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Instrument configuration for native *N*-linked oligosaccharide characterization by HPAE-PAD/MS

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Keywords

Glycosylation, glycoprotein, released *N*-glycan, biosimilar, monoclonal antibody, mAb, biotherapeutics, IgG, glycan, carbohydrate, biotherapeutic, ion chromatography, IC-MS, HRAM MS, Q Exactive, CarboPac PA200 column

Goal

Provide application installation instructions for coupling HPAE-PAD analyses to the Thermo Scientific™ Q Exactive™ family of mass spectrometers. This technical note focuses on using this set up to profile *N*-linked glycans released from glycoproteins.

Introduction

The biopharmaceutical industry requires reliable, high throughput workflows for *N*-linked glycan analyses. Many of the commonly used analytical approaches are based on glycan derivatization, which is known to introduce issues such as differential loss of sialic acid and incomplete labeling. Preparation of native glycans is not subject to these concerns. This approach starts with a fast in-solution glycoprotein digestion, followed by a rapid removal of protein, reductant, surfactant, and salt using graphitized carbon. Using this technique, 96 samples can be processed in approximately two hours. These native glycans can then be analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). This new approach supports bias-free, in-depth characterization of *N*-linked glycans released from glycoproteins. This characterization is required for biological products according to The International Conference on Harmonization's Q6b guidelines.[1] Therefore, the present method is ideally suited for biopharmaceutical quality control (QC) including for antibody-derived glycans.

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This technical note describes a a novel workflow combining facile release of *N*-linked glycans, rapid glycan purification, and analysis by HPAE-PAD coupled to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer. High-performance anion-exchange chromatography, a type of ion chromatography (IC), supports simultaneous separation and detection of neutral and sialylated (charged) glycans without the need for derivatization. The chromatographic resolution of glycans is based on the number of sialic acid units, branch and positional isomerism, and the presence/absence of core or outer arm fucose. The Thermo Scientific™ Dionex™ ICS-5000⁺ HPIC™ system can be coupled to a Thermo Scientific™ Dionex™ UltiMate™ WPS TBPL Thermostatted Biocompatible Standard Well Plate Autosampler and a Q Exactive Orbitrap mass spectrometer independent of the PAD to separate and characterize native *N*-linked glycans. Because the HPAE separation employs high-pH eluents, post-column removal of the sodium ion is required prior to introduction into the mass spectrometer. A “tee” is placed immediately after the column to divert partial flow to each of the detectors, thus supporting both PAD and MS detection. Approximately 45% of the total flow goes to the PAD detector, and 55% to the MS through an electrochemically-regenerated desalter designed for this application.

The resulting PAD glycan profiles are reproducible, and the Q Exactive MS and MS/MS acquisition supports identification of the native glycans, including several isobaric forms, as these are virtually all separated by the Thermo Scientific™ Dionex™ CarboPac™ PA200 column. Where direct mass data is inconclusive, MS/MS spectra with diagnostic fragments allow for highly reliable annotation of the glycans and their isomers. A high resolution separation of native human α-1-acid glycoprotein (hu-AGP) glycans revealed 53 distinct glycans illustrating the exceptional resolving power of the combined HPAE and MS analyses. This method supports highly informative glycan analysis without introducing labeling bias.

