Separation of Asparagine-Linked (*N*-Linked) Oligosaccharides from Human Polyclonal IgG Using the CarboPac PA200 Column

INTRODUCTION

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a widely used technique for determining an extensive set of carbohydrates including, but not limited to, monosaccharides, disaccharides, oligosaccharides, smaller polysaccharides, sugar acids, such as sialic acids, sugar alcohols, sugar phosphates, and sugar nucleotides. One of the first published applications of HPAE-PAD was the separation of *N*-linked oligosaccharides released from mammalian glycoproteins. These early separations used the CarboPac® PA1 column.[1] In 1992 Dionex introduced the CarboPac PA100 to improve oligosaccharide resolution. The improved resolution was mainly the result of increased peak efficiency. Dionex Application Note 67[2] shows the improved efficiency of the PA100 relative to the PA1 by making a direct comparison of separations of neutral linear oligosaccharides with the two columns. Since its introduction, the PA100 has become the standard for neutral and charged oligosaccharide separations. Dionex Technical Note 42[3] demonstrates the separation of sialylated *N*-linked oligosaccharides using the PA100.

The importance of characterizing the *N*-linked oligosaccharides from recombinant glycoproteins used as therapeutics and other applications necessitated even more oligosaccharide peak resolution.[4] To meet this demand, Dionex introduced the CarboPac PA200 column. The PA200 is a pellicular anion-exchange column that mostly differs from the PA100 by a reduction in the resin bead size from 8.5 to 5.5 µm and a decrease in the latex bead size from 275 nm to 43 nm. This resin is packed in a 3 × 250 mm column body rather than the standard 4 × 250 mm format. In addition to increased resolution, this column also reduces eluent consumption and waste generation (optimum flow rate is 0.5 mL/min compared to 1.0 mL/min for the PA100), and requires less acetate to elute a given oligosaccharide compared to the PA100, allowing faster separations and making subsequent acetate removal a bit easier. Dionex Application Update 150[5] shows the benefits of higher resolution and faster separations using the PA200 for the same maltodextrins separated with the PA1 and PA100 in Application Note 67.

This application note shows how the PA200 improves *N*-linked oligosaccharide separations. The PA200 is used to profile *N*-linked oligosaccharides released from human polyclonal IgG by the enzyme PNGase F or endoglycosidase H (Endo H). Human polyclonal IgG has a mixture of neutral and charged *N*-linked oligosaccharides that is generally more complex than the set of *N*-linked oligosaccharides found on a typical monoclonal IgG. This note also shows how subsequent exoglycosidase digestions can be used to assist in understanding and identifying oligosaccharide structure. The CarboPac PA200 is the new standard for achieving high resolution HPAE-PAD *N*-linked oligosaccharide separations and delivers the resolution necessary to ensure that the *N*-linked oligosaccharides from one lot of a recombinant glycoprotein are comparable to the *N*-linked oligosaccharides from a second lot.

EQUIPMENT

ICS-3000 chromatography system consisting of:

- SP single gradient pump module
- DC detector and chromatography module with single or dual heating zone and 6-port injection valve
- ED electrochemical detector equipped with cell containing a disposable Au working electrode and a combination pH–Ag/AgCl reference electrode
- AS Autosampler with Sample Tray Temperature Controlling option, and 1.5 mL sample tray

Chromeleon® 6.8 Chromatography Workstation

Centrifuge (Eppendorf® 5400 series)

SpeedVac™ evaporator

Heated water bath

Vacuum pump (for eluent preparation)

*This application can also be performed on older Dionex systems equipped for HPAE-PAD.

