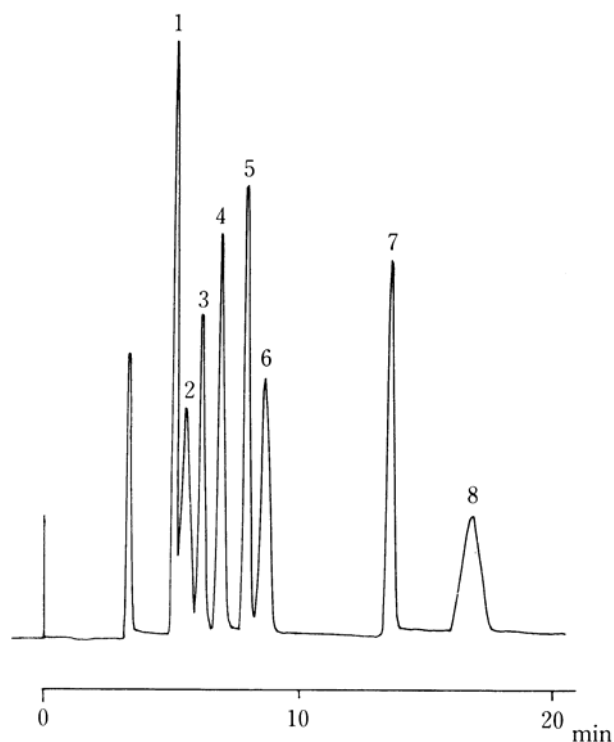


Figure 11 Separation of monosaccharides and disaccharides

Column: T SKgel Amide-80
 4.6mm ID × 25cm
 Eluent: ACN/water = 80/20
 Flow rate: 1.0mL/min
 Temperature: 80 °C
 Detection: RI
 Sample: 1. rhamnose
 2. fucose
 3. xylose
 4. fructose
 5. mannose
 6. gucose
 7. scrose
 8. matose

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

As shown in Figure 10 for the analysis of cyclophoran, the degrees of polymerization from 17 to 24 are separated.

4-3 Separation of a Mixture of Monosaccharides and Disaccharides

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

4-4 Separation of Glycosides (crude stevioside)

Figure 12 shows the measurement of polar impurities, which are usually difficult to measure in reversed-phase chromatography. This type of separation is simplified by using a normal phase TSKgel Amide-80 column.

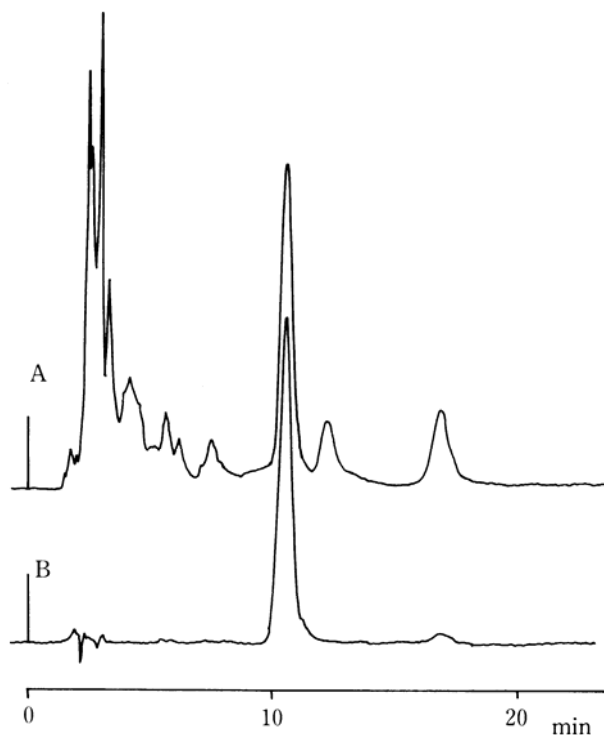


Figure 12 Chromatogram of crude and purified stevioside

Column: T SKgel Amide-80
 4.6mm ID × 25cm
 Eluent: ACN/water = 80/20
 Flow rate: 1.0mL/min
 Temperature: 25 °C
 Detection: UV @ 210nm