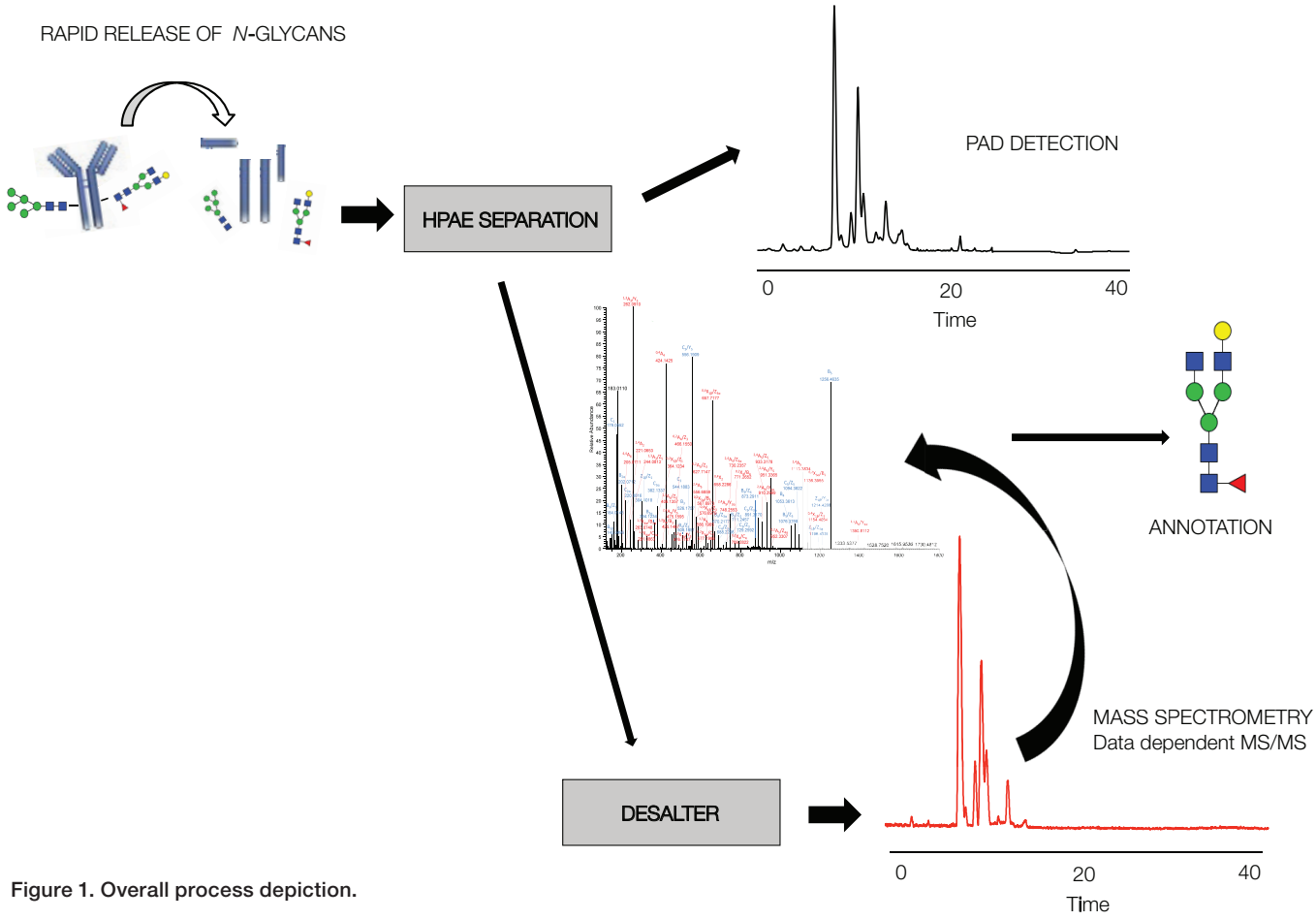


Figure 1. Overall process depiction.

Table 1. Consumables.

