Analysis of Exoglycosidase Digestions of *N*-Linked Oligosaccharides Using HPAE-PAD

INTRODUCTION

Analysis of protein glycosylation is an important part of glycoprotein characterization. Oligosaccharides can be linked to a protein through a serine or a threonine as *O*-linked glycans, or through an asparagine as *N*-linked glycans. To analyze the structure of oligosaccharides, various chromatographic and spectroscopic techniques are often required.

High-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) is one of the most powerful and commonly used techniques for protein glycosylation analysis. Using this technique, oligosaccharides and monosaccharides can be quickly separated and their structures can frequently be identified by comparing them with authentic carbohydrate standards. Separations of oligosaccharides are based on their fine structural differences such as the composition and the sequences of the oligosaccharides, linkage isomerism, degree of sialylation, and degree of branching. Separations of monosaccharides are mainly dependent upon the difference between their pK_a values. Such differences usually allow baseline resolution of common neutral and amino sugars found in mammalian glycoproteins.

HPAE-PAD, in combination with different endo- and exoglycosidases, and appropriate oligosaccharide standards, allows step-by-step structural analysis of oligosaccharides. Treatment of a glycoprotein with an endoglycosidase releases the attached oligosaccharides from the protein. Profiles of the released oligosaccharides can be obtained using HPAE-PAD. Subsequent digestion of these oligosaccharides with various specific exoglycosidases generates structural information. Since intact enzymes are not commonly eluted at the regions where mono- and oligosaccharides are eluted, these enzymatic digestions can be injected directly into the analytical system for separation and identification of the released carbohydrates.

This technical note describes the use of exoglycosidases to obtain structural information about *N*-linked oligosaccharides commonly found in mammalian glycoproteins. HPAE-PAD is used to confirm the identity of the released monosaccharides, as well as the remaining di-, tri,- or oligosaccharides. Empirical rules used to predict oligosaccharide elution order are included in the last section of this technical note.

EQUIPMENT

Dionex DX 500 HPLC system consisting of:
- GP40 Gradient Pump
- ED40 Electrochemical Detector
- AS3500 Autosampler

PeakNet Chromatography Workstation

CONDITIONS AND METHODS

Columns: CarboPac™ PA1 Analytical Column (4 x 250 mm)
Guard Column (4 x 50 mm)
CarboPac PA-100 Analytical Column (4 x 250 mm)
Guard Column (4 x 50 mm)

Flow Rate: 1.0 mL/min

Pulse Setting for ED40 Detector:

Time	Potential	Integration
0.00	0.05	
0.20	0.05	Begin
0.40	0.05	End
0.41	0.75	
0.60	0.75	
0.61	–0.15	
1.00	–0.15	

Eluent A: 250 mM Sodium acetate

Eluent B: 500 mM Sodium hydroxide

Eluent C: Deionized water, 17.8 MΩ-cm resistance or better

Eluent D: 100 mM Sodium hydroxide

Methods:

Method*	Column	Time (min)	A (%)	B (%)	C (%)	D (%)
1	CarboPac PA-100	0	8	20	72	0
		5	8	20	72	0
		65	80	20	0	0
		65.05	8	20	72	0
		70	8	20	72	0
2	CarboPac PA-100	0	0	50	50	0
		4	0	50	50	0
		27	32	50	18	0
		32	32	50	18	0
		32.1	0	50	50	0
		37	0	50	50	0
3	CarboPac PA1	0	0	0	84	16
		3	0	0	84	16
		28	0	0	40	60
		28.05	0	50	50	0
		38	0	50	50	0
		38.05	0	0	84	16
		48	0	0	84	16

* Method 1: For separations of sialylated oligosaccharides
Method 2: For separations of neutral oligosaccharides
Method 3: For separations of mannose, GlcNAc, chitobiose, and fucosylated chitobiose

REAGENTS AND STANDARDS

Sodium hydroxide, 50% w/w (Fisher Scientific, Fair Lawn, New Jersey, USA)

Anhydrous sodium acetate (Fluka BioChemica, Ronkonkoma, New York, USA)

The following exoglycosidases and standards were obtained from Oxford GlycoSystems, Rosedale, New York, USA:

Chitobiose

6-α-Fucosyl chitobiose (fucosylated chitobiose)

α-Fucosidase from *Bovine epididymi*

β-Galactosidase from *Streptococcus pneumoniae*

β-*N*-Acetylhexosaminidase from *Streptococcus pneumoniae*

α-Mannosidase from Jack bean

β-Mannosidase from *Helix pomatia*

Neuraminidase from *Vibrio cholerae*

The following monosaccharide and oligosaccharide standards were obtained from Dionex Corporation, Sunnyvale, California, USA:

GP 03, GP 05, GP 07, GP 10, GP 11, GP 12, GP 14, GP 15, GP 16, GP 17, GP 18, FT 02, PI 01. (See Figure 1 for structures)

N-Acetylneuraminic acid (Dionex Standards: Mono, NANA)

The following monosaccharides were obtained from Sigma Chemical Company, St. Louis, Missouri, USA:

D(+)Galactose

N-Acetyl-D-Glucosamine (GlcNAc)

α-L(–)Fucose

D-Mannose, 99%, mixture of anomers (Aldrich Chemical Company, Milwaukee, Wisconsin, USA).

PREPARATION OF SAMPLES AND SOLUTIONS

Eluent A: 250 mM Sodium Acetate

Dissolve 20.5 g of anhydrous sodium acetate into a final volume of 1.0 L of deionized water. Filter the eluent through a 0.2 µm filter, and then degas the eluent for 5 minutes before use.