5. Comments about Operating Conditions

5-1 Solvent in the Sample Solution

Generally in normal phase partition chromatography, polarity of the sample solvent can have a marked effect on band broadening. Table 1 shows the results of one such experiment. The results indicate that band broadening is increased when the polarity of the sample solvent is higher than the polarity of the mobile phase. Therefore, it is suggested, if possible, to either dissolve the sample in mobile phase, or if the sample is dissolved in water to add organic solvent to obtain the approximate composition of the mobile phase. If the composition of the solvent in a sample solution differs from that of the mobile phase, any possible negative effect will be magnified as the volume injected is increased.

5-2 Eluent Composition and Pressure Drops

As with any HPLC column, the pressure drop over the column depends on the composition of the eluent, the flow rate and temperature. When working under normal phase conditions the pressure drop over the column increases with increasing water content of the mobile phase. Normally with an acetonitrile/water system, it is recommended that the water content is not increased to more than 60%. When using a higher water content, it is recommended to operate the TSKgel Amide-80 column at a low flow rate.

5-3 Column Oven and Injection Valve

When analyzing a reducing sugar using TSKgel Amide-80, the column oven needs to be set at about 80°C. Often under these conditions the sample contains an organic solvent with a boiling point of 100°C or lower. If a column oven with a built-in injection valve is used, quantification will probably be hampered by solvent expansion or vaporization. Thus it is recommended that the injection valve be installed instead at a section of the instrument that is operated at room temperature and that the valve and column be connected by a short piece of 0.2mm ID stainless tubing.

5-4 Adsorption of Sample Components and Cleaning Methods

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

In the case of TSKgel Amide-80 columns, adsorption of hydrophobic substances without charge rarely occurs. Certain polysaccharides can be adsorbed very strongly

on TSKgel Amide-80 packing material. To remove such strongly retained substances, running an acetonitrile/water system (45/55, v/v) at flow rate of 0.5mL/min for about 3 hours is normally sufficient to elute these contaminants off the column. In addition, the baseline usually becomes stable shortly after cleaning the column with solvent containing a 5% higher water content than the mobile phase that was used for measurement.

(2) Removal of Cationic Substances

Since TSKgel Amide-80 uses silica gel as the base material, residual unbonded silanol groups with a negative charge remain on the packing material surface and can bind to cationic substances under a non-buffered mobile phase. These cationic substances can be eluted and removed by adding a trace amount of salt to the mobile phase. For example, running the acetonitrile/50mM phosphate buffer, pH 6.0, (50/50) at a flow rate of 0.5mL/min for about 3 hours as a cleaning solvent will remove cationic substances in most cases.

Table 1: Effect of Sample Solvent Composition

Sample solvent Acetonitrile/water (v/v)	Mannitol		Sucrose	
	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 showed several shoulders. The highest point of the peak was used as the elution volume.

Separation conditions

Column: T SKgel Amide-80
4.6mm ID × 25cm
Eluent: ACN/water = 75/25 (v/v)
Flow rate: 1.0mL/min
Detection: RI
Temperature: 25 °C
Sample: mannitol, sucrose, 2.0mg/mL, 20µL each

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. It has overcome the weaknesses of conventional normal phase partition chromatography packing materials and achieves high precision as well as favorable reproducibility.

In addition to TSKgel Amide-80 columns, Tosoh offers several other columns for saccharide analysis. TSKgel Sugar AX series (anion exchange method – with use of saccharide-boric acid complex formation), TSKgel SCX (H⁺ type), TSKgel PW-type for aqueous gel filtration chromatography (including PW_{XL} series for oligosaccharide/polysaccharide separation), and TSKgel NH₂-60 (containing an aminopropyl chemical bonding type silica gel) for normal phase partition chromatography can also be used for saccharide analysis.

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Acknowledgement

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