Analysis of Various Oligosaccharides Using HILIC Mode

Introduction

An oligosaccharide is a polysaccharide containing at least two monosaccharides bonded by glycosidic linkages. It is known that the oligosaccharides are utilized by gut bacteria inside animal bodies, and increasing the bacteria number is beneficial for the digestive health of animals. Thus, functionality benefits of the oligosaccharides have been focused in some industries.

Size Exclusion Chromatography (SEC) is one of the known methods for oligosaccharide analysis. However, separations become more difficult as the size of the analyte (degree of polymerization) becomes larger. On the other hand, Hydrophilic Interaction Liquid Chromatography (HILIC) can provide better separation for the larger oligomers than the SEC mode does. Thus, the methods using the HILIC mode are also commonly used these days.

Shodex HILICpak VN-50 series columns are specifically developed for the separation of high-molecular weight oligosaccharides that were difficult to be analyzed by previously available HILIC columns. It is packed with multi-porous polyvinyl alcohol polymers modified with diol functional groups (Fig. 1).

Fig. 1. Schematic diagram of VN-50 packing material

The polymer-based packing material makes the column durable to the high pH conditions (the maximum working pH is 13). Since the modified diol functional group is nonionic, the VN-50 series column does not adsorb (retain) acidic sugars inside the column which is an often-observed problem with amino columns. Thus, the column is feasible for analyzing various oligosaccharides.

This technical article introduces analysis of five oligosaccharides including nonionic oligosaccharides such as dextrin, N-acetylchitooligosaccharides (polymer of N-acetylglucosamine) and xylooligosaccharides; cationic oligosaccharides, chitosan-oligosaccharides (polymer of glucosamine); and anionic oligosaccharides, oligogalacturonic acids.

Figure 2 shows structures of oligosaccharides analyzed in this study. Different oligosaccharides exhibit different characteristics because of their constituent sugars, thus eluent compositions and column temperature etc. were optimized for each analysis.

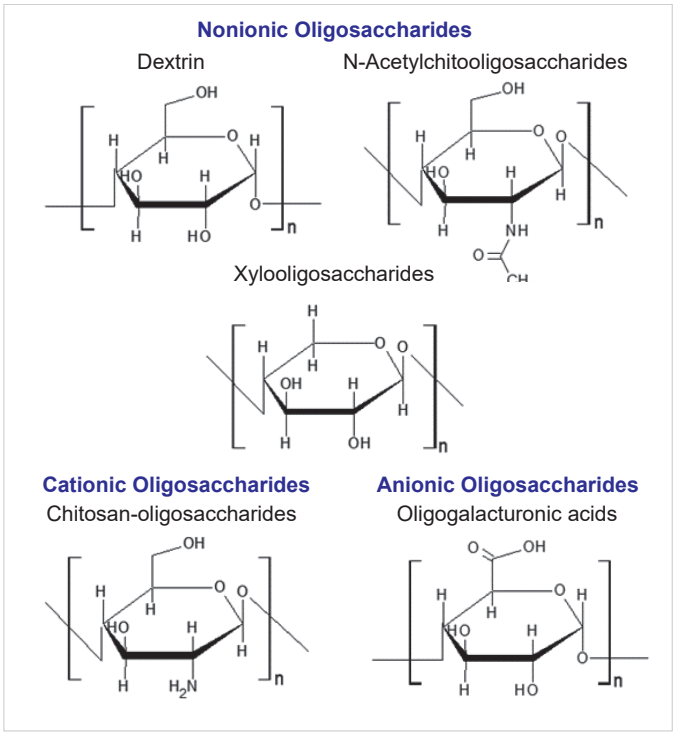


Fig. 2. Structures of oligosaccharides studied

Experimental

1. Preparation of oligosaccharides samples

The oligosaccharides analyzed were commercial standard or food ingredients grades. The solid samples were dissolved in water, and then equal amount of acetonitrile was added. The separations of all peaks were improved when the sample contained 50 % acetonitrile, compared to when 100 % water alone was used to prepare the sample (data not shown). When impurities were observed, the sample was centrifuged at 5,000 × g for 5 minutes to remove the impurity and the supernatant was used for the analysis.