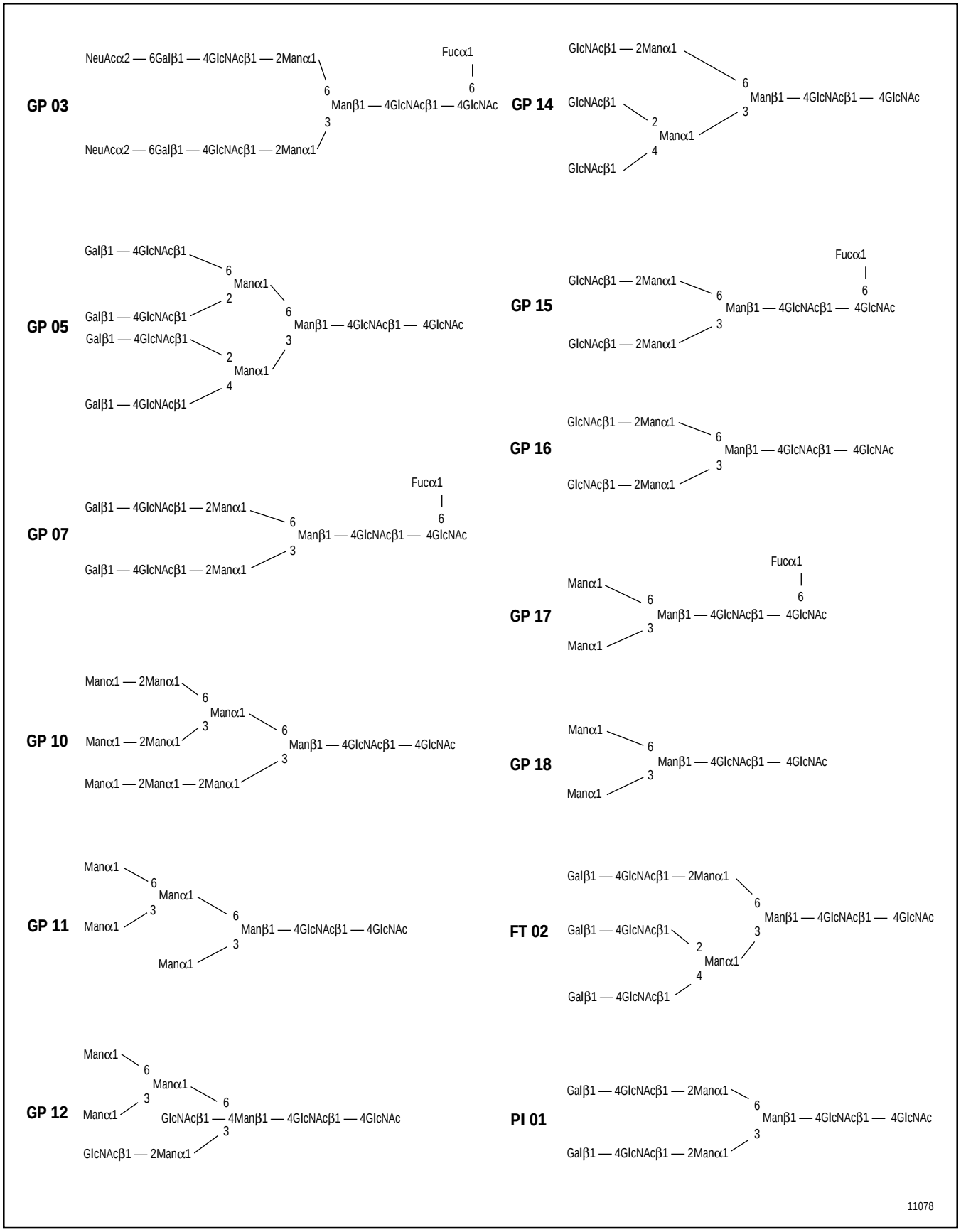

Figure 1 *Structures of Dionex oligosaccharide standards.*

Eluent B: 500 mM Sodium Hydroxide

Filter 1.0 L of deionized water through a 0.2 µm filter. Then degas the deionized water for 5 minutes. Add 26 mL of 50% w/w sodium hydroxide to a final volume of 1.0 L of the degassed water.

Eluent C: Water

Filter 1.0 L of deionized water through a 0.2 µm filter. Then degas the deionized water for 5 minutes before use.

Eluent D: 100 mM Sodium Hydroxide

Filter 1.0 L of deionized water through a 0.2 µm filter. Then degas the deionized water for 5 minutes. Add 5.2 mL of 50% w/w sodium hydroxide to a final volume of 1.0 L of the degassed water.

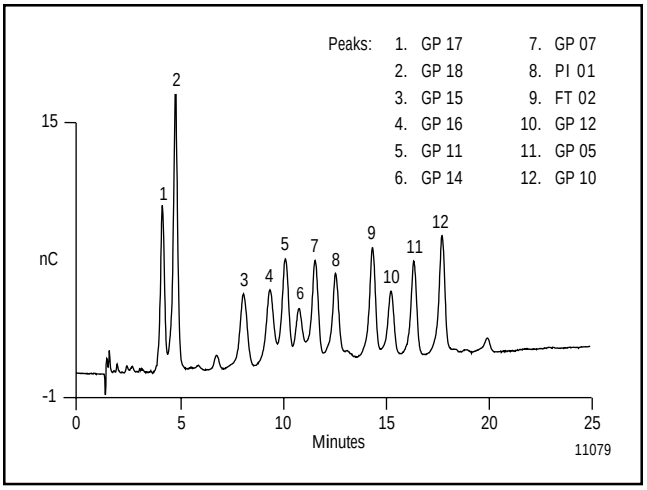

Figure 2 *Separation of 12 neutral oligosaccharide standards. 1 µg of each standard was dissolved in 250 µL of water. Injection volume: 10 µL; separation: Method 2, Column: CarboPac PA-100.*

Exoglycosidase Stocks

α-Fucosidase from Bovine epididymis: Dissolve 0.1 U of the enzyme in 40 µL of the 5X incubation buffer (500 mM sodium citrate-phosphate, pH 6.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

