

HPAE-PAD *N*-linked oligosaccharide profiling of IgG

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Keywords

Glycan, electrochemical detection, recombinant glycoprotein, monoclonal antibody, mAb, ion chromatography, biotherapeutics, glycan analysis

Goal

To develop an HPAE-PAD method to profile the major neutral and charged asparagine-linked (*N*-linked) oligosaccharides from IgGs, including monoclonal antibodies

Introduction

Profiling the *N*-linked oligosaccharides of a glycoprotein is one of the important analyses used for glycoprotein characterization. This is especially true when a glycoprotein is being produced as a human therapeutic. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is one of the techniques commonly used to characterize glycoprotein glycosylation, including the *N*-linked oligosaccharides of the protein.^{1,2} Monoclonal antibodies (mAbs) are IgG-type immunoglobulins and are the most common protein therapeutic. They are glycosylated with each constant region heavy chain containing a conserved *N*-linked glycosylation site. This glycosylation has been shown to impact complement-dependent cytotoxicity, effector function, serum clearance, and other biological processes. Hence, determining the *N*-linked glycosylation of an mAb is an important part of its characterization, and the glycosylation of each lot of a mAb biotherapeutic must be comparable to the glycosylation of the mAb used in its clinical trials. This technical note

reports an HPAE-PAD method to profile IgG *N*-linked oligosaccharides and *N*-linked high-mannose type oligosaccharides that are atypical for human polyclonal IgG but sometimes found on mAbs. This HPAE-PAD method is orthogonal to other oligosaccharide methods applied to IgG.³ More information on the development and application of the separation described in the technical note can be found in Rohrer, et al.³

Experimental Equipment

Thermo Scientific™ Dionex™ ICS-5000+ HPLC™ system* consisting of:

- Dionex SP Single Pump or Dionex DP Dual Pump module
- Dionex DC Detector/Chromatography module
- Dionex AS-AP Autosampler
- Dionex ED Electrochemical Detector (P/N 079830)
- Dionex Electrochemical Cell (P/N AAA-061756)
- Dionex pH - Ag/AgCl Reference Electrode (P/N 061879)
- 10 µL PEEK™ Sample Injection Loop (P/N 042949)

*A Dionex ICS-3000 or Dionex ICS-5000 system can also be used.

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Sodium hydroxide (NaOH), 50% w/w (Fisher Scientific™ P/N SS254-500)

- Sodium acetate, anhydrous, electrochemical grade (P/N 059326)
- G0 (NGA2) (Fisher Scientific P/N NC0145854)
- G1F (NA2G1F) (Fisher Scientific P/N NC9603959)
- MAN-6 (Fisher Scientific P/N NC9071406)
- G2bF (NA2FB) (Fisher Scientific P/N NC0145860)
- MAN-9 (Fisher Scientific P/N 50-355-869)
- MAN-5 (Fisher Scientific P/N 50-355-864)
- A1F (Fisher Scientific P/N NC9603958)
- A2F (Fisher Scientific P/N NC9698597)
- Thermo Scientific™ Pierce™ Human Polyclonal IgG (P/N PA1-31154)
- PNGase F, 15,000 units, 500,000 U/mL, a Unit is defined as the amount of enzyme required to remove >95% of carbohydrate from 10 µg of denatured RNase B in 1 h at 37 °C in a total reaction volume of 10 µL (New England BioLabs®, P/N P0705S). Enzyme is supplied with: 10x glycoprotein denaturing buffer (5% SDS, 10% β-mercaptoethanol), 10x G7 buffer (0.5 M sodium phosphate, pH 7.5 at 25 °C), and 10% NP-40.
- α2-3,6,8 neuraminidase (New England BioLabs)
- β1,4 galactosidase (New England BioLabs)
- α-L-fucosidase (bovine kidneys, Sigma-Aldrich®)
- Maltose, (Sigma-Aldrich, P/N M-5885)
- Maltotriose, (Sigma-Aldrich, P/N M-8378)
- Maltotetraose, (Sigma-Aldrich, P/N M-8253)