2. Preparation of oligogalacturonic acid samples

The oligogalacturonic acid was prepared from commercial oligogalacturonic acid standard. Pectinase was used to degrade the oligogalacturonic acid following the below preparation procedure.

1.8 mL of 50-mM ammonium formate buffer (pH 4.0) was added to 20 mg of polygalacturonic acid. The polygalacturonic acid was dissolved in a warm bath at 70 °C. The container was kept at 40 °C. The enzyme solution was prepared by dissolving 1 mg of pectinase in 1 mL of 50-mM ammonium formate buffer (pH 4.0). 200 µL of the prepared enzyme solution was added to the galacturonic acid at 40 °C to let the enzyme reaction to proceed. After 40 minutes, 500 µL of the mixture and 500 µ L of acetonitrile were added to deactivate the pectinase. The sample was then, centrifuged at 5,000 × g for 5 minutes to remove the impurities. The supernatant was then used for the analysis.

3. Analytical conditions

LC/MS system used was Shimadzu Prominence-i. The column used was Shodex HILICpak VN-50 2D (2.0 mm I.D. x 150 mm; particle size 5 µm; pore size 100 Å). The detector used was an ELS. To separate the oligosaccharides distributed in a wide molecular weight range, gradient method was used.

Results and Discussion

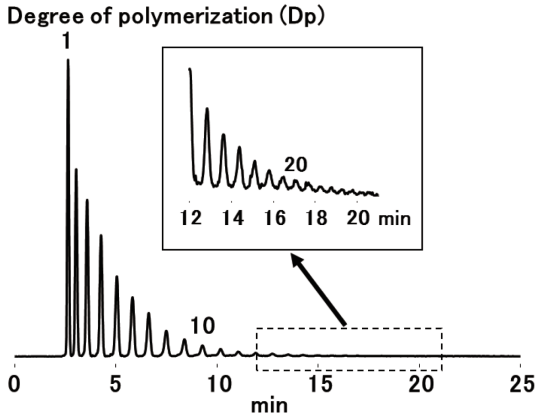
1. Analysis of nonionic oligosaccharides

The dextrin prepared in 1:1 water/acetonitrile was analyzed. The dextrin of up to around 25 mer were well separated. Figure 3 shows the obtained chromatogram and the analytical conditions used.

The sugars with reducing group terminals exist as α and β isomers. Under certain HPLC conditions where the rate of α and β isomer conversion is low, the isomers are separated inside the column. This is called anomer separation and can be observed as peak splitting and/or peak broadening in the chromatogram. If anomer separation was observed even when using the mentioned conditions (water/acetonitrile eluent and column temperature at 40 °C), follow below methods to suppress the separation.

(1) Keep the same eluent (water/acetonitrile), but increase the column temperature to 60 °C.
(2) If improvement was not observed by following (1), then use basic solvent instead of water for the eluent.

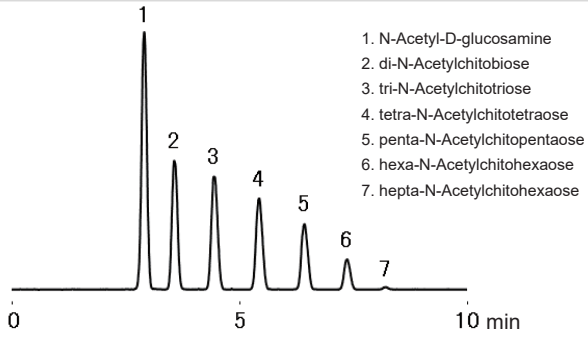
Under these conditions, the conversion rate of α and β isomers becomes faster and they will not separate inside the column.