β-Galactosidase from *Streptococcus pneumoniae*: Dissolve 40 mU of the enzyme in 40 µL of the 5X incubation buffer (500 mM sodium acetate, pH 6.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

β-*N*-acetylhexosaminidase from *Streptococcus pneumoniae*: Dissolve 30 mU of the enzyme in 40 µL of the 5X incubation buffer (500 mM sodium citrate-phosphate, pH 6.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

α-Mannosidase from Jack bean: Dissolve 2 U of the enzyme in 40 µL of the 5X incubation buffer (500 mM sodium acetate with 10 mM of Zn^{2+}, pH 5.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

β-Mannosidase from *Helix pomatia*: Dissolve 0.2 U of the enzyme in 40 µL of the 5X incubation buffer (500 mM sodium citrate-phosphate, pH 4.0) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

Neuraminidase from *Vibrio cholerae*: Dissolve 0.2 U of the enzyme in 40 µL of the 5X incubation buffer (50 mM sodium acetate with 4 mM $CaCl_2$, pH 5.5) that is supplied with the enzyme kit. Then dilute this mixture to 200 µL with water.

Aliquots of the above enzyme stocks were used with different substrates to generate the desired digested products. See figure legends for digest conditions.

Carbohydrate Standards

Fucose, galactose, GlcNAc, and mannose (concentration: 1 mg/mL each): To prepare each sugar stock, dissolve 1 mg of the sugar in 1 mL of water.

N-acetylneuraminic acid (NANA, supplied as 25 nmol dry powder, final concentration: 50 nmol/mL): Add 500 µL of deionized water to 25 nmol of the sugar as supplied.

Chitobiose (supplied as 20 µg dry powder, final concentration: 100 µg/mL): Add 200 µL of deionized water to 20 µg of the sugar as supplied .

Fucosylated chitobiose (supplied as 20 µg dry powder, final concentration: 40 µg/mL): Add 500 µL of water to 20 mg of the sugar as supplied.

Dionex standards (supplied as 15 µg dry powder, final concentration: 30 µg/mL each): to prepare each oligosaccharide stock, add 500 µL of water to 15 µg of the sugar as supplied.

RESULTS AND DISCUSSION

1. Gradient Methods and Columns

Separations of sialylated oligosaccharides have previously been demonstrated using a CarboPac PA-100 column and a 20–200 mM sodium acetate gradient in the presence of 100 mM sodium hydroxide.[1–3] Using a different linear gradient, a better separation of neutral oligosaccharides can be achieved, as shown in Figure 2. With this method, 12 neutral oligosaccharides, representing oligomannose-type, complex-type and hybrid-type oligosaccharides, were resolved.

Using a CarboPac PA1 column and Method 3, mannose, GlcNAc, fucosylated chitobiose and chitobiose can also be separated (mannose and GlcNAc are approximately 75% resolved). Method 3 is particularly useful for identification of the chitobiose core, and the released mannose and GlcNAc residues in an enzymatic digestion.

2. Exoglycosidase Digestions of Complex-Type Oligosaccharides

Neuraminidase

Both neutral and sialylated *N*-linked oligosaccharides can be released from glycoproteins using the amidase peptide-*N*-glycosidase F (PNGase F). A profile of the sialylated and the neutral oligosaccharides can be obtained using Method 1 and a CarboPac PA-100 column.[4] The terminal sialic acids can then be released from the nonreducing end of the sialylated oligosaccharides by subsequent treatment of the PNGase F digest with neuraminidase. The resulting neutral oligosaccharides can be separated from each other, and identified by comparing their retention times with appropriate oligosaccharide standards.

An example of a neuraminidase digestion and identification of the released products is shown in Figures 3a–3e. Figure 3b shows the separation of a digestion of a disialylated, core-fucosylated, biantennary complex oligosaccharide incubated with neuraminidase (from *Vibrio Cholerae)* at 37 °C for 24 hours. Peak 1 coelutes with an asialo, fucosylated biantennary oligosaccharide standard, as shown in Figure 3c. Thus, the peak represents the oligosaccharide substrate with its sialic acids removed. Peak 2, the released *N*-acetylneuraminic acid (NANA), is also identified as it coelutes with a NANA standard (see Figure 3d).