REAGENTS AND STANDARDS

Deionized water, Type 1 reagent-grade, 18.2 MΩ-cm resistivity

Sodium acetate, HPLC grade (CH_3COONa, Aldrich, P/N 71185; or Dionex, P/N 059326)

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

Glacial acetic acid (ACS Grade or better)

Neuraminidase, recombinant (cloned from *Clostridium perfringens*) (New England BioLabs) (P/N P0720S)

PNGase F, glycerol-free (New England BioLabs)

β-Galactosidase (*S. pneumoniae*)—(Oxford Glycosystems—currently available from other sources)

N-acetylneuraminic acid (Neu5Ac) (Ferro Pfanstiehl Laboratories—see Dionex Technical Note 41)

N-glycolylneuraminic acid (Neu5Gc) (Ferro Pfanstiehl Laboratories—see Dionex Technical Note 41)

OligoStandard™ Sialylated *N*-Linked Alditols (Dionex, P/N 043064)

Sterile assembled micro-centrifuge tubes with screw cap, 1.5 mL (Sarstedt 72.692.005)

Filter unit, 0.2 µm nylon (Nalgene® Media-Plus with 90 mm filter, Nalge Nunc International, P/N 164-0020) or equivalent nylon filter for eluent preparation.

1.5 mL polypropylene autosampler vials, with caps and slit septa (Dionex vial kit P/N 061696)

SAMPLE

Human Polyclonal IgG (Sigma-Aldrich)

CONDITIONS

Column:	CarboPac PA200 Analytical, 3 × 250 mm (P/N 062896) CarboPac PA200 Guard, 3 × 50 mm (P/N 062895)
Eluents:	A: 100mM Sodium hydroxide B: 100 mM Sodium hydroxide, 0.5 M sodium acetate
Method:	0–5 min 99% A, 1% B 5–60 min 1 to 36% B
Flow Rate:	0.5 mL/min
Column Temperature:	30 ºC
AS Tray Temperature:	15 ºC
Inj. Volume:	9 µL
Inj. Loop:	10 µL
Detection:	PAD, conventional or disposable Au WE
Waveform:	See Table 1.
Run Time:	75 min (return to initial conditions for 15 min prior to injection).

Table 1. Waveform A, Four-Potential Carbohydrate Waveform[6]				
Time (sec)	Potential (V) (Ag/AgCl reference)	Gain*	Ramp*	Integration
0.00	+ 0.1	Off	On	Off
0.20	+ 0.1	On	On	On
0.40	+ 0.1	Off	On	Off
0.41	- 2.0	Off	On	Off
0.42	- 2.0	Off	On	Off
0.43	+ 0.6	Off	On	Off
0.44	- 0.1	Off	On	Off
0.50	- 0.1	Off	On	Off

*These parameters are not used on older model Dionex chromatography systems.

PREPARATION OF SOLUTIONS AND REAGENTS

100 mM Sodium Hydroxide (Eluent A)

It is essential to use high-quality water of high resistivity (18 MΩ-cm or better) that contains as little dissolved carbon dioxide as possible. Biological contamination should be absent. Dilute 10.4 mL of a 50% (w/w) sodium hydroxide solution into 2 L of water to prepare a 0.1 M sodium hydroxide solution. After preparation, keep the eluent blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times. See Dionex Technical Note 71 for more details on eluent preparation for HPAE-PAD carbohydrate analysis.[7]

100 mM Sodium Hydroxide/1 M Sodium Acetate (Eluent B)

Measure approximately 800 mL of water into a 1 L graduated cylinder. Add a stir bar and begin stirring. Weigh out 82.0 g of anhydrous, crystalline sodium acetate. Add the solid sodium acetate steadily to the briskly stirring water to avoid the formation of clumps, which are slow to dissolve. After the salt dissolves, remove the stir bar with a magnetic retriever. Using a plastic pipette, measure 5.2 mL of 50% (w/w) sodium hydroxide and add it to the acetate solution. Rinse the pipette by drawing up the acetate solution into the pipette and dispensing it back into the graduated cylinder several times. Add water to the solution to reach a final level of 1000 mL. Replace the stir bar and stir briefly to mix. Vacuum filter through a 0.2 µm nylon filter. This may be slow, as the filter may clog with insolubles from the sodium acetate. After preparation, keep this eluent blanketed under helium at 34 to 55 kPa (5 to 8 psi) at all times. See Dionex Technical Note 71 for more details on eluent preparation for HPAE-PAD carbohydrate analysis.[7]