Sample : 5 mg/mL dextrin (in 50 % CH_3CN), 10 µL
Column : Shodex HILICpak VN-50 2D
Eluent : (A) H_2O, (B) CH_3CN
Linear gradient
70 - 50 % B (0 - 30 min)
Flow rate : 1.0 mL/min
Detector : ELS
Column temp. : 40 ºC

Fig. 3. Analysis of dextrin

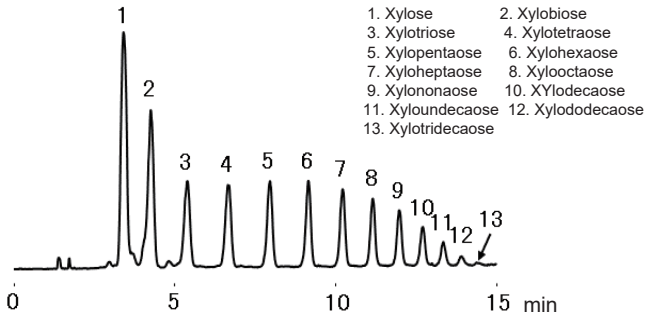
Analysis of N-acetylchitooligosaccharides with water/acetonitrile eluent and column temperature at 40 °C exhibited anomer separation. However, by increasing the column temperature to 60 °C, up to 7 mer oligosaccharides were separated (Fig. 4).



Sample : 5 mg/mL N-acetylchitooligosaccharides (in 50 % CH_3CN), 10 µL
Column : Shodex HILICpak VN-50 2D
Eluent : (A) H_2O, (B) CH_3CN
Linear gradient
80 - 50 % B (0 - 10 min)
Flow rate : 1.0 mL/min
Detector : ELS
Column temp. : 60 ºC

Fig. 4. Analysis of N-acetylchitooligosaccharides

Analysis of xylooligosaccharide with water/acetonitrile eluent and column temperature at 40 °C exhibited anomer separation. By increasing the column temperature to 60 °C, improvement of the peak splitting was observed, but the peaks were broad. Thus, we replaced the water with 0.1 % aqueous ammonium solution and analyzed the xylooligosaccharide at 40 °C. The peak shapes were improved and up to 13 mer oligosaccharides were observed (Fig. 5). Moreover, the use of aqueous ammonium solution as a basic volatile eluent allows the use of not only ELS detector but corona CAD and MS for detection.

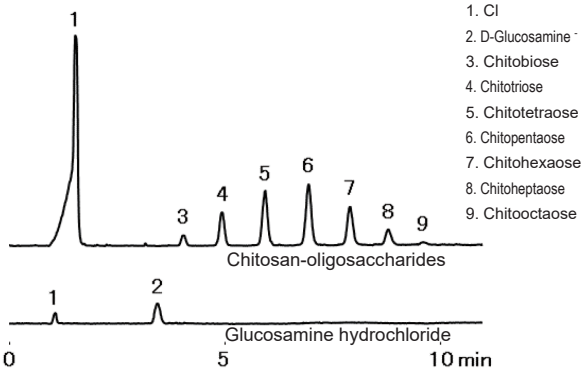


Sample : 5 mg/mL xylooligosaccharides (in 50 % CH_3CN), 10 µL
Column : Shodex HILICpak VN-50 2D
Eluent : (A) 0.1 % NH_3 aq., (B) CH_3CN
Linear gradient
80 - 50 % B (0 - 20 min)
Flow rate : 1.0 mL/min
Detector : ELS
Column temp. : 40 ºC

Fig. 5. Analysis of xylooligosaccharides

2. Analysis of cationic oligosaccharide

Analysis of chitosan-oligosaccharides with water/acetonitrile eluent and column temperature at 40 °C exhibited very broad peaks. The peak shapes did not improve by increasing the column temperature to 60 °C nor the temperature change made the peaks even broader. Thus, we hypothesized the cause of the peak broadening was from ionic dissociation instead of anomer separation. The dissociation occurs in the amino functional group of the chitosan-oligosaccharides. This dissociation can be suppressed by using basic condition. We used 0.1 % aqueous ammonium solution/acetonitrile as the eluent and analyzed it at 40 °C. Since glucosamine monomer was not detected in the chitosan-oligosaccharides standard used, we analyzed glucosamine hydrochloride separately. This enabled to obtain good peaks and separation of 8-mer oligosaccharides (Fig. 6).

