

Improved Neutral Oligosaccharide Separation on the CarboPac PA200 Column with NaOH Concentration Adjustment



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INTRODUCTION

Analysis of the glycosylation patterns of glycoprotein therapeutics (such as antibodies) is of great importance, as their glycans are essential to the functions of these glycoproteins. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a powerful technique that delivers high-resolution carbohydrate separations and sensitive detection without sample labeling. CarboPac[®] columns are a family of strong anion-exchange columns developed by Dionex Corporation for HPAE-PAD. These columns separate mono- and disaccharides, sugar alcohols, sugar acids including sialic acids, and oligosaccharides, including homopolymer oligosaccharides that differ only in length.^{1,2} The CarboPac PA200 column, the newest member of the CarboPac column family, is designed for high-resolution separations of oligosaccharides.³ Here, the authors demonstrate method development on this column for oligosaccharide separations. HPAE-PAD separations of oligosaccharides typically use eluents consisting of a fixed concentration of NaOH and a gradient of sodium acetate. The authors investigated adjusting the NaOH concentration from the typical value of 100 or 150 mM to improve resolution of neutral *N*-linked oligosaccharides. Reducing the NaOH concentration and optimizing the sodium acetate gradient resulted in improved profiling of *N*-glycans from glycoproteins, including monoclonal and polyclonal antibodies. The optimized gradient separates two fucosylated Man₃GlcNAc₄Gal position isomers commonly found on monoclonal antibodies that have not been separated by any published HPLC method.

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EXPERIMENTAL CONDITIONS

Chemicals

Oligosaccharide standards (Dextra labs)

PNGase F, β 1-4 Galactosidase (New England Biolabs)

Deionized water, Type 1 reagent grade, 18.2 M Ω cm resistivity

Sodium acetate, HPLC grade (Dionex, P/N 053926)

Sodium hydroxide, 50% (w/w) (Fisher Chemicals, P/N SS254-500)

Human IgG (Sigma)

Monoclonal antibody (gift from a biotechnology company)

N-glycan samples (gift from a biotechnology company)

Instrument

Analyses were performed on a Dionex ICS-3000 chromatography system, which includes an ICS-3000 DP gradient pump, AS autosampler, and ICS-3000 DC column compartment with an electrochemical cell. The carbohydrates were detected by pulsed amperometry with a gold electrode and an Ag/AgCl reference electrode using the standard 4-potential waveform developed at Dionex (Dionex Technical Note 21).

Chromatography was controlled by Chromeleon[®] Chromatography Data System software (Dionex Corporation).

Eluents were prepared by following the recommendations in Dionex Technical Note 71.

Columns

CarboPac PA200 analytical column (3 \times 250 mm, Dionex, P/N 062896)

CarboPac PA200 guard column (3 \times 50 mm, Dionex, P/N 062895)

RESULTS

A typical gradient for oligosaccharide separations on the CarboPac PA200 is from 10 or 20 mM up to 200 mM NaOAc in 100 or 150 mM NaOH. This is effective for separating sialylated *N*-linked glycans. However, some glyco-biologists have observed that these conditions are not always effective for neutral *N*-linked glycan separations. Here, the authors show that decreasing the NaOH concentration can greatly improve the resolution of the neutral *N*-linked oligosaccharides. Six neutral and two sialylated *N*-linked oligosaccharide standards were used in this study (Table 1). These eight oligosaccharides are typically found on mono- and polyclonal antibodies and other glycoproteins. Figure 1 shows that the six neutral oligosaccharides are well-resolved with 40–60 mM NaOH.