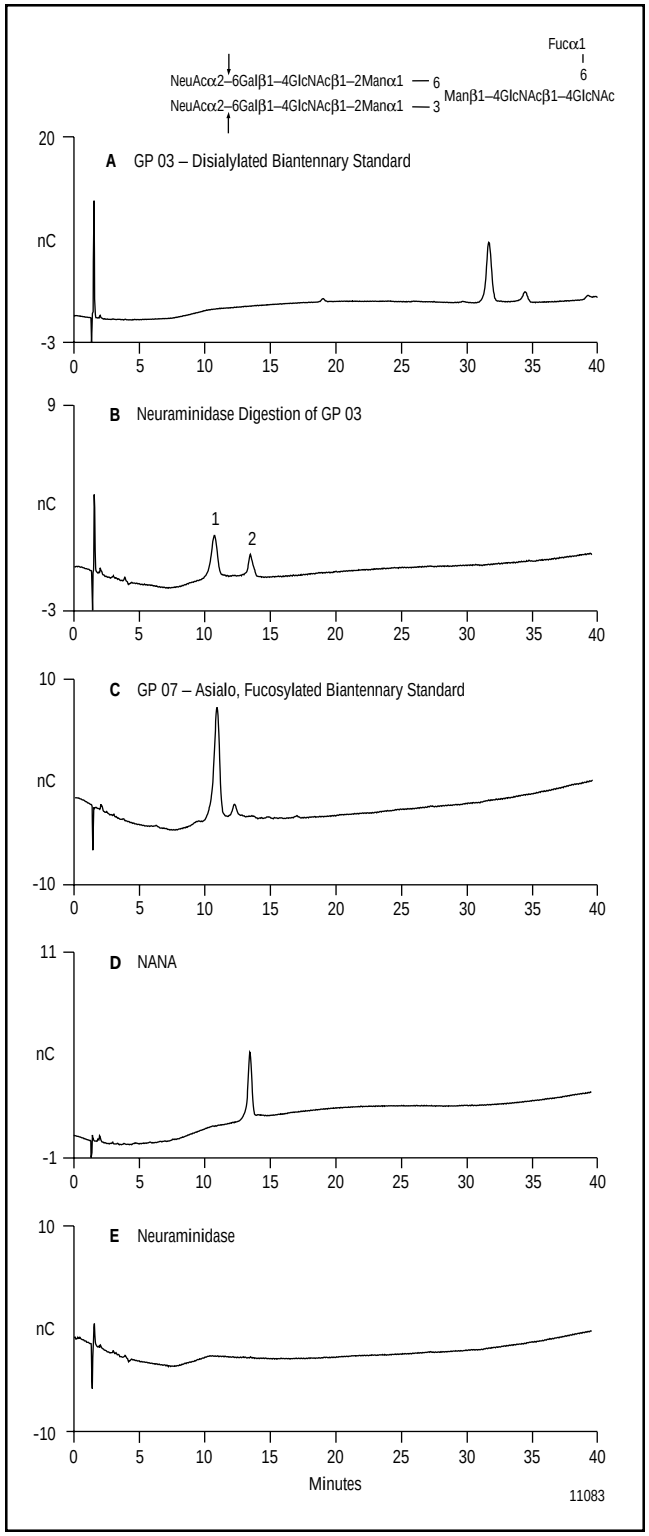

Figure 3 *(a) Disialylated biantennary oligosaccharide standard (Dionex standard GP 03); (b) separation of a neuraminidase digestion of a disialylated, biantennary complex type oligosaccharide with a fucosylated core (Dionex standard GP 03). Digest conditions: 30 µL of GP 03 was mixed with 5 mU of neuraminidase, and incubated at 37 °C for 24 hours. Injection volume: 10 µL; (c) an asialo, bi-antennary complex oligosaccharide with a fucosylated core (Dionex standard GP 07); (d) NANA; and (e) Neuraminidase. Separation: Method 1, Column: CarboPac PA-100.*

β-*Galactosidase*

Galactose is frequently present at the nonreducing end of neutral, complex-type oligosaccharides. These neutral oligosaccharides can be branched and the degree of galactosylation may vary. It has been demonstrated that neutral oligosaccharides with different degrees of galactosylation can be separated using the CarboPac PA-100 column.[5]

Different galactosidases can be used to determine if an oligosaccharide is galactosylated at the nonreducing terminal and to identify the linkages of the galactose residues. For example, a terminal galactose linked to a GlcNAc (Galβ1—›4 GlcNAc) can be removed by treating the oligosaccharide with β-galactosidase from *Streptococcus pneumoniae*. After an asialo, fucosylated biantennary oligosaccharide was incubated with the β-galactosidase for 24 hours at 37 °C, two species were generated as shown in Figure 4b. Peak 2 coelutes with an asialo, agalacto, core-fucosylated, biantennary oligosaccharide standard shown in Figure 4c. The parent oligosaccharide and a galactose standard are shown in Figures 4a and 4d, respectively. The results indicate that peaks 1 and 2 represent the released galactose residues and the agalacto biantennary oligosaccharide product (with the galactose residues removed), respectively.

β-N-*Acetylhexosaminidase,* β-*Mannosidase, and* α-*Mannosidase*

All complex-type oligosaccharides have a common trimannosyl chitobiose core (see oligosaccharide structure in Figure 5) — a mannose residue linked (β1—›4) to the chitobiose (two GlcNAc residues) — with two additional mannose residues linked to the mannose (proximal to the chitobiose) through α1—›6 and α1—›3 linkages, respectively. Two additional GlcNAc residues can also be linked to the two terminal mannoses of the trimannosyl chitobiose core. To release the terminal GlcNAc residues and the mannose residues from the chitobiose core, several exoglycosidases can be used simultaneously. A terminal GlcNAc linked to a trimannosyl chitobiose core through a GlcNAcβ1—›2 Man linkage can be removed using β-*N*-acetylhexosaminidase from *Streptococcus pneumonia* (optimal incubation pH = 5). The mannose residues present in the trimannosyl chitobiose core can also be removed using β-mannosidase from *Helix pomatia* (optimal incubation pH = 4.5) and α-mannosidase from Jack bean (optimal incubation pH = 5).