Conditions

Columns:	Thermo Scientific™ Dionex™ CarboPac™ PA200, 3 × 250 mm (P/N 062896) Dionex CarboPac PA200 Guard, 3 × 50 mm (P/N 062895)				
Eluents:	A) DI water B) 100 mM NaOH C) 200 mM sodium acetate in 100 mM NaOH				
Eluent Gradient:	Table 1				
Flow Rate:	0.5 mL/min				
Injection Volume:	5 µL partial loop injection, loop size 10 µL				
Temperature:	30 °C (column and detector compartments)				
Detection:	Pulsed Amperometric, Gold on Polyester disposable working electrode (P/N 060139) with 2 mil gasket (P/N 060141), Gold on PTFE disposable working electrode (P/N 066480) using the same gasket, or conventional Au electrode (P/N 079850) with 1 mil gasket (P/N 045972)				
Background:	~18 nC (using the carbohydrate 4-potential waveform)				
Noise:	~50 pC				
System Backpressure:	~2900 psi				
Carbohydrate 4-Potential Waveform for the ED:					
<i>Time (s)</i>	<i>Potential (V)</i>	<i>Gain</i>	<i>Region*</i>	<i>Ramp*</i>	<i>Integration</i>
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	
*Settings required in the Dionex ICS-3000/5000, but not used in older Thermo Scientific Dionex systems.					
Reference Electrode Mode:	Ag (Ag/AgCl reference)				

Table 1. Eluent program used to separate IgG N-linked oligosaccharides.

Time (min)	%A (Deionized Water)	%B (100 mM NaOH)	%C (200 mM Sodium Acetate in 100 mM NaOH)
0	50	50	0
30	0	100	0
35	0	100	0
50	0	0	100
60.0	0	0	100
60.1	50	50	0
75	50	50	0

Eluents

100 mM sodium hydroxide

It is essential to use high quality deionized (DI) water of high resistivity (18 MΩ·cm or better) that contains as little dissolved carbon dioxide as possible. Biological contamination should be absent. Plastic tubing in the water system should be minimized, as it often supports microbial growth, which can be a source of carbohydrate contamination. It is extremely important to minimize contamination with carbonate, a divalent anion at pH >12, because it binds strongly to the columns and interferes with carbohydrate chromatography, causing a loss of resolution and efficiency. Commercially available sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used. A 50% (w/w) sodium hydroxide solution is much lower in carbonate and is the preferred source for sodium hydroxide. Dilute 5.2 mL of a 50% (w/w) sodium hydroxide solution into 1 L of DI water to prepare a 0.1 M sodium hydroxide solution. After preparation, keep the eluent blanketed under UHP grade nitrogen or UHP grade helium at 34 to 55 kPa (5 to 8 psi) at all times.

200 mM sodium acetate in 100 mM sodium hydroxide

Measure approximately 800 mL of DI water into a 1 L graduated cylinder. Add a stir bar and begin stirring. Weigh out 16.4 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. Vacuum filter through a 0.2 µm nylon filter. Transfer to a 1 L volumetric flask. Using a plastic serological pipette, measure 5.2 mL of 50% (w/w) sodium hydroxide and add it to the acetate solution. Add degassed DI water to the mark. After preparation, keep this eluent blanketed under nitrogen or helium at 34 to 55 kPa (5 to 8 psi) at all times. More details on eluent preparation for HPAE-PAD can be found in Technical Note 71.⁴

Preparation of standards

Mix of maltose standards

Prepare the maltose, maltotriose, and maltotetraose stock standard solutions (10 mg/mL) in DI water. Dilute 100 µL of each stock standard solution to 1 mL to prepare 1 mg/mL standard solutions. Prepare the mixed retention time standard by mixing 997 µL DI water with 1 µL of each 1 mg/mL maltose, maltotriose, and maltotetraose solutions, to obtain a 1 µg/mL solution of each carbohydrate.

Individual N-linked oligosaccharide standards

Dissolve each oligosaccharide standard in a volume of DI water to produce a 100 µg/mL solution. Use each of these stock solutions to prepare mixed standard solutions where the concentration of each oligosaccharide is 1 µg/mL.

Sample preparation

PNGase F digestion

Add 20 µL of 1:10 dilution of PNGase F enzyme preparation to 200 µL protein (≤ 11 mg/mL) and 200 µL DI water. Incubate the mixture at 37 °C for 20 h. Dilute this sample 1:1 with DI water prior to chromatography. For the digestion control, substitute the 200 µL protein with 200 µL DI water (i.e., 400 µL total DI water).

Neuraminidase digestion

Dilute the purchased neuraminidase with pH 5 sodium acetate buffer to yield a 5000 U/mL solution. Prepare the digest by adding equal volumes this neuraminidase solution and the PNGase F digest (i.e., released oligosaccharides). This mixture is incubated at 37 °C for 1 h.

α -L-Fucosidase digestion

Dilute the purchased fucosidase with 2000 µL DI water. Prepare the digest by adding equal volumes of this fucosidase solution and the sample. This mixture is incubated at 37 °C for 20 h. For partial digestion (i.e., incomplete release of fucose) terminate the digestion at 6.5 h. Typically, 15 µL of each is used. Digested standards have a starting concentration of 1 µg/mL.