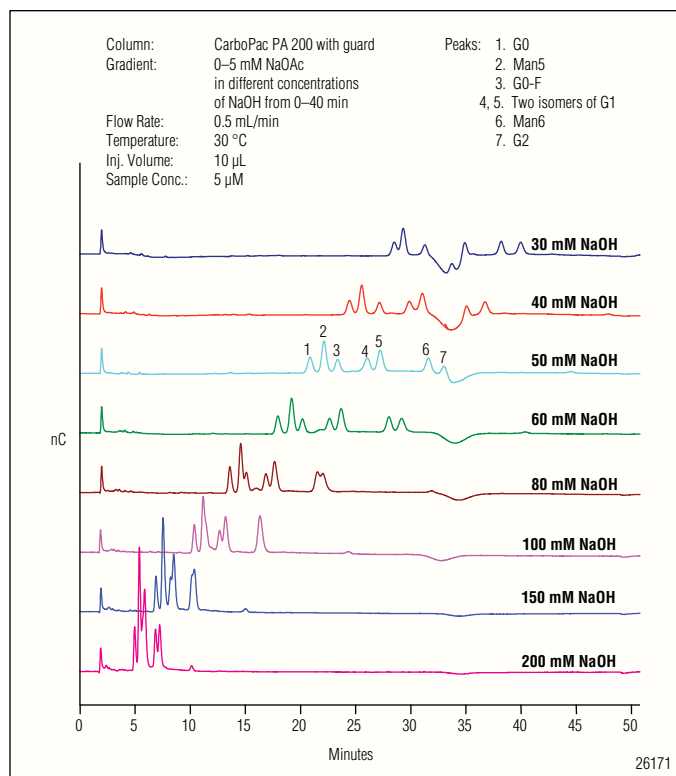


Figure 1. Effect of NaOH concentration on the separation of neutral oligosaccharides on the CarboPac PA200 column.

Standard	Standard			
	Mannose	Galactose	<i>N</i> -Acetylglucosamine	Sialic acid Fucose
Man 5				
Man 6				
G0-F				
G2				
G1				
G0				
A1				
A2				

Table 1. Structures of the oligosaccharide standards used in this study.

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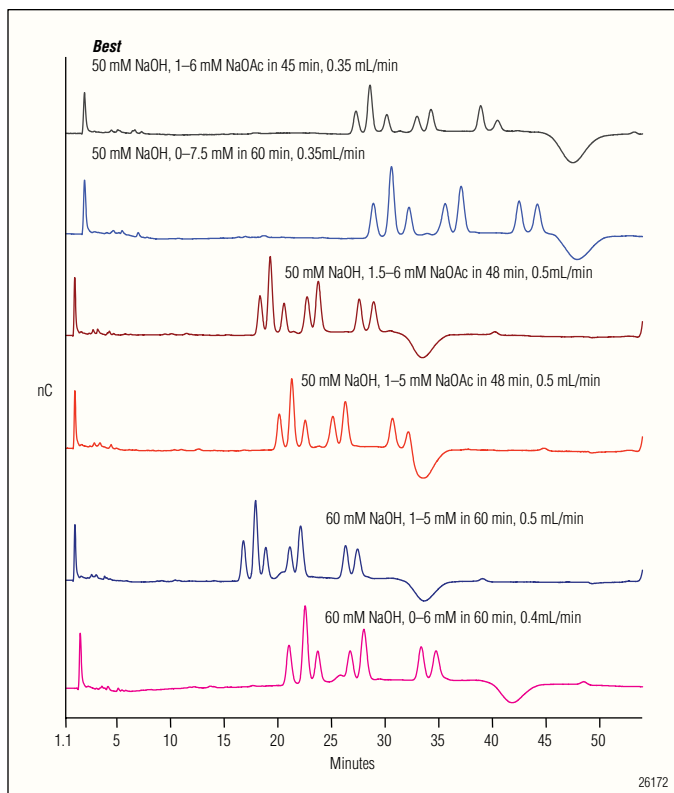


Figure 2. Optimization of the gradient for neutral oligosaccharide separation on the CarboPac PA200 column.

Under certain conditions, the oxygen dip (~34 min in Figure 1) interfered with the peak detection. The dip in the baseline is due to the dissolved oxygen introduced into the column upon sample injection being greater than the oxygen concentration of the eluent. The intensity of the dip is 1–2 nC and usually negligible. It can be minimized by setting a >20 μ L cut volume in the injection command. Here, the authors adjusted the gradient and flow rate to move the dip away from the sample peaks. The gradient of 1–6 mM NaOAc in 50 mM NaOH from 0–45 min at 0.35 mL/min were the best conditions, keeping the oxygen dip far enough away from the sample peaks while maintaining high resolution for the oligosaccharides (Figure 2).