25 mM Sodium Acetate Buffer pH 5.5

Prepare 200 mL of 0.025 M sodium acetate (0.41 g anhydrous sodium acetate dissolved in 200 mL water) and 200 mL 25 mM acetic acid (0.29 mL glacial acetic acid (17.4 M) added to 150 mL water and brought to a final volume of 200 mL). Prepare the pH 5.5 buffer by combining 89 mL of the 0.025 M sodium acetate and 11 mL of the 0.025M acetic acid.

PREPARATION OF SAMPLES

Human Polyclonal IgG *N*-linked Oligosaccharides

200 µL of 10 mg/mL human polyclonal IgG (in water) was treated with 20 µL 10X G7 buffer (included with the PNGase F purchase) and 20 µL PNGase F, which had been diluted 1:100 with water. This sample was incubated at 37 °C for 20 h, microcentrifuged, and the supernatant analyzed directly.

Desialylated Human Polyclonal IgG *N*-linked Oligosaccharides

20 µL of the PNGase F-digested IgG sample above was treated with 20 µL 25 mM sodium acetate buffer pH 5.5, and 2 µL neuraminidase. This sample was incubated at 37 °C for 20 h and the supernatant analyzed directly. Note: The sodium citrate buffer supplied with the enzyme was not used; instead, the above sodium acetate buffer was used.

Desialylated Human Polyclonal IgG *N*-linked Oligosaccharides and Degalactosylated Human Polyclonal IgG *N*-linked Oligosaccharides

20 µL of the PNGase F and neuraminidase-digested IgG sample and 40 µL of the PNGase F-digested IgG sample were treated with 5 µL and 10 µL of a 1 mU/µL galactosidase solution (vial containing 40 mU of lyophilized product was reconstituted in 40 µL 25 mM sodium acetate buffer pH 5.5), respectively. These samples were incubated at 37 °C for 25 h and the supernatants analyzed directly.

RESULTS AND DISCUSSION:

Figure 1 shows a comparison of the asparagine-linked (*N*-linked) oligosaccharides released from bovine fetuin, reduced to form the oligosaccharide alditols, and separated on the CarboPac PA100 and CarboPac PA200 columns. The smaller bead size of the PA200 resin results in more efficient peaks and therefore better oligosaccharide resolution compared to the PA100 column. The oligosaccharides are also eluted with less sodium acetate and due to the lower flow rate (0.5 mL/min rather than 1.0 mL/min) the efficiency of online desalting with carbohydrate membrane desalter should be improved. There is less eluent used and less waste generated. We have observed the same resolution improvement for *N*-linked oligosaccharides released from human transferrin, human alpha-1-acid glycoprotein, and bovine ribonuclease B.

The improvement in oligosaccharide resolution is more dramatic when the PA100 and PA200 separations of *N*-linked oligosaccharides released from human polyclonal IgG are compared (Figure 2). The improved resolution of the oligosaccharides between 5 and 15 min is especially beneficial for the oligosaccharides released from recombinant monoclonal antibodies, which have less heterogeneity than human polyclonal IgG.[8] The majority of the *N*-linked oligosaccharides from human IgG are biantennary.

HPAE-PAD used in combination with exoglycosidases can yield information about oligosaccharide structure (Figure 3). Treating the PNGase F digest of human polyclonal IgG with neuraminidase reveals that the later eluting peaks (18–22 min) contained Neu5Ac. The retention times of those peaks suggest they each contained a single Neu5Ac. The disappearance of small peaks between 27 and 29 min suggest that they are disialylated oligosaccharides. Chromatography of a standard mixture containing *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) shows that the only sialic acid released from these oligosaccharides is Neu5Ac, as is expected from a glycoprotein of human origin. A recombinant glycoprotein expressed in Chinese hamster ovary cells or a glycoprotein from another mammal may contain Neu5Gc.

