Application Note

High pressure stable SEC phases for high-resolution oligosaccharide analysis

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High-resolution gel permeation chromatography (GPC) analysis methods have been developed for oligosaccharides.[1–3] Some of the very high resolving GPC materials in this special field, however, have flow rate restrictions due to a low upper pressure limit, leading to long analysis times.

To overcome the disadvantage of long analysis times, a GPC material has been developed that allows very high resolution oligosaccharide analysis in a very short time.[4] Results of the latest improvements to the material are presented here.

Experimental

PSS-WinGPC software (**Polymer Standards Service GmbH, PSS,** Mainz, Germany) controlled an isocratic HP1100 HPLC system (**Agilent Technologies,** Palo Alto, CA) equipped with an external RI71 refractive index detector (**Shodex,** New York, NY). Injection volume: 20 µL, sample concentration: 5 g/L eluent, flow rate: 1.00 mL/min, temperature: 20 or 80 °C. Columns: PSS-MCX 100 Å, 5 µm; 1.000 Å, 5 µm; 10^5 Å, 10 µm; 10^6 Å, 10 µm (8 × 300 mm). GPC columns and polymeric and oligomeric standards were obtained from **PSS.** The MCX 100-Å, 5-µm column and MCX 1000-Å, 5-µm 8 × 300 mm columns are stable up to 200 bar pressure. The typical backpressure at 1 mL/min and 20 °C is 40–60 bar. Thus, the highest allowable flow rate is approx. 3 mL/min. Typical plate counts are 35–50,000 plates/m.

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Results and discussion

Porosities

Using a special kind of synthesis and treatment, a rigid, high pressure stable spherical high-resolution strong cation exchange (SCX) material with unique properties was developed (**PSS**). The material was tailored to specific customer requirements regarding particle size and porosity. From these size exclusion chromatography (SEC) materials, GPC columns (8 × 300 mm, stainless steel) were packed. The columns were characterized by GPC calibration curves. For this reason, narrow molecular weight distributed pullulan standards (special non-branched sugars) with a molecular weight of 180–1,600,000 D were used for characterization of the SCX materials. GPC calibration curves were obtained (*Figure 1*). The calibration curves illustrate the relationship between molecular weight respective to molecular size and elution volume. Information regarding details of the pore characteristics was thus obtained. This characterization is limited to between 180 and 1,600,000 D due to the limited availability of pullulan calibration standards.

The SCX GPC materials (PSS-MCX) were developed in specific pore sizes to meet the requirements of individual user needs. The porosity characteristics of PSS-MCX 100, 1000, 10^5, and 10^6 Å are presented here (Figure 1). From the calibration curves, the different porosities can be distinguished very clearly. The 100-Å material separates pullulans from 180 to 6000 D. This material has obvious advantages for applications in which a small exclusion limit and/or a large separation gel surface is important. The 1000-Å material separates the molecular weight range from 180 to 50,000 D. The separation volume is large, from V_e = 5.9 to 10.2 mL. The GPC calibration curve for small saccharides is very flat; thus, this column is very promising for GPC applications in which high chromatographic resolution for oligomers is needed. The 10^5-Å column separates molecular weights up to >1,600,000 D but has poor resolution for oligomers. The 10^6-Å column also separates molecular weights up to >1600,000 D with poor resolution for oligomers. From the shape of the 10^6-Å calibration curve, the authors conclude that the exclusion limit, and thus the pore size, is larger than for the PSS-MCX 10^5-Å material.