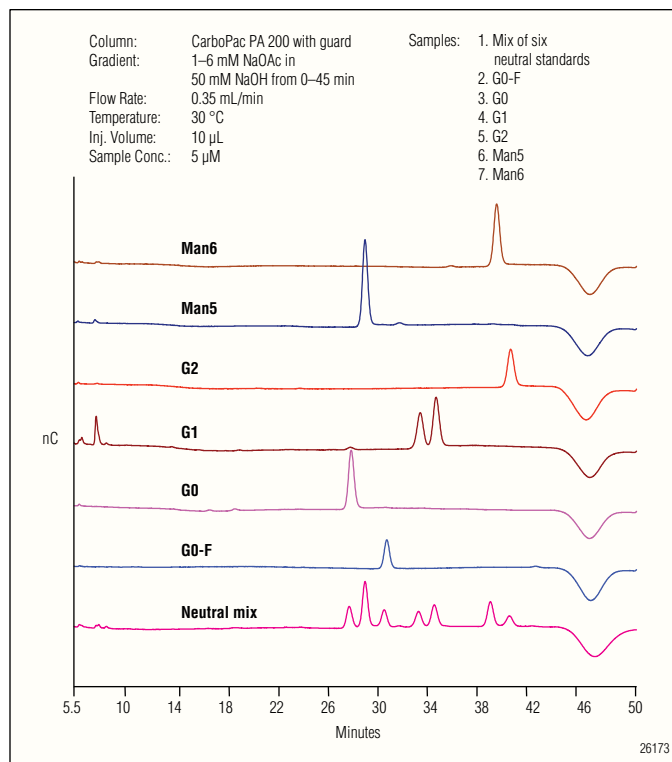


Figure 3. Analysis of the neutral oligosaccharide standards with the optimized gradient.

Each of the neutral standards was eluted with the optimized gradient and compared with the profile of the neutral standard mixture (Figure 3). The G1 standard showed two peaks, and they are probably the two position isomers of G1. To confirm the identities of these two peaks, G1 was incubated with β 1-4 galactosidase for 24 h. After the digestion, only a minor peak appeared at one of the two original peaks' position, and a peak eluted at the same time as the G0 standard. These results show that the two original G1 peaks had terminal galactose and after the galactose cleavage both were converted to G0. These elutions were performed with a faster gradient instead of the optimized gradient, as the oxygen dip did not interfere with the G0 and G1 peaks using the faster gradient.

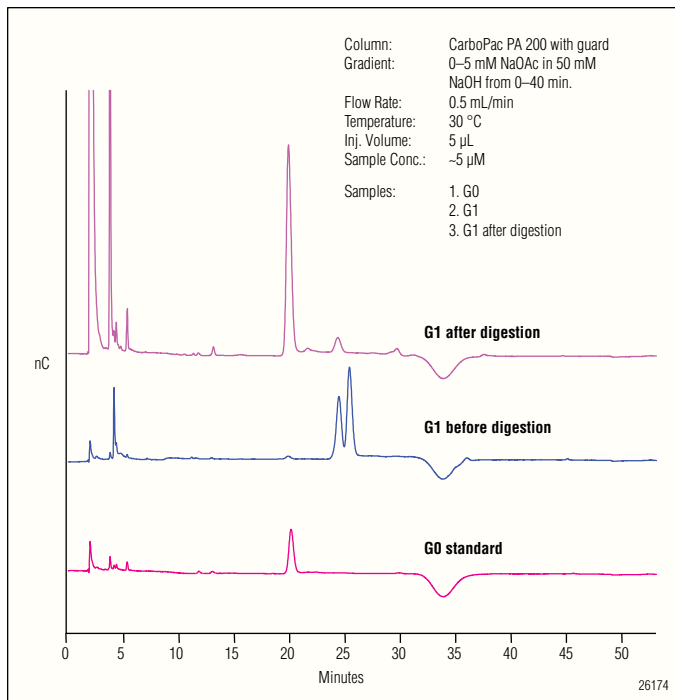


Figure 4. Elution of G1 before and after digestion with galactosidase.