Among the *N*-linked oligosaccharides known to occur on human IgG are those that have branches that terminate in a β-linked galactose. We treated the PNGase F digest of human polyclonal IgG with β-galactosidase to reveal these structures. Figure 4 shows that many of the neutral *N*-linked oligosaccharides of human IgG have at least one terminal galactose. This also suggests that the two major monosialylated oligosaccharides are biantennary,

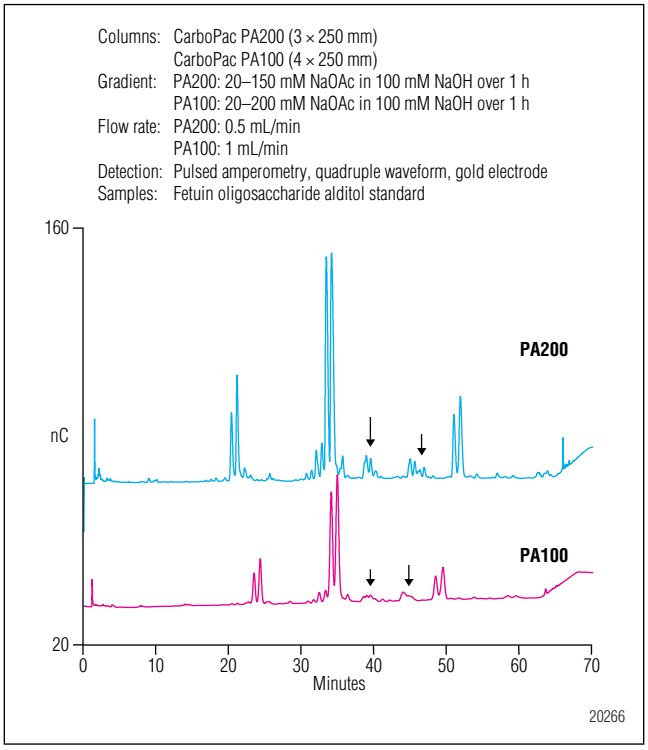

Figure 1. Fetuin oligosaccharide profiles: CarboPac PA200 vs CarboPac100 column.

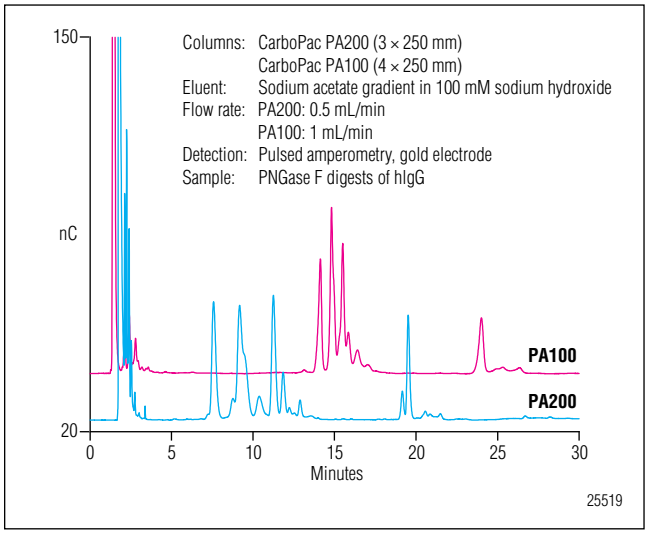

Figure 2. Human polyclonal lgG N*-linked oligosaccharides CarboPac PA200 vs CarbPac PA100 column.*