β -Galactosidase digestion

Dilute the purchased galactosidase with 950 µL DI water to yield an 800 U/mL solution. Treat PNGase F digests and the neuraminidase and fucosidase digests of the PNGase F digests with 16 U (20 µL) of galactosidase and incubate at 37 °C for 1 h. To prepare a mixture of standards containing bisecting *N*-acetyl glucosamine, treat the G2bF standard and the G2bF standard previously digested with fucosidase with 16 U of enzyme and incubate at 37 °C for 1 h. The digest solution has a standard concentration of 1 µg/mL.

System setup

System decontamination (base and EDTA washes)

Decontamination is recommended when routinely working with the low sample amounts reported in this technical note. This process removes or minimizes small peaks that could interfere with identification of low abundance oligosaccharides. Wash the system and PEEK tubing with 1 M NaOH, prior to installing the Dionex CarboPac PA200 column, following procedures in the Dionex CarboPac column manual (Document No. 031992). The 1 M NaOH is prepared by diluting 52 mL of 50% sodium hydroxide to 1 L with DI water as described in TN71. Place all four eluent lines to the 1 M hydroxide bottle, and wash all four lines and the proportioning valve by proportioning the pump 25/25/25/25 for 1 h at 0.5 mL/min. To make sure all surfaces come in contact with sodium hydroxide, rotate the injector valve. This can be done using the 'Toggle Injection Valve' tab on the Autosampler panel in the Thermo Scientific™ Chromeleon™ CDS software. Following the base wash, repeat the process with DI water. Then wash the system with 6.5 mM (2.4 g/L) disodium EDTA for 1 h at 0.5 mL/min. For the EDTA wash, the gold electrode is removed from the cell, and replaced by a spacer block (P/N 062158). Note that if using a disposable electrode, the spacer block is already installed. The EDTA wash is followed by rinsing with DI water for 1 h at 0.5/min.

Plumbing the chromatography system

To plumb the chromatography system, use black PEEK (0.010 inch, P/N 052306) tubing between the pump and the injection valve, and red PEEK (0.005 inch, P/N 052310) tubing after the injector valve. Install a gradient mixer (GM-4) between the pump and the injection valve. Install about 10 cm of red PEEK tubing after the detector (for backpressure that reduces detector noise) before connecting it to the waste line. In a typical set up, the total length of red PEEK tubing between the injection valve and the detector cell is approximately 120 cm. The approximate length of tubing from injection valve to the base of the heat exchanger is 19 cm; inside the heat exchanger, 47 cm; from heat exchanger to guard column, 25 cm; guard column to analytical column, 10 cm; and analytical column to detector, 20 cm. For the best agreement of index values between chromatography systems the individual lengths of red PEEK tubing between the injection valve and detector cell should be equal.

Guidelines and recommendations

General

Replace the autosampler flush water and the rear seal wash water weekly.

Evidence of biological growth/contamination of eluents or in wash/flush water first appears in the 40–50 min region in the chromatogram of a DI water injection. (Figure 1, chromatogram B). If contamination is observed, repeat the base and EDTA wash (described in the System setup section), with the column set removed, and prepare fresh eluents.

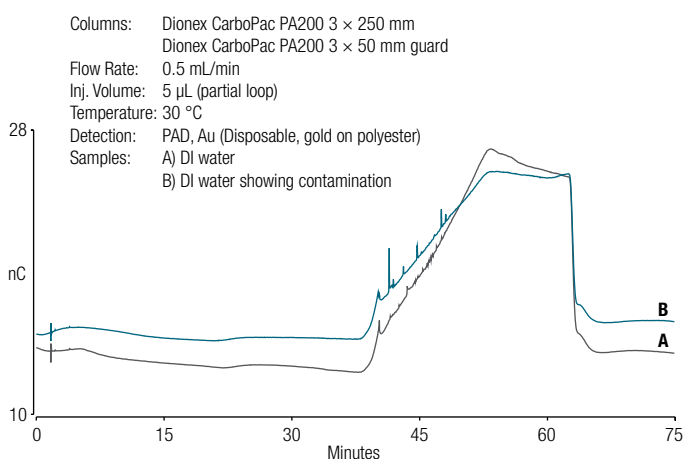


Figure 1. DI water injections illustrating system peaks associated with system contamination. A) DI water injected on a system that does not show signs of contamination. B) DI water injected on a system showing signs of contamination.

Autosampler

Autosampler performance affects reproducibility. To ensure injection reproducibility, first check that the flush line, sample syringe, and sample vials are free of air bubbles. Additional autosampler troubleshooting information is available in the Dionex AS-AP Autosampler manual.

Data analysis in the Chromeleon CDS software

Retention time ladder and retention index values

Retention indexes are used to correct retention time variations between systems and analysts, and facilitate peak assignment. They are calculated by the Chromeleon CDS software based on the variables entered for retention time standards in the component table of the Processing Method Editor. In the Chromeleon CDS software, Retention Index (Ret. Index or RI) for a component is tabulated in the Processing Method Editor. On the Component Table window, right-click to open the Table Columns window. Select Ret. Index and move it up as a visible variable.