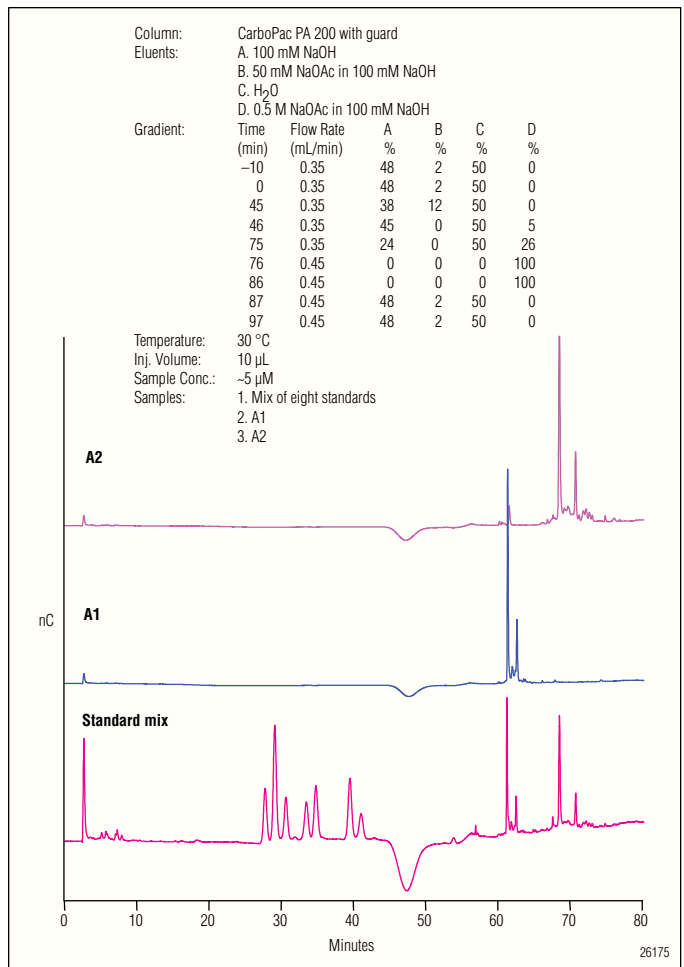


Figure 5. Extended gradient to include separation of sialylated oligosaccharides.

The optimized gradient can also be extended to include elution with higher NaOAc concentrations, so that sialylated oligosaccharides can also be separated in the same injection. As Figure 5 shows, neutral standards and two sialylated standards (A1 and A2) can be resolved in one run.

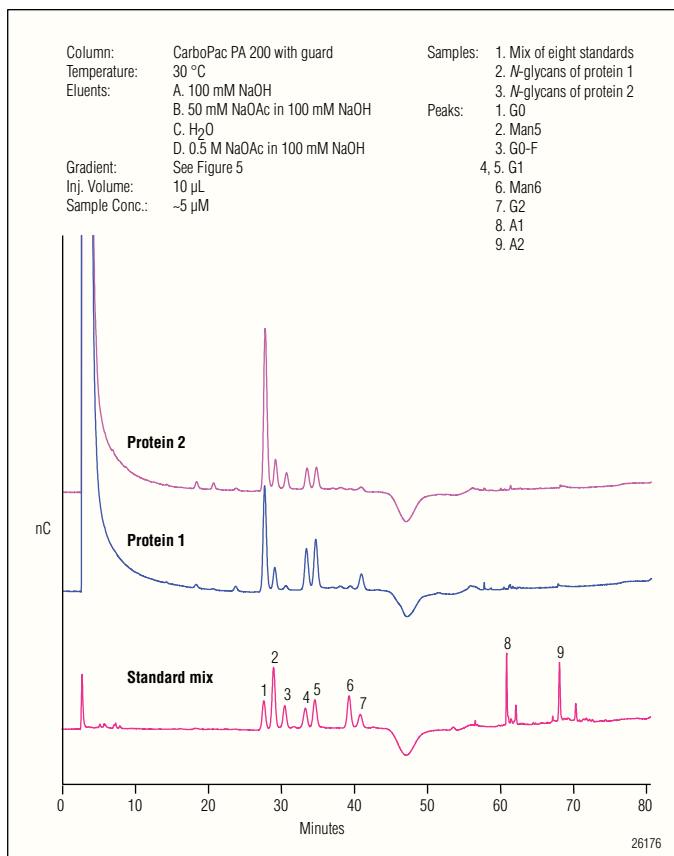


Figure 6. Profiling of *N*-linked oligosaccharides released from two glycoproteins.