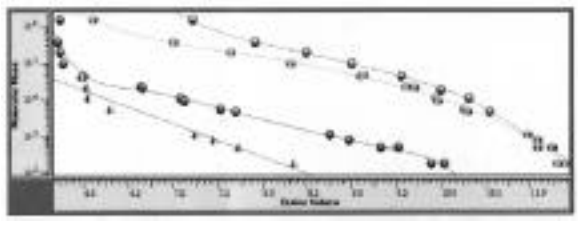

Figure 1 *Calibration curves of pullulans (180–1,600,000 D) obtained from PSS MCX GPC columns (8 × 300 mm). Eluent: 0.05% NaN_3 in H_2O, flow: 1 mL/min, T = 20 °C, 10^5 Å, 10^6 Å (upper curve).*

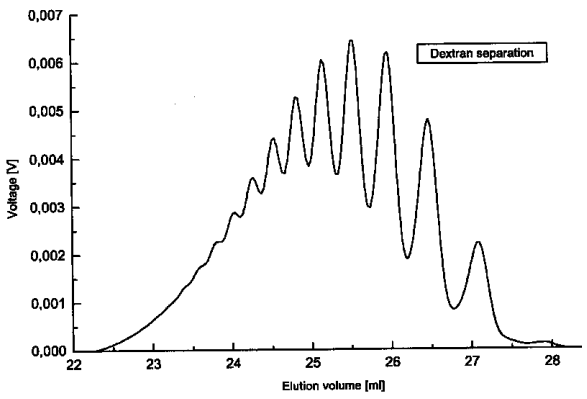

Figure 2 *Analysis of an oligomeric dextran mixture, GPC column combination: PSS MCX 1000 Å, 5 µm, 3× (8 × 300 mm). Eluent: 0.05% NaN_3 in H_2O, flow 0.5 mL/min, T = 80 °C, RI detection.*

When a large separation range is needed for the analysis of small and very large polysaccharides, a column combination of MCX 1000 Å and MCX 10^5 Å or a column combination of MCX 1000 Å with 10^5 Å and 10^6 Å is recommended. This combination allows the analysis of oligomeric and polymeric saccharides up to >1,600,000 D.[4] Of course, the very high resolution seen with the oligomeric saccharides will be reduced if large porous columns are combined with small porous columns. The reason for this concerns the smaller percentage of small pores in these GPC column combinations. A high percentage of smaller pores normally leads to high resolution for oligomeric analytes.

High-resolution oligosaccharide analysis

During the authors' experiments, the PSS-MCX 1000-Å, 5-µm 8 × 300 mm GPC column was tested for high-resolution oligosaccharide analysis. By combining three MCX 1000-Å, 5-µm 8 × 300 mm columns (thus 8 × 900 mm for the total separation) and using an eluent flow of 0.5 mL/min and a column temperature of 80 °C, they obtained the remarkable result shown in *Figure 2*.

The investigated oligosaccharide mixture (oligodextrane) was resolved up to dp 11 in 1 hr. Further experiments determined that oligo-dextrines gave a significantly higher oligomeric resolution than oligo-dextranes (the structural differences between the dextrines with different molecular weights are larger than between the dextranes). These results will be detailed in a later publication.

Several experiments were conducted for systematic optimization of flow and temperarure conditions on the oligomeric separation. The results are presented below.

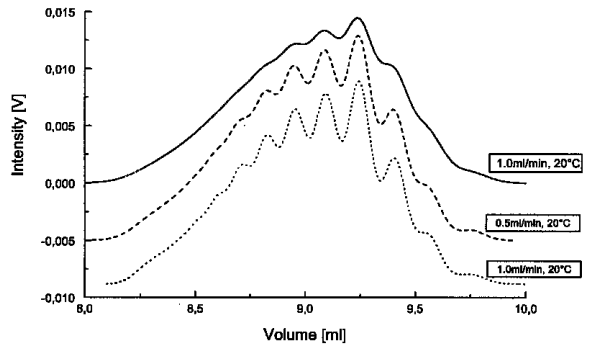

Figure 3 *Elution profiles of an oligomeric dextran mixture (180, 342, 504 D) at 20 °C. Flow rates: 1.0, 0.5, and 0.25 mL/min. GPC column: PSS MCX 1000 Å, 5 µm (8 × 300 mm). Eluent: 0.05% NaN_3 in H_2O, T = 20 °C, RI detection.*