Product Name	Device Description	Part Number
Thermo Scientific™ Dionex™ HPAE-PAD/MS Assembly Kit	Dionex IC tubing assembly kit for the Dionex HPAE-PAD/MS detection kit Includes the components described below	302854
Thermo Scientific™ Dionex™ HPAE-PAD/MS Assembly Kit Components	Eluent Preheater to Injector tubing	302841
	Pump to Injector tubing	302842
	Injector to guard column tubing	302843
	Guard column to analytical column tubing	302844
	Analytical column to 1/16" micro Tee tubing	302845
	Micro "Tee" to ED detector cell tubing	302846
	Micro "Tee" to ERD 500 tubing	302847
	ERD 500 to MS probe inlet tubing	302848
	Back pressure/flow adjusters (3 options)	302849 302850 302841
	MicroTee Assembly PEEK- 1/16 inch (Install immediately after column outlet)	302852
	ERD 500, 2 mm	60-085089
Thermo Scientific™ Dionex™ CarboPac™ PA200 Guard Column	Guard column, 3 x 50 mm	062895
Thermo Scientific™ Dionex™ CarboPac PA200 Column	Separation column, 3 x 250 mm	062896
Electrochemical Cell	Includes knob and support block	072044
Ag/AgCl Reference Electrode	Reference electrode	061879
Au on PTFE Electrodes	Working electrode, package of six (used for carbohydrate analyses) included six 2 mil PTFE gaskets	066480
2 mil Gasket	If purchased separately, package of two PTFE gaskets	060141
pH Buffer, pH 7	Reference electrode pH calibration standard	SB107-500**
pH Buffer, pH 10	Reference electrode pH calibration standard	SB115-500**

Equipment and supplies

Thermo Scientific Dionex ICS-5000⁺ HPIC system with:

- Dual Pump (DP)
- Electrochemical detector (ED)
- Electrochemical cell, Reference electrode and disposable gold working electrode (Au on PTFE)
- Detector compartment with temperature control
- WPS TBPL autosampler with tray temperature control option (P/N 5823.0020), PEEK needle (P/N 6820.3025 (15 µL) or 6820.3023 (3.5 µL), PAEK stator (P/N 6820.0034), Valcon E3 rotor (6820.0035, PAEK), 2 mL vial tray (6820.4070) and 1.5 (P/N 079812) or 0.3 mL (P/N 055428) vials.
- 4 L Bottle for supplying DI to the ERD 500 (P/N 063292)
- Thermo Scientific™ Chromeleon™ CM7.2 SR4 Chromatography Data System (CDS) software
- Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer*
- Fisher Scientific™ Isotemp™ Digital Dry Bath/Block Heater (Fisher Scientific P/N 88-860-21)

*This method can be applied to any Q Exactive mass spectrometer

Reagents and standards

- Deionized (DI) water, Type I reagent grade,18 MΩ-cm resistivity or better
- Sodium Hydroxide, 50% w/w (Fisher Scientific P/N SS254-500)
- Sodium Acetate, anhydrous, electrochemical grade (P/N 059326)
- 10x PBS buffer (Sigma Aldrich P/N P5493)
- HEPES (Sigma Aldrich P/N H3375-100G)
- Sodium perfluorooctanoate (PFOA, Alfa Aesar P/N L16988)
- N-glycanase® Ultra (PNGase F, ProZyme, Inc, P/N GKE-5020D)
- 96 well PCR plate (Fisherbrand, P/N 14230237)
- HyperSep Hypercarb Filter Plate, 40 µL bed volume (Thermo Fisher Scientific, Rockford, IL, P/N 60110-504)

Sample preparation

Glycoprotein solutions are dissolved to 10 mg/mL in 1x PBS buffer. Afterwards, 20 µL of glycoprotein solutions were dispensed in the wells of a 96 well PCR plate. In order, 2 µL of 0.5 M HEPES buffer (pH 7.9), 2 µL of 200 mM TCEP solution and finally 2 µL of 12% (w/w) PFOA detergent solution was added and mixed thoroughly by pipette action. This mixture was incubated at 95 °C for 5 min to denature the protein. Two µL of undiluted PNGase F are added to the mixture followed by incubation at 50 °C for 5 min. After deglycosylation, the glycans were purified using graphitized carbon in a filter plate format.

High-performance anion-exchange chromatography

A Dionex ICS-5000⁺ HPIC system with WPS autosampler performs the glycan separations on a 3 × 250 mm Dionex CarboPac PA200 column with PAD detection at 0.5 mL/min flow rate. Desalting is accomplished with a Thermo Scientific™ Dionex™ ERD 500 Electrolytically Regenerated Desalter using 350 mA current and 3.5–4.0 mL/min regenerant water flow. The column flow is split immediately post column and 250–300 µL/min (50-60% of the total flow) was diverted to the suppressor and into the MS.