For the current oligosaccharide analysis, we assign three retention time standards to be used as reference peaks, i.e., we use the ladder of maltose, maltotriose, and maltotetraose, which are assigned arbitrary retention indices of 200, 300, and 400. In the Ret. Index column of these peaks, enter 200, 300, and 400. The RI values of all other peaks remain empty; they are calculated by means of interpolation, and listed in the Interactive Results window.

By default, the Chromeleon CDS software calculates the following linear retention index:

where,

$$RI_S = RI_Z + ([RI_{Z+1} - RI_Z] \times [t_S - t_Z] / [t_{Z+1} - t_Z])$$

RI = Retention index (e.g., in this case, RI = 200, 300, 400 is entered)

S = Substance

Z = number of the marker

The following is true:

$$t_Z < t_S < t_{Z+1}$$

To specify which relationship and standards the Chromeleon CDS software uses for calculating the retention index, right-click on the Retention Index column, in the Interactive Results window to define the properties. Enter `peak.ri("lin","Extr-IncExp KI","refSample","injection.evaluate("\injection.type\") = \"Check Standard\"){}` for

Formula. This formula specifies a linear ("lin") relationship between the retention times and the defined retention index and also specifies extrapolation for sample peaks with retention times outside of the three retention time standards (Figure 2).

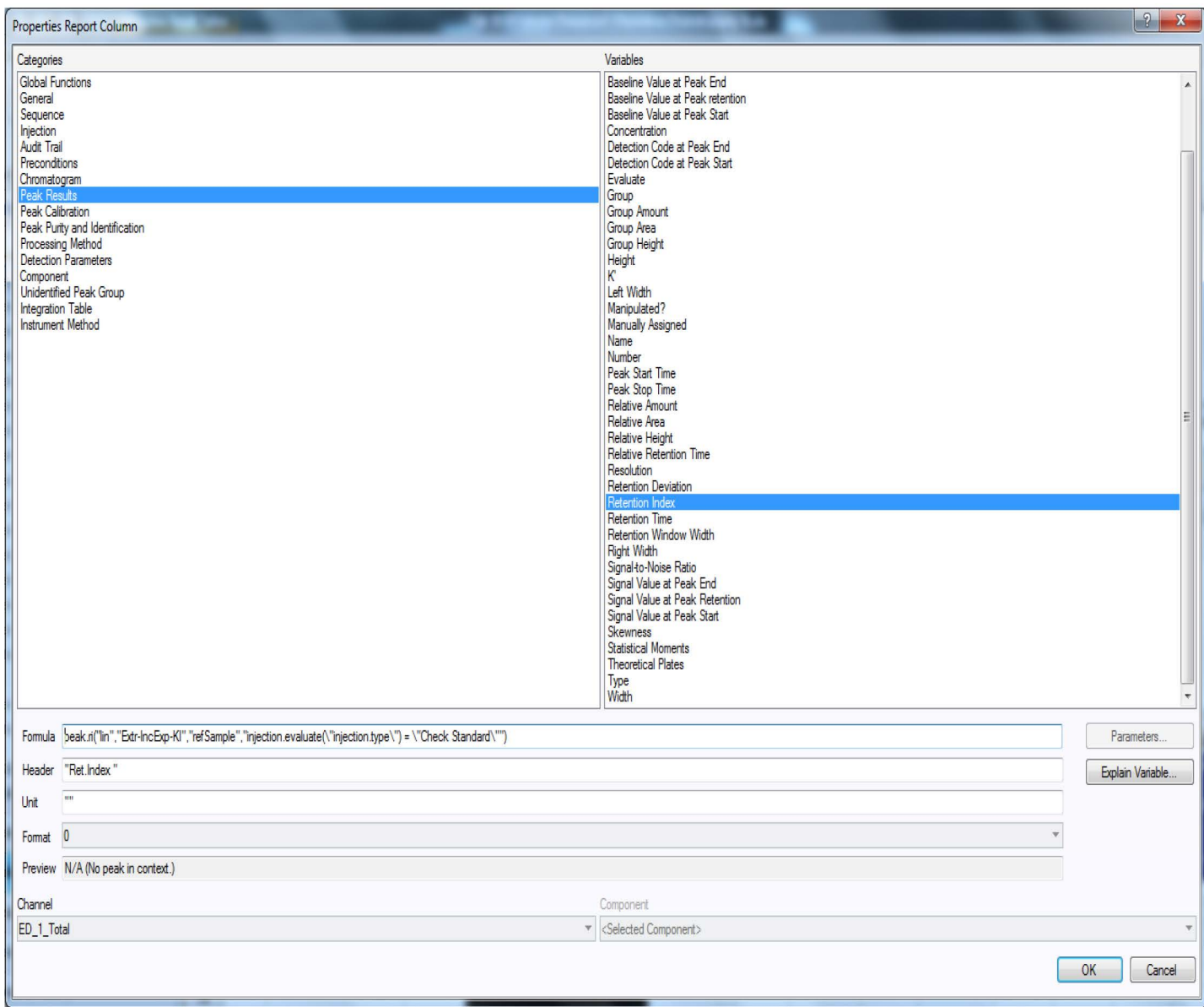


Figure 2. Chromeleon CDS software window showing the retention index formula.

These settings may also be set within the Retention Index dialog as shown in Figure 3. For oligosaccharide analysis, the following are checked: (i) Include expected retention times for not detected component, (ii) Subtract dead time (Kovats index), and (iii) Extrapolate if the peak's retention time is outside the retention time reference range.

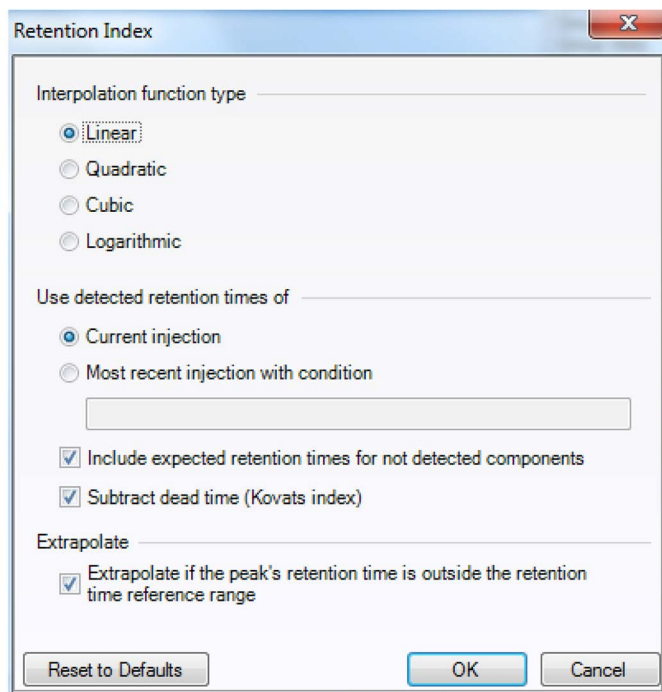


Figure 3. Chromeleon CDS software window showing the parameters of Retention Index.