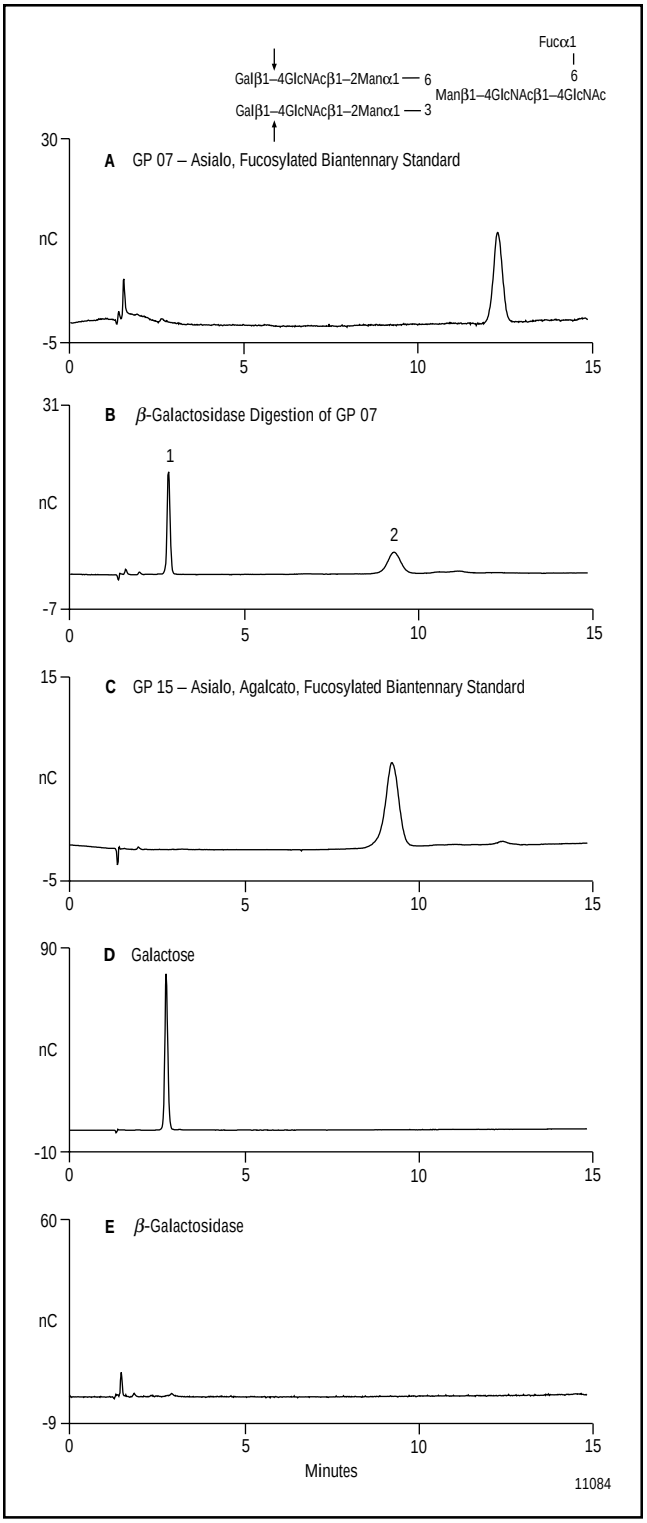

Figure 4 *(a) Asialo, biantennary complex oligosaccharide with a fucosylated core (Dionex standard GP 07). (b) Separation of a* β*-galactosidase digestion of an asialo, biantennary complex-type oligosaccharide with a fucosylated core (Dionex standard GP 07). Digest condition: 30 µL of GP 07 was mixed with 2 mU of* β*-galactosidase. The mixture was incubated at 37 °C for 24 hours. Injection volume: 10 µL. (c) Asialo, agalacto, fucosylated biantennary oligosaccharide (Dionex standard GP 15). (d) Galactose. (e) Galactosidase. Separation: Method 2, Column: CarboPac PA-100.*

Figure 5b shows an example of such a digestion — an asialo, agalacto, biantennary complex oligosaccharide was treated with all three enzymes at 37 °C for 24 hours. The substrate peak at 9.5 minutes disappears completely and several species appear in the region between 1.5 and 4 minutes. To identify these species, the digestion was also separated using Method 3 and a CarboPac PA1 column for resolution of the chitobiose, the mannose and the GlcNAc residues. As shown in Figure 5c, peaks 1 and 2 coelute with a GlcNAc standard shown in Figure 5d, and a mannose standard shown in Figure 5e, respectively. Peaks 1 and 2, thus represent the released GlcNAc and mannose residues, respectively. Peak 3 coelutes with a chitobiose standard shown in Figure 5f, indicating that it represents the released chitobiose.

Figure 5g shows a chromatogram of an incubation of *β-N*-acetylhexosaminidase, *α*-mannosidase, and *β*-mannosidase at 37 °C for 24 hours. No peak was detected between 5 to 30 minutes, indicating that none of the three exoglycosidases is acting as a hydrolysis substrate during the incubation.

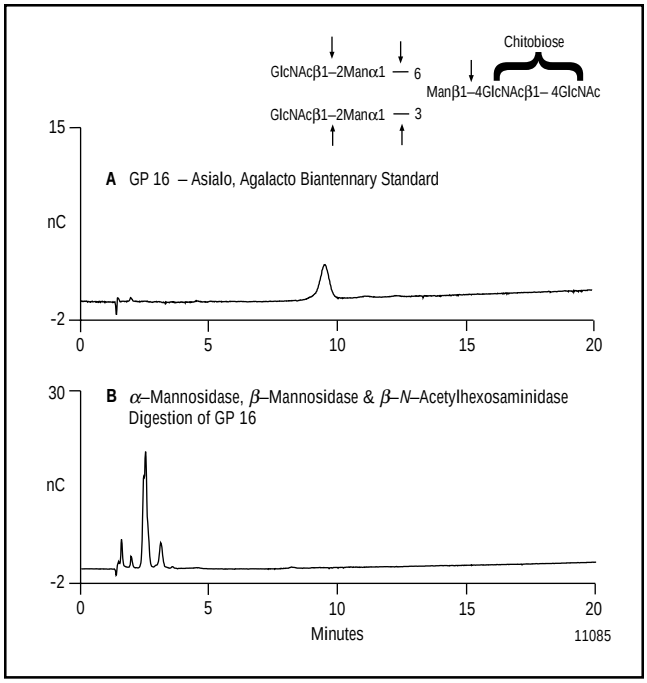

Figure 5 *(a) Asialo, agalacto biantennary complex oligosaccharide (Dionex standard GP 16). (b) Separation of a* β-N-*acetylhexosaminidase,* α*-mannosidase, and* β*-mannosidase digestion of an asialo, agalacto, biantennary complex oligosaccharide (Dionex standard GP 16). Digest condition: 30 µL of GP 16 was mixed with 1.5 mU of* β-N-*acetylhexosaminidase, 20 mU of* β*-mannosidase, and 100 mU of* α*-mannosidase. The mixture was incubated at 37 °C for 24 hours. Injection volume: 18 µL; Separation: Method 2, Column: CarboPac PA-100.*