Mass spectrometry

A Q Exactive Orbitrap mass spectrometer used in negative ion electrospray mode was coupled to the IC system. The spray voltage was 3.2 kV and the capillary temperature was set to 320 °C. The probe heater temperature was 200 °C. The sheath and auxiliary gas flow were 30 and 10 respectively (arbitrary units). MS spectra were acquired at *m/z* 400-2000 over the chromatographic separation at 70,000 FWHM resolution. The MS automatic gain control (AGC) target was set to 3×10^6 and the maximum injection time was 120 msec. Data dependent MS/MS experiments for the top 10 ions were performed using a normalized collision energy (NCE) of 30 kV. For MS/MS experiments the AGC target value was 1×10^5, the maximum injection time was 300 msec and resolution was 17,500 FWHM. The number of microscans in both MS and MS/MS experiments was 1, quad isolation window was 1.5 *m/z*. The underfill ratio and intensity threshold values were set at 5 % and 2×10^4 respectively. For data acquisition and processing, Thermo Scientific™ Xcalibur™ software 3.0 was used. MS/MS experiments were evaluated using SimGlycan v 5.0 software (PREMIER Biosoft, Palo Alto, CA). Support for SimGlycan is available at Support@premierbiosoft.com

Eluent preparation

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.

250 (100) 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 20.5 g (8.2 for 100 mM) of anhydrous, electrochemical grade sodium acetate. Add the solid sodium acetate steadily to the stirring water to avoid clump formation. 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 (3 to 6 psi) at all times. Guidance to help avoid problems with eluent preparation for HPAE-PAD can be found in Technical Note 71.[2]

System configuration and method details

Experimental conditions

Column	Dionex CarboPac PA200 (3 × 250)
Eluents	A: DI Water B: 100 mM NaOH C or D: Eluent B with 0.1 M or 0.25 M Sodium Acetate
Flow Rate	0.5 mL/min
Column Temperature	30 °C
Detector Temperature	30 °C
Injection Volume	1–10 µL (microliter pickup option)
Detection	PAD: 4-potential carbohydrate waveform 2 Hz (see box below)
Reference Electrode	Ag/AgCl
Working Electrode	Au on PTFE 0.002” gasket
Desalting	ERD 500 2 mm
Mass Spectrometry	Q Exactive MS Scan conditions are provided in the Mass Spectrometry Section below

Table 2. 4-Potential carbohydrate waveform (Waveform A*)

Time (s)	Potential (V)	Integration
0.00	+0.10	—
0.20	+0.10	Begin
0.40	+0.10	End
0.41	-2.0	—
0.42	-2.0	—
0.43	+0.6	—
0.44	-0.1	—
0.50	-0.1	—

*Waveform A is discussed in TN 21.[3]

Separation conditions

Gradient 1: Used to resolve neutral and sialylated glycans from IgG samples

A: DI water
B: 100 mM NaOH
C: 100 mM sodium acetate in 100 mM NaOH

Time (min)	A (%)	B (%)	C (%)	Slope
0.00	74	24	2	5
5.00	74	24	2	5
17.50	47	46	7	5
44.00	5	0	95	4
48.90	5	0	95	5
49.00	74	24	2	5
74.00	74	24	2	5

Gradient 2: Used to resolve glycans released from glycoproteins harboring primarily charged (sialylated) glycans

A: DI H_2O
B: 100 mM NaOH
C: 250 mM sodium acetate in 100 mM NaOH

Time (min)	B (%)	C (%)	Curve
0.00	93.60	6.40	5
60.00	52.00	48.00	5
75.00	52.00	48.00	5
75.10	93.60	6.40	5
90.10	93.60	6.40	5

Gradient 3: An improved gradient used to resolve glycans from mAbs or other glycoproteins with predominantly neutral glycans

A: DI H_2O
B: 100 mM NaOH
C: 100 mM sodium acetate in 100 mM NaOH
D: 250 mM sodium acetate in 100 mM NaOH