where one oligosaccharide has one branch with a terminal *N*-acetylglucosamine and the other branch with a terminal Neu5Ac (peak 8), and the second oligosaccharide has terminal galactose and terminal Neu5Ac on its two branches (peak 9). Other studies suggest that both structures fucose linked to the reducing terminal *N*-acetylglucosamine in an α1,6 linkage ("core fucose").[8] An oligosaccharide with core fucose will elute earlier than the same oligosaccharide without core fucose.[9] Peak 2, eluting just after the main peak 1, in the degalactosylated sample may be the defucosylated version of the main peak. Treatment with an α-fucosidase either before or after galactosidase treatment should reveal the oligosaccharides containing fucose.

If we first treat the PNGase F digest of human polyclonal IgG with neuraminidase and then treat it with β-galactosidase (Figure 5), we get a similar result as Figure 4 with the notable absence of the sialylated oligosaccharides. Peaks 1 and 2 are undoubtedly the same oligosaccharides as peaks 1 and 2 in Figure 4. Either peak 3 or 4 is Neu5Ac. After treatment with the neuramindase, there are a number of neutral oligosaccharides eluting close together and some partial co-elutions. The released Neu5Ac elutes in the same region. Although resolution of this region of the chromatogram is improved compared to the CarboPac PA100, it is possible that increasing the sodium hydroxide concentration from 100 to 250 mM could offer further improvement.[10]

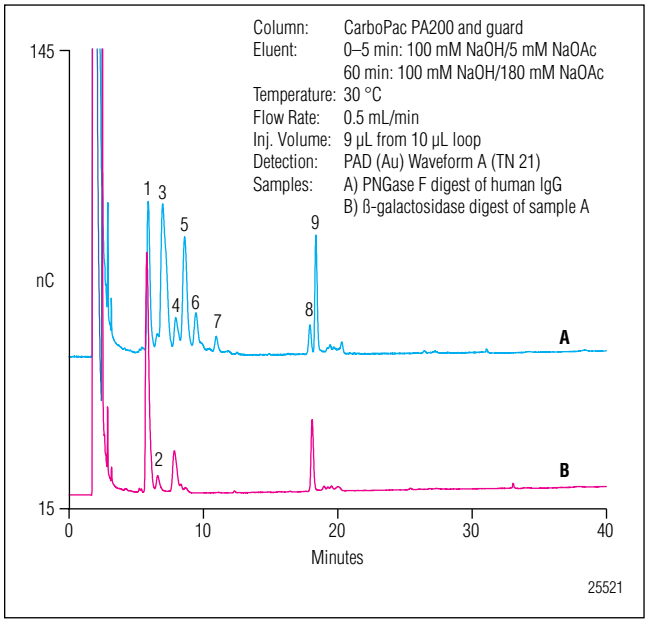

Figure 4. Degalactosylated N*-linked oligosaccharides from human polyclonal lgG separated on a CarboPac PA200 column.*

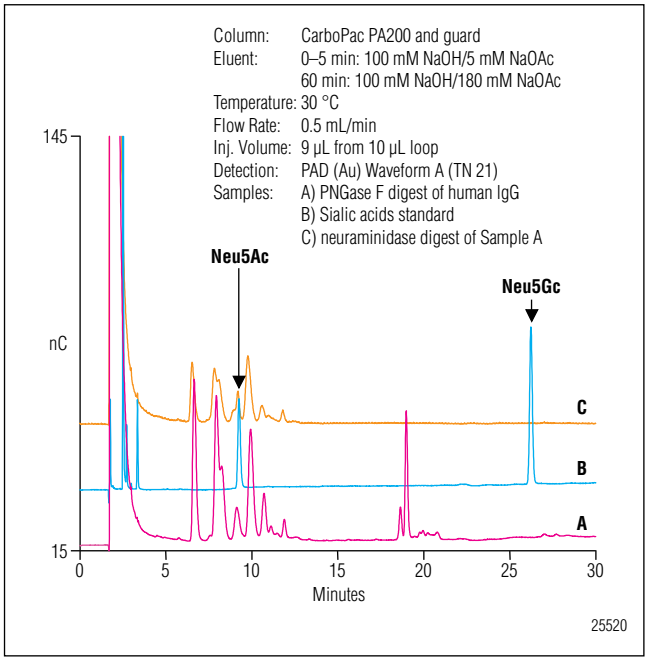

Figure 3. Monitoring release of sialic acids from human polyclonal lgG N*-linked oligosaccharides by HPAE-PAD.*