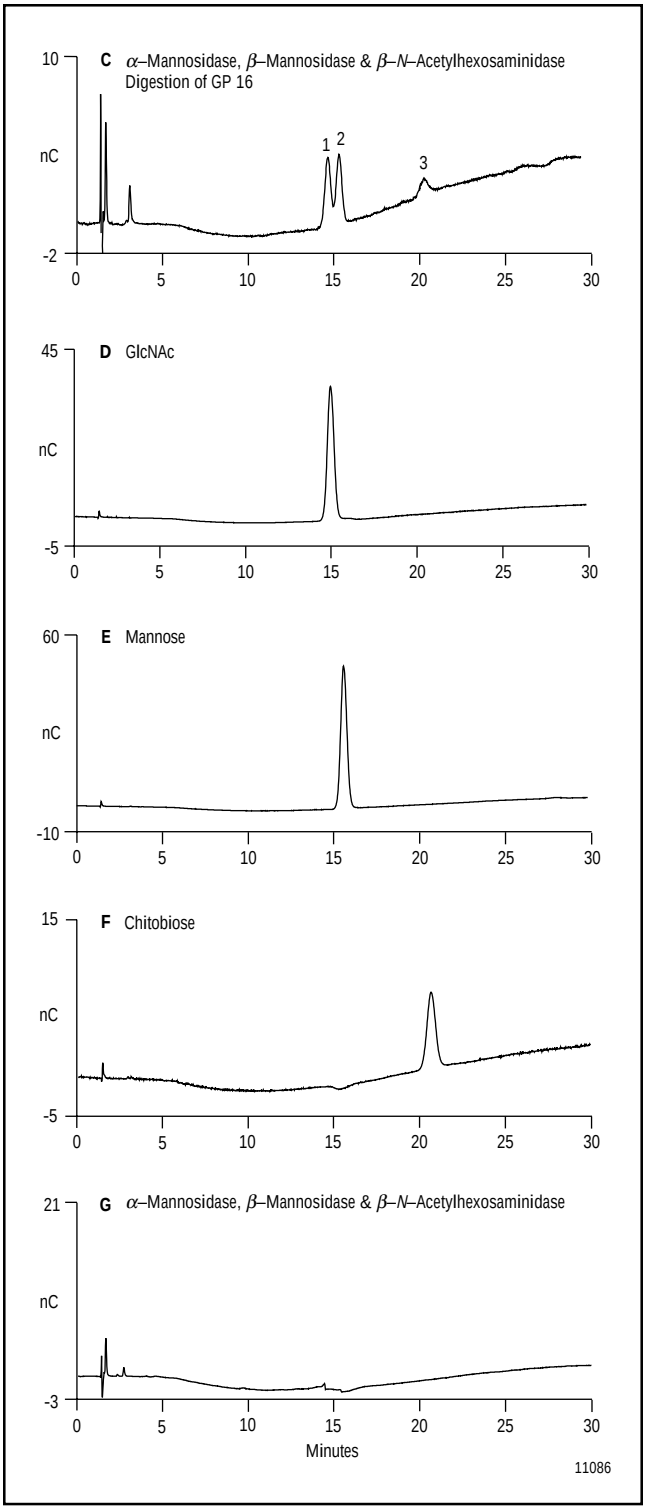

Figure 5 (cont.) *(c) Separation of a* β-N-*acetylhexosaminidase,* α*-mannosidase, and* β*-mannosidase digestion of an asialo, agalacto, biantennary complex oligosaccharide (Dionex standard GP 16). Digest condition: Identical digest used for Figure 5b. Injection volume: 10 µL; (d) GlcNAc; (e) Mannose; (f) Chitobiose; (g)* β-N-*acetylhexosaminidase,* α*-mannosidase, and* β*-mannosidase. Separation: Method 3, Column: CarboPac PA1.*

3. Exoglycosidase Digestion of Fucosylated Oligosaccharides

α-*Fucosidase*

Frequently, an *N*-linked oligosaccharide (oligomannose-, complex- or hybrid-type) is fucosylated at the GlcNAc residue proximal to the asparagine at the glycosylation site of the protein backbone. This fucose can be removed independently using α-fucosidase without removing any monosaccharides attached to the nonreducing terminal of the glycan. Thus, using one exoglycosidase, analysts can determine if an oligosaccharide is fucosylated. This method is particularly useful when a collection of fucosylated and afucosylated oligosaccharides is present in the same sample. Disappearance of one or more peaks after fucosidase digestion, and identification of the afucosylated products and fucose allow an analyst to identify the fucosylated oligosaccharides present in the original sample.

Figure 6b shows the results of an α-fucosidase (from Bovine epididymis) digestion of a fucosylated $Man_3GlcNAc_2$ oligosaccharide (GP 17) after a 24-hour incubation at 37 °C. Peak 1 coelutes with a fucose standard shown in Figure 6d. Peak 2 coelutes with a $Man_3GlcNAc_2$ oligosaccharide standard shown in Figure 6c. The results suggest that the fucosylated $Man_3GlcNAc_2$ oligosaccharide was converted to its corresponding afucosylated analog in a 24-hour incubation.

4. Digestion of Oligomannose-Type Oligosaccharides

α-*Mannosidase &* β-*Mannosidase*

Treatment of the oligomannose-type oligosaccharides with appropriate α- and β-mannosidases simultaneously can release all the mannose residues from the chitobiose core. Figure 7b shows a separation of a digestion of a fucosylated $Man_3GlcNAc_2$ oligosaccharide (GP 17) with β-mannosidase (from Jack bean) and α-mannosidase (from *Helix pomatia*). The substrate peak at 4.1 minutes disappears completely, and several peaks elute in the region between 1 to 4 minutes. This digest was also analyzed using method 3 and the CarboPac PA1 column for better separation of the fucosylated chitobiose and the released mannose residues. As shown in Figures 7c and 7d, peak 2 coelutes with a fucosylated chitobiose standard, and it also elutes approximately 1.5 minutes earlier than the chitobiose peak (see Figure 5f).