van Deemter and temperature conditions

Flow rate and GPC column temperature typically have large influences on the quality and resolution of GPC separations. The quality of GPC separations is influenced primarily by diffusion processes. These processes have an influence on the analyte equilibrium adjustment between mobile phase and pore volume. Good equilibrium adjustment is important in order to obtain a good separation. To achieve good equilibrium, low viscosity of the solvent (heating of the GPC column) and low velocity of the analyte (lowering of the eluent flow rate) are both important. These influences were investigated using the example of an oligomeric dextran mixture that was measured under different conditions on PSS-MCX 1000-Å, 5-µm 8 × 300 mm GPC columns. Results from measurements performed at temperatures of 20 and 80 °C and at flow rates of 0.25, 0.5, and 1.0 mL/min are shown in *Figures 3* and *4*.

From these elution profiles, the authors concluded, as expected, that separation performance is increased by lowering the flow rate and increasing temperature. The influence of flow rates respective to flow velocities on the separation performance (expressed as *H*, theoretical plate height) is described using the van Deemter curve respective to the van Deemter equation:

$$H = A + B/v + C*v$$

The height, *H*, of a theoretical plate depends on

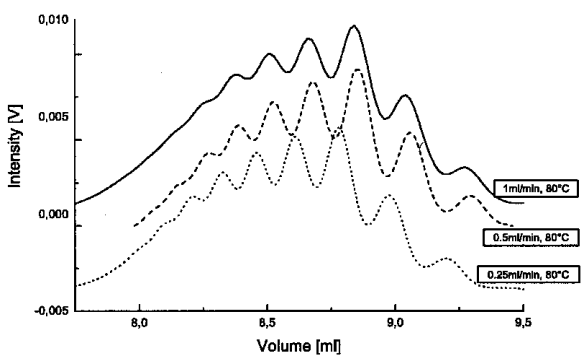

Figure 4 *Elution profiles of an oligomeric dextran mixture (180, 342, 504 D) at 0 °C. Flow rates: 1.0, 0.5, and 0.25 mL/min. GPC column: PSS MCX 1000 Å, 5 µm (8 × 300 mm). Eluent: 0.05% NaN_3 in H_2O, T = 80 °C, RI detection.*

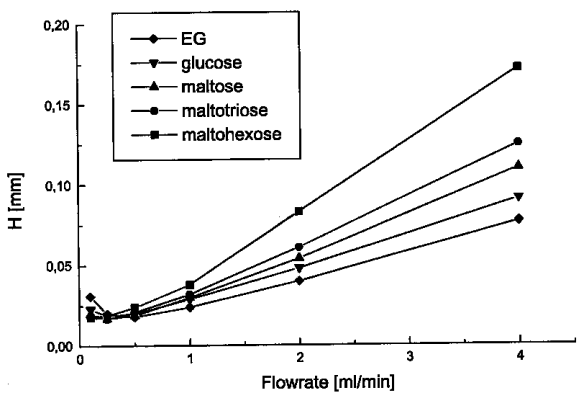

Figure 5 *H (u′) curve for different-sized sugars at T = 20 °C. PSS-MCX 1000 Å, 5 µm (8 × 300 mm). Eluent: 0.05% NaN_3 in H_2O.*

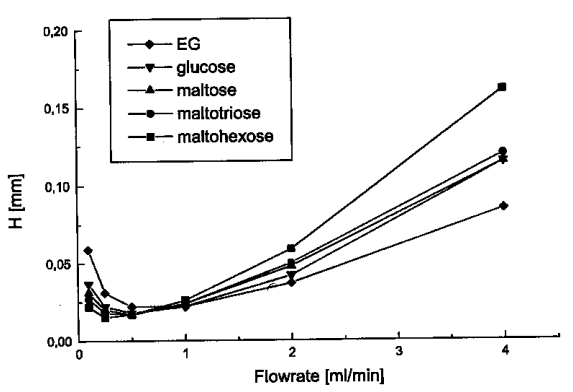

Figure 6 *H (u′) curve for different-sized sugars at T = 80 °C. PSS-MCX 1000 Å, 5 µm (8 × 300 mm). Eluent: 0.05% NaN_3 in H_2O.*