For setting the other parameters, click on the Parameters tab. Click “*Most recent injection with condition*” to use the retention times of the most recent injection that meets a certain condition. The condition is entered in the box, injection.evaluate(“injection. Type”) = “Check Standard”.

When injecting the combined maltose, maltotriose, and maltotetraose standard as a retention time standard it is important that the injection be labeled as a check standard in the sequence. The Chromeleon CDS software will use the check standard that is injected before the samples for the reference retention times. It is recommended that sets of samples be bracketed with this retention time standard to correct for any changes that may occur during a sequence.

Chromatographic parameters of the retention time standards

Typical chromatographic parameters for the retention time standards were as follows:

- (i) maltose, retention time was 10.8–13.6 min, asymmetry was 0.9–1.06
- (ii) maltotriose, retention time was 30.7–37.5 min, asymmetry was 0.9–1.16
- (iii) maltotetraose, retention time was 42–42.7 min, asymmetry was 0.9–1.19

These data were from over 100 injections of the mix of standards and three column production lots on two systems.

Mechanical polishing of conventional gold working electrodes

New conventional (nondisposable) working electrodes should first be polished with coarse powder (P/N 36319). Apply 2–3 mL of water on a fresh polishing pad and polish for 1 min. This step removes the coarse polishing powder particles embedded in the gold material. Repeat the above steps with the fine polishing compound (P/N 36318) on a fresh piece of polishing cloth. Assemble the ED cell including an electrode with a 1 mil gasket. If necessary, wait for at least 2 h for the response to stabilize. Future working electrode polishing may be necessary if detection sensitivity is lost and working electrode fouling is suspected (i.e., system contamination, autosampler problems, reference electrode problems, and cell leaking have been ruled out). Note: Do not polish disposable electrodes. If using disposable gold electrodes, use the 2 mil gasket supplied with the electrodes.