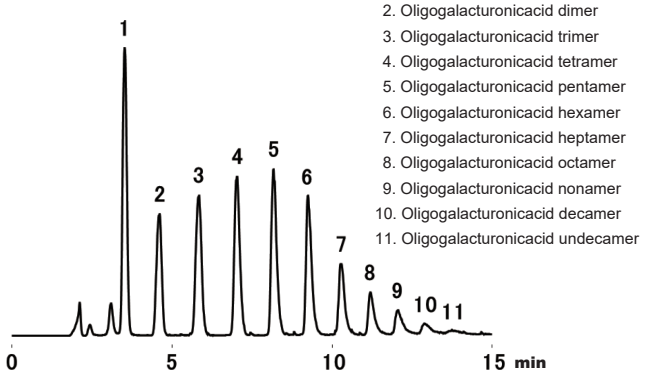


Sample : 0.1 mg/mL glucosamine hydrochloride and 5-mg/mL chitosan-oligosaccharides (in 50 % CH_3CN), 10 µL
Column : Shodex HILICpak VN-50 2D
Eluent : (A) 0.1 % NH_3 aq., (B) CH_3CN
Linear gradient
75 - 60 % B (0 - 10 min) → 60 % B (10 - 15 min)
Flow rate : 1.0 mL/min
Detector : ELS
Column temp. : 40 ºC

Fig. 6. Analysis of glucosamine hydrochloride and chitosan-oligosaccharides

3. Analysis of anionic oligosaccharides

Analysis of oligogalacturonic acid with water/acetonitrile eluent and column temperature at 40 °C exhibited broad peaks. We assumed ionic dissociation could be the cause of the peak broadening, as oligogalacturonic acid is an ionic oligosaccharide like chitosan-oligosaccharides. The galacturonic acid contains a carboxylic functional group. Thus, to prevent the ionic dissociation, an acidic eluent should be used. We used 1 % formic acid instead of water for the eluent. Sharp peaks were obtained under the modified condition and up to 11-mer oligosaccharides were observed (Fig. 7).



Sample : 5 mg/mL Oligogalacturonic acids (in 50 % CH_3CN), 10 µL
Column : Shodex HILICpak VN-50 2D
Eluent : (A) 1 % HCOOH aq., (B) CH_3CN
Linear gradient
55 - 35 % B (0 - 10 min) → 35 % B (10 - 15 min)
Flow rate : 0.6 mL/min
Detector : ELS
Column temp. : 40 ºC

Fig. 7. Analysis of oligogalacturonic acids

Conclusions

In this technical article, analytical conditions were optimized for the separation of three nonionic oligosaccharides, one cationic oligosaccharide, and one anionic oligosaccharide using the Shodex HILICpak VN-50 4D. Good separations were obtained by optimizing the analytical conditions while considering the characteristics of each oligosaccharide: For the nonionic oligosaccharides, it is important to suppress anomer separation, while for ionic (cationic and anionic) oligosaccharides, it is important to suppress ion dissociation. Table 1 summarizes the optimized analytical conditions for each oligosaccharide and the expected effect of the choice. The analytical conditions developed here should be applicable for the analysis of other oligosaccharides. The HILICpak VN-50 series column has high tolerance towards a wide range of pH, and thus it is feasible analyzing various types of oligosaccharides.

Table 1. Various oligosaccharides and their recommended analytical conditions

Ionic characteristics of oligosaccharide	Oligosaccharide	Constituent monosaccharide	Eluent	Column Temp.	Expected effect
Nonionic	Dextrin	Glucose	H_2O/CH_3CN	40 ºC	--
	N-Acetylchito oligosaccharides	N-Acetylglucosamine	H_2O/CH_3CN	60 ºC	Prevention of anomer separation
	Xylooligosaccharides	Xylose	0.1 % NH_3 aq. /CH_3CN	40 ºC	Prevention of anomer separation
Cationic	Chitosan Oligosaccharides	Glucosamine	0.1 % NH_3 aq. /CH_3CN	40 ºC	Prevention of amino functional group dissociation
Anionic	Oligogalacturonic acids	Galacturonic acids	1 % HCOOH aq. /CH_3CN	40 ºC	Prevention of carboxylic functional group dissociation

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