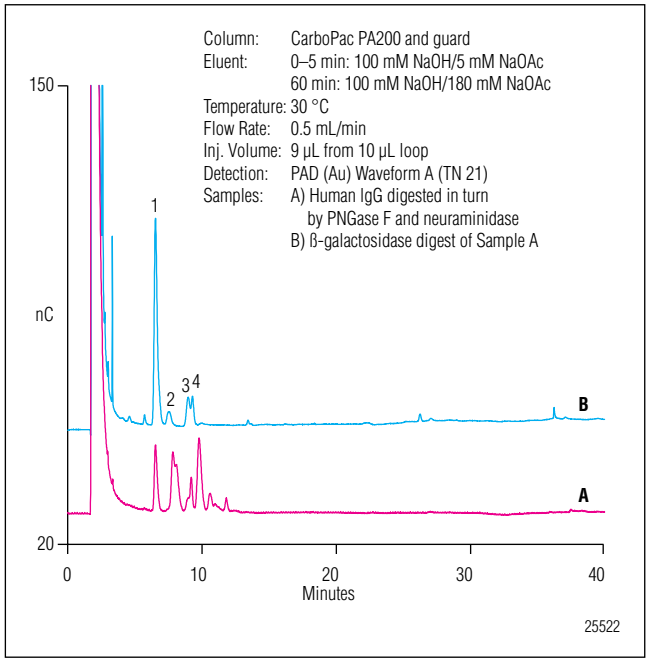

Figure 5. Desialylated and degalactosylated N*-linked oligosaccharides from human polyclonal lgG separated on a CarboPac PA200 column.*

CONCLUSION

The smaller bead size of the CarboPac PA200 column improves resolution of closely-eluting oligosaccharides. The improved resolution is beneficial for characterization of a variety of *N*-linked oligosaccharides, especially those released from recombinant monoclonal antibodies. Further optimization of hydroxide eluent concentration may improve resolution further. The smaller column diameter of the PA200 reduces eluent consumption and waste generation while improving online desalting.

PRECAUTIONS

PNGase F is sold in glycerol-containing and glycerol-free preparations. For HPAE-PAD glycerol-free preparations are recommended. Figure 6 shows chromatograms of the N-linked oligosaccharides released from human polyclonal IgG using PNGase F with and without glycerol. The peaks from the glycerol-containing PNGase F treatment elute a bit earlier than the same peaks from the glycerol-free PNGase F treatment, and the peaks are less efficient. This suggests that the glycerol overloads the column slightly.

Enzyme digests are performed in salt-containing buffers (e.g. the neuraminidase digest was executed in sodium acetate buffer). Injecting too large a volume of these digests will overload the column with the buffer anion, altering the chromatography. A slight column overload will be similar to the effect shown with the glycerol-containing PNGase F. For most buffers and samples, injection volumes under 50 µL should yield good chromatography. Injection volumes over 50 µL should be tested. Tests can use a Neu5Ac standard or similarly inexpensive compound rather than valuable sample or a more costly oligosaccharide standard.