Results and discussion

Separation

The separation in this technical note was designed to separate the 14 most abundant uncharged (neutral) *N*-linked oligosaccharides and the major sialic acid-containing (charged) oligosaccharides of human serum polyclonal IgG, as well as the high-mannose type oligosaccharides sometimes found on recombinant IgGs. The 14 neutral oligosaccharides make up nearly 100% the neutral oligosaccharides of the IgG, and the six charged oligosaccharides represent approximately 84% of the charged oligosaccharides of the IgG.⁵ A separation meeting those criteria should be able to separate the majority of *N*-linked oligosaccharides found on mAbs, each of which typically has less oligosaccharide microheterogeneity than polyclonal IgG. A review of the published HPAE-PAD methods for IgG *N*-linked oligosaccharide separation, and a check of the better methods in our lab, revealed that there was some improvement needed to meet our goal. The higher quality published separations used 50 or 55 mM NaOH and a low concentration gradient of sodium acetate.^{6,7} The separation shown here starts at 50 mM NaOH and uses a linear gradient of NaOH to 100 mM over 30 min and is held at 100 mM for 5 min before the introduction of 15 min gradient of sodium acetate to 200 mM to separate the charged oligosaccharides. The final gradient conditions are maintained for 15 min before conditions are returned to initial conditions for 15 min prior to the next injection. These conditions achieved the desired separation of the neutral oligosaccharides, but did impact the appearance of the chromatogram. Electrochemical detection is sensitive to pH, so the gradient of hydroxide causes a positive slope in the baseline. Additionally, a method starting with a hydroxide eluent that does not contain sodium acetate, and then switching to a sodium acetate-containing eluent, will exhibit a change in the baseline that has the appearance of a peak when the sodium acetate is introduced. This is the result of the column changing from the hydroxide form to the acetate form. The small peak represents the eluted hydroxide that was bound to the column. The sodium acetate gradient also causes a rise in the baseline as electrochemical detection is also sensitive to ionic strength. These baseline changes are insignificant when working at high analyte concentrations but are noticeable when using the low quantities of oligosaccharides we chose to work with in this technical note. The initial work suggested that the disposable gold on polyester and the conventional gold

working electrodes gave better baseline performance than the gold on PTFE disposable working electrode, but in subsequent work we observed no significant difference in performance among the three working electrodes. For recommendations on using disposable gold working electrodes, please see Technical Note 110.⁹

Figure 4 shows a separation of the *N*-linked oligosaccharides released from a commercial source of human serum IgG. The peaks are identified by their oligosaccharide codes. The structures that correspond to those codes are shown in Figure 5. Oligosaccharide peaks were identified by a combination of separation of standards, treatment of standards with exoglycosidases, treatment of samples with exoglycosidases, knowledge of the most abundant IgG oligosaccharides, and the empirical rules of HPAE-PAD *N*-linked oligosaccharide separations.⁹ The primary method of peak identification is shown in the fourth column of Table 2 (page 10). Figure 6 shows an example of how exoglycosidases were used to identify oligosaccharide peaks. The A1F standard, which contains the two positional isomers of A1F (*N*-acetylneuraminic acid on either the Man α 1,6 or Man α 1,3 branch), was treated with fucosidase. The chromatogram shown in orange illustrates chromatography of the standard with the two isomers labeled A1F and A1F'. The chromatogram shown in blue illustrates chromatography of the standard after treatment with the fucosidase. This should result in the disappearance of the A1F and A1F' peaks, and the appearance of two new peaks, A1 and A1', which elute later than A1F and A1F' according to the empirical rules of HPAE-PAD *N*-linked oligosaccharide separations.⁹

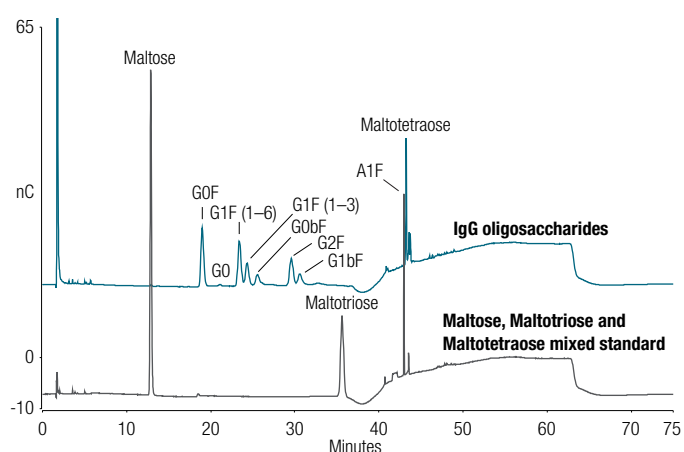


Figure 4. Separation of the PNGase F digest human serum IgG (teal trace) and the retention index standard (gray trace). The conditions are listed in the Conditions section of this technical note.