Time (min)	A (%)	B (%)	C (%)	D (%)	Curve
0.0	50.0	46.5	3.5	0.0	5
17.0	47.0	44.0	9.0	0.0	6
50.0	0.0	0.0	100.0	0.0	5
50.1	0.0	0.0	35.0	65.0	5
54.9	0.0	0.0	35.0	65.0	5
55.0	50.0	46.5	3.5	0.0	5
70.0	50.0	46.5	3.5	0.0	5

Instrument setup and installation

The Dionex ICS-5000⁺ HPIC system is configured for electrochemical detection, operating under high-pressure conditions up to 5000 psi. To install this application, connect the Dionex WPS autosampler and the Dionex ICS-5000⁺ HPIC system modules as shown below using the HPAE-PAD/MS Assembly Kit.

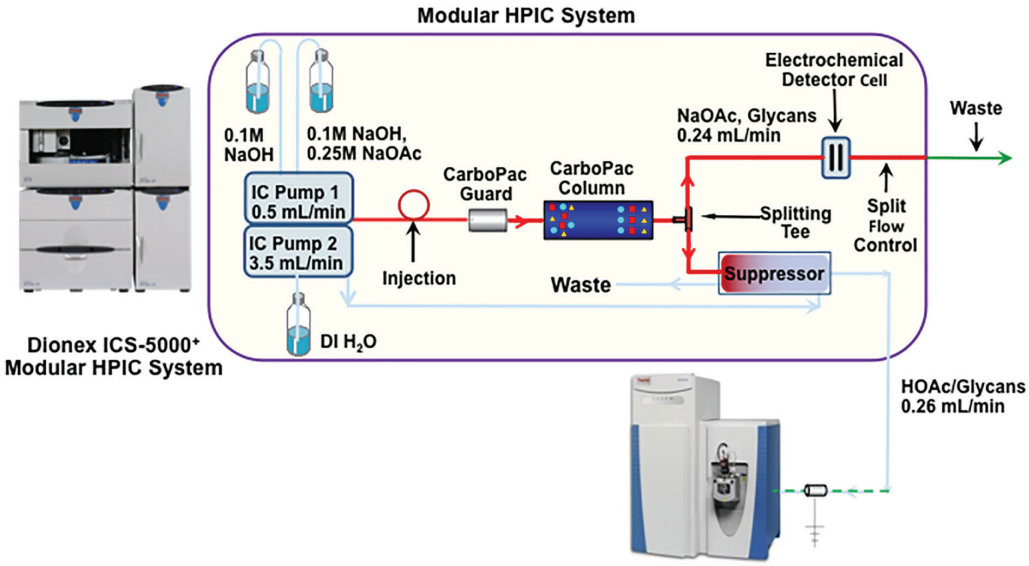
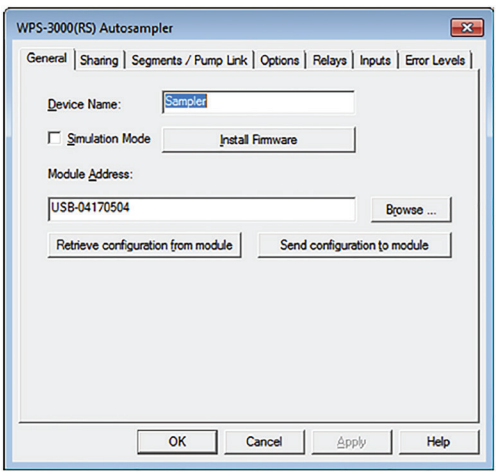


Figure 2. Configuring the IC system for connection to the Q Exactive mass spectrometer.