N-linked glycans were cleaved from two glycoproteins with PNGase F. After overnight digestion, protein was removed by cold ethanol precipitation. The released *N*-linked glycans were vacuum dried and then suspended in deionized water before separation using a CarboPac PA200 column with the optimized gradient. The major species were well resolved and their retention times matched those found in the standard mix (Figure 6).

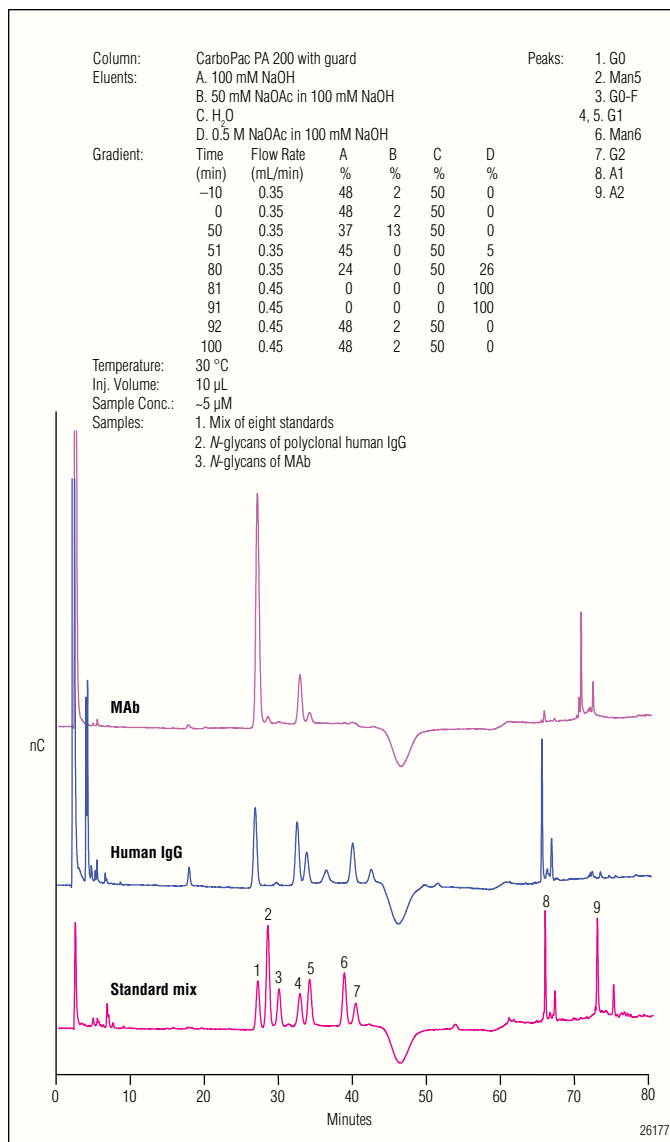


Figure 7. Profiling of *N*-linked oligosaccharides released from polyclonal human IgG and a MAb.

Commercially available polyclonal antibody (human IgG) and a monoclonal antibody (MAb) were also treated with PNGase F. After the digestion, the samples were diluted with water and injected directly to the column. Standards were dissolved in the same final buffer and injected to compare with the profiles of these samples. The polyclonal human IgG sample had more types of neutral glycans than the Mab sample, which contained mostly G0, and some G1. The human IgG had A1 glycans but little A2, while the Mab had more A2.

CONCLUSIONS

The resolution of neutral *N*-linked oligosaccharides on the CarboPac PA200 column is greatly improved by decreasing the concentration NaOH in the eluent. When optimizing oligosaccharide separations on the CarboPac columns, NaOH concentration is an important factor to consider.

An efficient gradient was developed for high-resolution profiling of neutral and sialylated *N*-glycans on the CarboPac PA200 column. The two G1 position isomers were also well-resolved with this gradient. This method is useful in the biotechnology and pharmaceutical industries for analysis of glycans released from glycoprotein therapeutics, especially monoclonal antibodies and other glycoproteins containing a significant number of neutral *N*-linked oligosaccharides.

REFERENCES

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