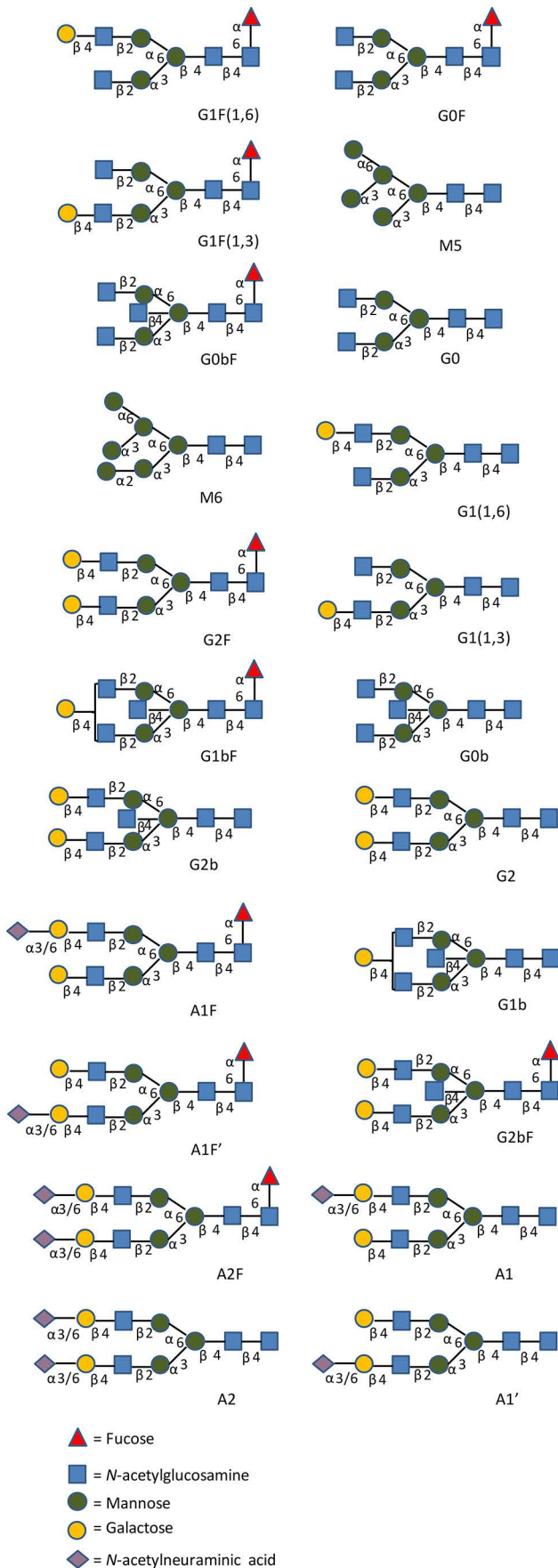


Figure 5. IgG oligosaccharide structures.

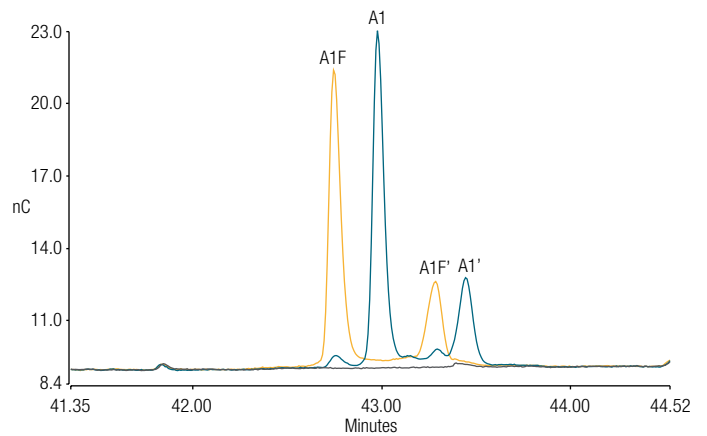


Figure 6. Fucosidase digestion of the A1F standard. The gray trace is the enzyme blank chromatogram, the orange trace is the A1F standard chromatogram, and the teal trace is the fucosidase digestion of the A1F standard

This is observed, although it appears the digestion did not proceed to completion as small peaks remain for A1F and A1F'. The chromatogram shown in black is a DI water injection that shows that the small peak at 41.85 min is not part of the A1F standard.