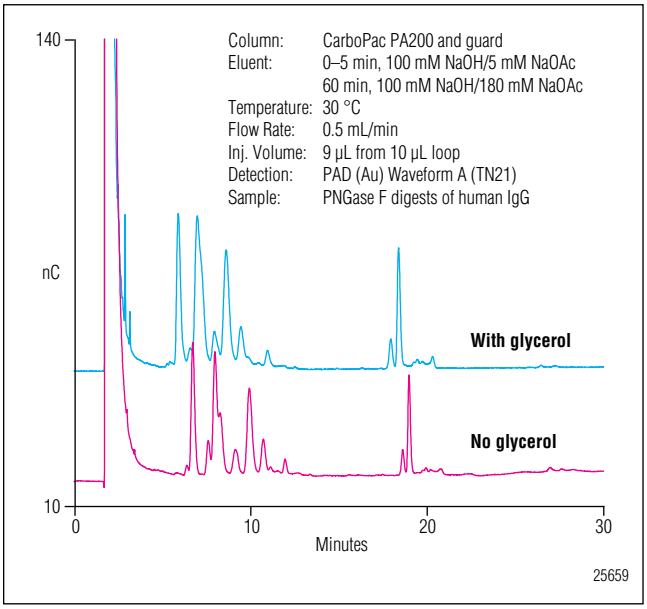

Figure 6. Effect of Glycerol on HPAE-PAD of human polyclonal IgG N-*linked oligosaccharides.*

REFERENCES

1. Hardy, M.R.; Townsend, R.R. Separation of Positional Isomers of Oligosaccharides and Glycopeptides by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection *Proc. Natl. Acad. Sci. USA* **1988,** *85,* 3289–3293.
2. Dionex Corporation. *Determination of Plant-Derived Neutral Oligo- and Polysaccharides;* Application Note 67, LPN 1474: Sunnyvale, CA, 2003.
3 Dionex Corporation. *Glycoprotein Oligosaccharide Analysis Using High-Performance Anion-Exchange Chromatography;* Technical Note 42, LPN 0932: Sunnyvale, CA, 1997.
4. Hardy, M. R.; Rohrer, J. S. High-pH Anion-Exchange Chromatography (HPAEC) and Pulsed Amperometric Detection (PAD) for Carbohydrate Analysis. In *Comprehensive Glycoscience*, Vol. 2; J.P. Kamerling, Ed.; Elsevier: Amsterdam, The Netherlands, 2007; pp 303–327.
5. Dionex Corporation. *Determination of Plant-Derived Neutral Oligo- and Polysaccharides Using the CarboPac PA200;* Application Update 150, LPN 1779: Sunnyvale, CA, 2005.
6. Dionex Corporation. *Optimal Settings for Pulsed Amperometric Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector;* Technical Note 21, LPN 034889-03: Sunnyvale, CA, 1998.
7. Dionex Corporation. *Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection;* Technical Note 71, LPN 1932-01: Sunnyvale, CA, 2007.
8. Weitzhandler, M.; Hardy, M.; Co, M.S.; Avdalovic, N. Analysis of Carbohydrates on IgG Preparations. *J. Pharm. Sci.* **1994,** *83,* 1670–1675.
9. Rohrer, J.S. Separation of Asparagine-Linked Oligosaccharides by High-pH Anion-Exchange Chromatography with Pulsed Amperometric Detection: Empirical Relationships Between Oligosaccharide Structure and Chromatographic Retention. *Glycobiology* **1995,** *5,* 359–360.
10. Cooper, G.A.; Rohrer, J.S. Separation of Neutral Asparagine-Linked Oligosaccharides by High-pH Anion-Exchange Chromatography with Pulsed Amperometric Detection. *Anal. Biochem.* **1995,** *226,* 182–184.

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S. (847) 295-7500
Canada (905) 844-9650

South America
Brazil (55) 11 3731 5140

Europe
Austria (43) 1 616 51 25 Benelux (31) 20 683 9768 (32) 3 353 4294
Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Sweden (46) 8 473 3380
Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

Asia Pacific
Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190
Taiwan (886) 2 8751 6655

www.dionex.com

LPN 2141 PDF 08/16