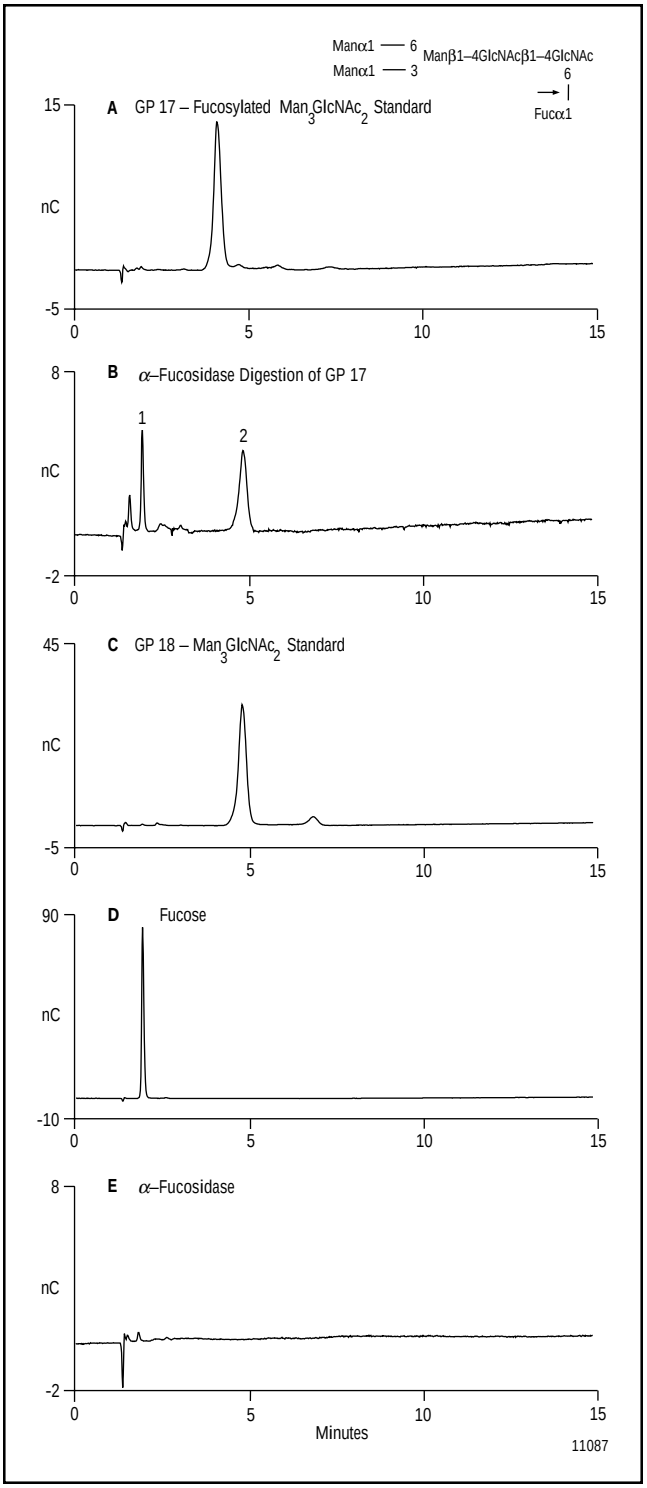

Figure 6 *(a) Fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). (b) Separation of an α-fucosidase digestion of a core fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). Digest condition: 30 µL of GP 16 was mixed with 5 mU of α-fucosidase. The mixture was incubated at 37 °C for 24 hours. Injection volume: 8 µL. (c) $Man_3GlcNAc_2$ (Dionex standard GP 18). (d) Fucose. (e) Fucosidase. Separation: Method 2, Column: CarboPac PA-100.*

This result agrees with the observation that fucosylated oligosaccharides elute earlier than their afucosylated analogs.

Figure 7f shows the chromatogram of an incubation of α- and β-mannosidases at 37 °C for 24 hours. No peak was detected between 5 and 25 minutes, indicating neither exoglycosidase is acting as a hydrolysis substrate during the incubation.

5. Factors Affecting Elution of Oligosaccharides Using the CarboPac PA-100 Column[1, 6–8]

Several factors affect the elution of these oligosaccharides (see Figure 2):