The major peaks in the human serum polyclonal IgG separation (Figure 4) are labeled. These are G0F, G0, the two isomers of G1F, G0bF, G2F, G1bF, and A1F. This preparation of human serum polyclonal IgG has very few afucosylated structures as demonstrated by the small peak for G0. Other human serum IgG preparations had significantly greater amounts of afucosylated structures.³ Though not shown, M5 and M6 are resolved from IgG oligosaccharides.³ Of the target neutral oligosaccharides, only G0bF was not resolved from the G1F.^{1,3,6} G0bF is a minor species and will not be found in mAbs produced with the currently used expression systems. This IgG preparation has little if any A2F and the high level of fucosylation in this preparation is also shown by the absence of the afucosylated sialylated structures. To shorten analysis time the separation was not designed to maximize resolution of the sialylated oligosaccharides. Sialylated oligosaccharides make up only about 10% of the oligosaccharides in human serum IgG and 90% are monosialylated.⁵ In human IgG, the sialic acids can be linked to either galactose $\alpha 2,3$ or $\alpha 2,6$. HPAE-PAD can resolve $\alpha 2,3$ and $\alpha 2,6$ isomers, but this separation did not attempt to resolve those isomers. A monoclonal antibody expressed in Chinese hamster ovary cells has only $\alpha 2,3$ linked sialic acids on its N-linked oligosaccharides. Figure 4 represents a PNGase F digest of 12 μ g of human polyclonal IgG. While this work demonstrates that the method is applicable to low sample quantities, using larger amounts (e.g., 5–10X) may be preferable to some analysts.

Table 2. Oligosaccharides and their Retention Index (RI) values.

Oligosaccharide Acronym	RI Value*	RI Value Confirmation**	Primary Oligosaccharide Identification Method
G0F	228	227	Galactosidase of the GIF standard
M5	232		Standard
G0	236	235	Standard
G1F(1-6)	247	246	Standard
G1F(1-3)	251	250	Standard
G0bF	259	255	Galactosidase of the G2bF standard
G1(1-6)	258		Fucosidase of the G1F standard
G1(1-3)	263		Fucosidase of the G1F standard
G0b	266		Galactosidase and Fucosidase of the G2bF standard
M6	269		Standard
G2F	274	273	Neuraminidase of the A2F standard
G1bF	281	277	Partial galactosidase of the G2bF standard
G2	290		Fucosidase of IgG
G1b	291		Fucosidase and partial galactosidase of the G2bF standard
G2bF	316		Standard
M7	323		Standard – multiple isomers
G2b	341		Fucosidase of the G2bF standard
M8	381		Standard – multiple isomers
M9	389,393		Standard – two peaks in this preparation
A1F	403,407	403, 411	Standard
A1F'	404,409		Standard
A1	405,410		Fucosidase of the A1F standard
A1'	405,410		Fucosidase of the A1F standard
A2F	424, 429		Standard
A2	426		Fucosidase of the A2F standard

*RI values of 200, 300, and 400 were assigned to maltose, maltotriose, and maltotetraose to create the values in this table as described elsewhere in this technical note.

**These values were from an analyst that set up a third system with a fourth Dionex CarboPac PA200 column about two years after collection of the RI values in the column to the left. Values were only measured in a human serum IgG separation.

To use this separation to tentatively identify *N*-linked oligosaccharides from unknown IgG samples, including mAbs, we measured retention index (RI) values for each oligosaccharide relative to maltose, maltotriose, and maltotetraose, which were assigned values of 200, 300, and 400, respectively. (For a description of this analysis please see the *Data analysis in the Chromeleon CDS software* section of this publication.) A chromatogram of these three standards is shown in Figure 4 and the RI values are listed in the second column of Table 2. These values should allow for more confidence when using this method on different chromatography systems with different eluent preparations, different Dionex

CarboPac PA200 columns, and different analysts. The values in the second column of Table 2 were produced by two analysts on two different systems with more than two eluent preparations, and three Dionex CarboPac PA200 columns from two different lots. During these experiments, retention time varied significantly more than RI values.³ Each of the aforementioned parameter variations still produced the separation of the IgG oligosaccharides and peak identification was unaffected. In other words, despite retention time variation, all peaks were separated and produced unique RI values (i.e., variation in a given experiment was to either lower retention or higher retention, and not to a mixture of lower

and higher retention). Throughout these experiments, the RI values varied no more than 2%. The greatest variables were the eluent preparation and the column. Nearly two years later, a third system was set up with a third analyst and a fourth Dionex CarboPac PA200 column. The separation in Figure 4 is from the third analyst and the RI values determined from the peaks in the separation of a PNGase F digestion of a commercial human polyclonal IgG sample are presented in the third column of Table 2. Note that these values differ less than 2% from the values determined two years earlier.

Conclusion

This technical note provides a detailed description of the setup and execution of an HPAE-PAD method to resolve the major *N*-linked oligosaccharides of IgGs, including mAbs. The method uses RI values established by the chromatography of three sugar standards to assist in identifying oligosaccharides across different systems, Dionex CarboPac PA200 columns, and eluent preparations. Low microgram quantities of IgG are required for chromatography. The same system can be used for monosaccharide, sialic acid, and other carbohydrate analyses.

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