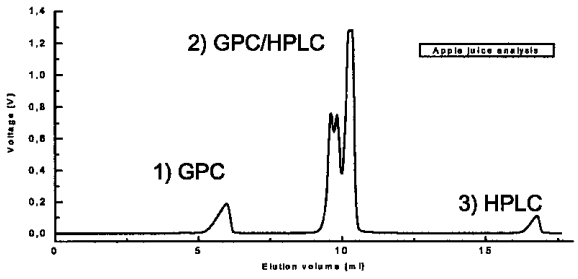

Figure 7 *Analysis of apple juice obtained from a PSS-MCX 1000 Å, 5 µm (8 × 300 mm) column. Eluent: 0.05% NaN_3 in H_2O, flow: 0.5 mL/min, T = 80 °C, RI detection: 1) Polymeric compounds (pectins), 2) sugars, 3) ethanol.*

v, the flow velocity, and A, B, and C, three constants associated with eddy diffusion (A), longitudinal diffusion (B), and mass transfer (C). The eddy diffusion is related to the different pathways that analytes use in the column packing due to the packing quality. The longitudinal diffusion is the diffusion of the analyte in the mobile phase. The mass transfer is the diffusion process of the analyte between the mobile phase and the pore volume. The eddy diffusion is independent from the flow velocity, *v*, while the longitudinal diffusion decreases with increasing *v* and mass transfer increases linearly with *v*. The van Deemter curve shows the influence of the sum of these three parameters. This curve shows the relationship of the theoretical plate height with the linear flow velocity. The plate height minimum shows the conditions for the best column performance. These curves were measured with MCX 1000-Å, 5-µm columns for different-sized analytes.

To calculate the flow velocity (mm/min) from the flow rate (mL/min) with MCX 1000-Å, 5-µm 8 × 300 mm columns, the flow rate needs to be multiplied by factor 0.515. At 20 °C, the plate height minimum is achieved for most of the investigated samples at a flow rate of 0.25 mL/min (*Figure 5*). At flow rates of 0.5 mL/min and higher, the plate heights increase. Increasing the temperature to 80 °C shifts the minimum plate height, and thus the best performance, to a flow rate of 0.5 mL/min. Furthermore, the minimum plate heights for separations at 20 °C and 0.25 mL/min are similar to the minimum plate heights achieved at 80 °C and 0.5 mL/min (*Figure 6*). This means that increasing the temperature from 20 °C to 80 °C allows an increase in flow rate at optimum flow conditions by a factor of 2 (0.5 instead of 0.25 mL/min). This reduces the separation time by 50% while maintaining high resolution.

If a higher resolution is needed, it can be achieved using a combination of three GPC columns instead of using one. This result was shown in Figure 2.

Combined HPLC and GPC analysis

In the case of very different molecules (differences in hydrodynamic size and large differences in lipophilicity), GPC and HPLC analysis can be performed in only one run. *Figure 7* shows the analysis of an apple juice. The GPC section shows a peak at 6 mL from the polymeric pectin and peaks at 10 mL from the mono- and disaccharides. After 10 mL, the HPLC section begins, and at 17 mL elution volume the ethanol peak occurs.

Conclusion

SCX columns were investigated for high-resolution oligosaccharide analysis. Within 1 hr, high-resolution GPC separations for oligomeric dextranes were obtained. The influence of temperature and flow rate on the separation behavior of oligosaccharides has been shown. In some cases (e.g., apple juice), combined GPC and HPLC analysis of analytes can be used.

References

1. Kato Y, O'Donnell JK, Fischer J. In: Wu C-S. ed. Column handbook for size exclusion chromatography. San Diego: Academic Press, 1999: 112.
2. Huber A, Praznikin W. In: Wu C-S. ed. Column handbook for size exclusion chromatography. San Diego: Academic Press, 1999: 459–97.
3. Williams A, Hagel L. In: Wu C-S. ed. Column handbook for size exclusion chromatography. San Diego: Academic Press, 1999: 27–74.
4. Dauwe C, Reinhold G. Sugar Industry 2000; 125:508–14.

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