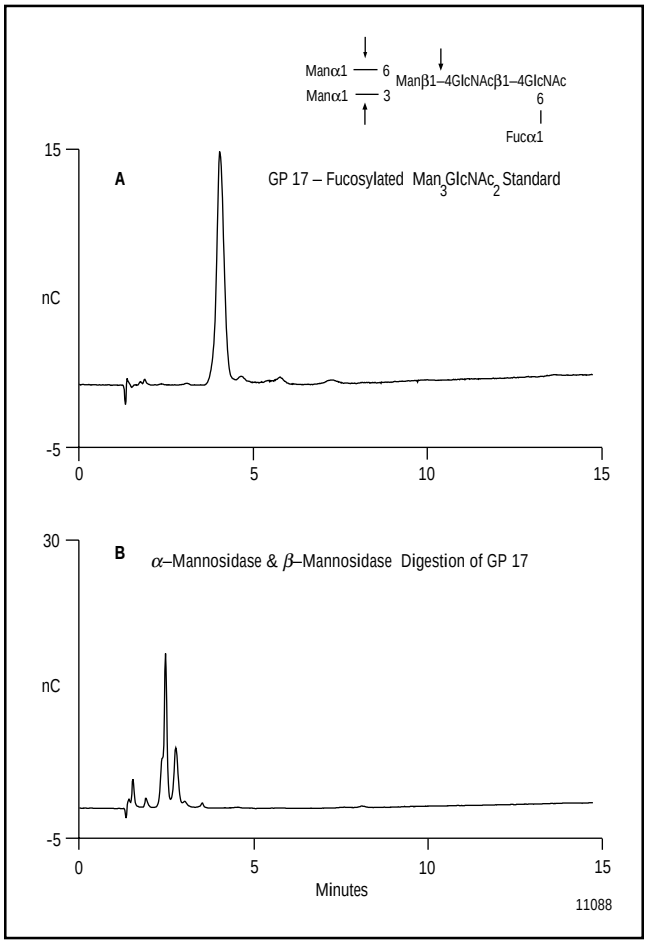

Figure 7 *(a) Fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). (b) Separation of an α-mannosidase and β-mannosidase digestion of a core fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). Digest condition: 10 µL of GP 17 was mixed with 10 mU of β-mannosidase and 100 mU of α-mannosidase. The mixture was incubated at 37 °C for 24 hours. Injection volume: 7 µL. Separation: Method 2, Column: CarboPac PA-100.*

1. Fucosylated oligosaccharides (peaks 1, 3, 7) elute earlier than their afucosylated analogs (peaks 2, 4, 8).
2. As the number of mannose residues in a high mannose oligosaccharide increases, its retention time also increases (peaks 2, 5, 12).
3. As the degree of branching increases, the retention time of the oligosaccharide increases (peaks 8, 9,11).
4. Removal of terminal galactose residues from a complex oligosaccharide reduces its retention time (peaks 3, 4, 6 vs. peaks 7, 8, 9).

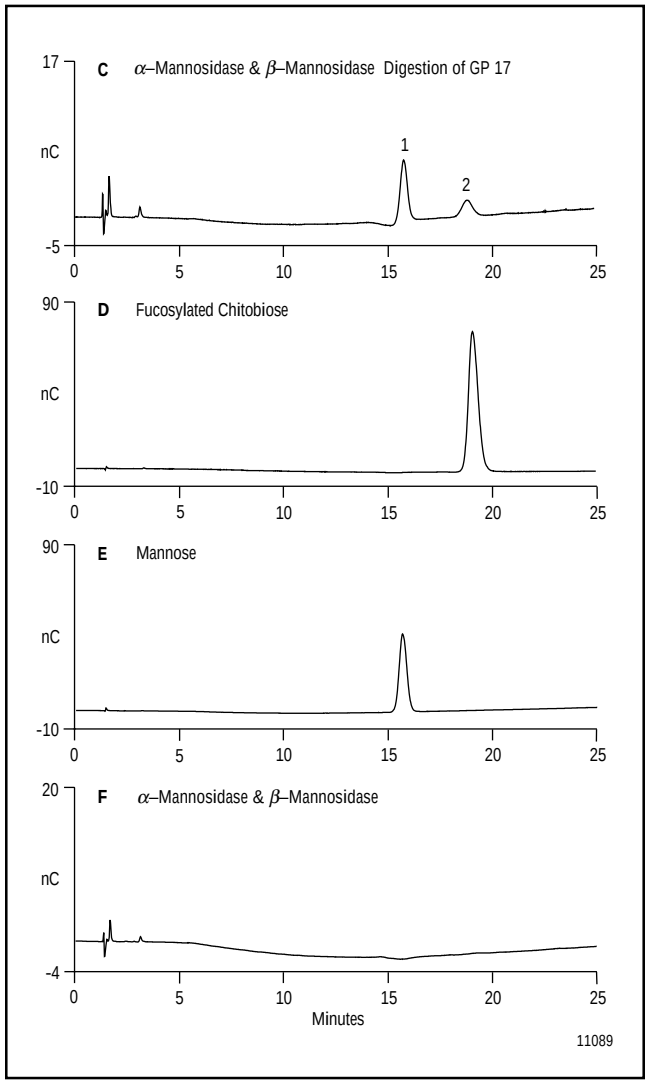

Figure 7 (cont.) *(c) Separation of an α-mannosidase and β-mannosidase digestion of a core fucosylated $Man_3GlcNAc_2$ oligosaccharide (Dionex standard GP 17). Digest condition: Identical digest used for Figure 7b. Injection volume: 7 µL. (d) Fucosylated chitobiose. (e) Mannose. (f) α-mannosidase and β-mannosidase. Separation: Method 3, Column: CarboPac PA1.*

Other factors have been known to affect the elution of oligosaccharides.[1, 6–8] These "rules" are summarized as follows:

1. A bisected oligosaccharide has a longer retention time compared to its non-bisected oligosaccharide analog.
2. Retention time of an oligosaccharide with a complete chitobiose decreases compared to its analog with one GlcNAc removed from the chitobiose.
3. An oligosaccharide with a Galβ1—›4 GlcNAc linkage elutes earlier than its analog with a Galβ1—›3 GlcNAc linkage; an oligosaccharide with a Neu5Acα2—›6 Gal linkage elutes earlier than its analog with a Neu5Acα2—›3 Gal linkage.
4. A nonreduced oligosaccharide has a longer retention time compared to its reduced analog.

SUMMARY

Using a combination of six exoglycosidases, terminal monosaccharides were released from oligosaccharides for analysis. Using the Dionex CarboPac PA1 and PA-100 columns, these released monosaccharides and the remaining oligosaccharides were separated and identified using HPAE-PAD. These oligosaccharides also elute in a predictable manner and several empirical rules can be used to predict the relative times at which they elute.

In every digestion, the appearance of the oligo- and monosaccharide products could be monitored directly by injecting the digestion into the analytical system. Identities of these products were confirmed by a comparison with appropriate standards. Thus, the high resolution of the CarboPac columns coupled with pulsed amperometric detection allows analysts to obtain structural information of *N*-linked glycans without extensive sample clean up.

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

Dionex Corporation
Salt Lake City Technical Center
1515 West 2200 South, Suite A
Salt Lake City, UT
84119-1484
(801) 972-9292

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