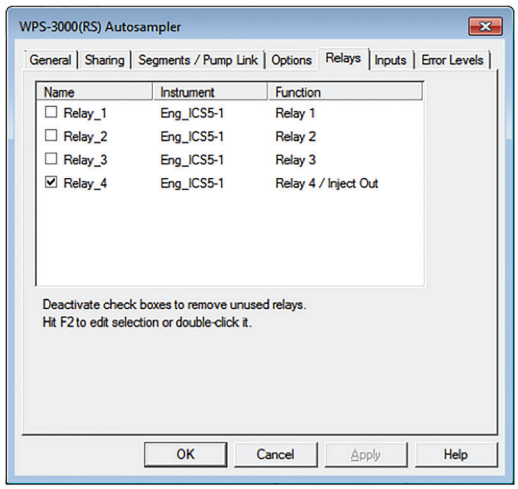
Electronic configuration

These configuration instructions assume that the Dionex ICS-5000⁺ HPIC system and Q Exactive Mass Spectrometer have been installed by Unity Lab Services (ULS) personnel and is demonstrated to be fully functional and in calibration.

To configure the coupled instruments, first start the Chromeleon Instrument Controller program and then select the link, *Configure Instruments*, which starts the Chromeleon Instrument Configuration Manager. Right-click on the computer name, select the Instrument to open a drop-down list of modules and select the WPS Autosampler.



Select the “Relays” tab and check the “Relay_4” box.



Click “OK” and make sure you then click the “Send Configuration to Module” button to install the changes you just made to the WPS sampler configuration. When the Autosampler configuration transfer is finished, click OK to complete the Sampler configuration, and save the new configuration by clicking on the “Save Installation on Instrument Controller” button on the Chromeleon Instrument Configuration Manager’s Menu Bar.

Install the relay interface cable from the WPS-300TB Ship-kit box to the “Digital I/O” connection 4, and the positive and negative *Digital Out* wires to the positive and negative relay inputs, respectively, on the Q Exactive mass spectrometer.

Electrochemical cell

Always wear gloves when handling the electrochemical cell. If this is a new ED cell, disassemble the cell and discard the shipping gasket.

Caution: Do not touch the working electrode with any paper products, as this can contaminate the working electrode.

The ED cell is a three-electrode cell: the counter electrode (cell body), a reference electrode (pH-Ag/AgCl), and a working electrode (conventional or disposable). The installation procedures are thoroughly discussed in the ED User’s Compendium for Electrochemical Detection.[4]

The fully assembled cell also includes a yoke block assembly to tighten the cell and a gasket to create a thin layer channel around the working electrode. The installation procedures below describe an electrochemical cell with a disposable working electrode; however, the procedures are similar when using a conventional working electrode. Different gaskets are available, but we used the 0.002” standard gaskets supplied with the disposable electrode kits. A support block is necessary when using a disposable working electrode, whereas the conventional working electrode is manufactured in a support block.

Note: Use 18 MΩ-cm DI water to prepare standards, eluents, and autosampler flush solutions. We recommend degassing of DI water used for eluents in HPAE carbohydrate methods. (Vacuum filtration can be an appropriate degassing method, but online-degassing is also an available option on the Dionex ICS-5000 DP pump module).

Installing the disposable electrode

Install the disposable working electrode over the gasket with the working surface (Au spot) facing the gasket. Install the support block firmly over the working electrode. Attach the yoke block by squeezing the opposing tabs and sliding it down to the cell body. Align the yoke block parallel to the cell body and rotate the yoke block sealing knob clockwise until you hear (or feel) three “clicks”. The cell with a conventional working electrode (P/N 061749) is assembled similarly with appropriate gasket (P/N 045972). For more information on successful use of disposable working electrodes see Technical Note 110.[5] The support block is not needed with the conventional working electrode.

Installing the pH-Ag/AgCl reference electrode into the electrochemical cell

First, condition the pH-Ag/AgCl reference electrode by removing the storage cap, rinsing the electrode with DI water to remove the potassium chloride solution, and calibrating the reference electrode in both pH 7 and pH 10 buffer solutions as described in the next section. The installation procedures are thoroughly discussed in the ED User’s Compendium for Electrochemical Detection.[4] Insert the reference electrode into the cell; ensure that no plugs are installed on the cell inlet and outlet (to avoid excess hydraulic pressure). Verify that the pH-Ag/AgCl reference electrode O-ring and spacer are present, then screw the pH-Ag/AgCl reference electrode into the reference electrode threads until it is finger-tight. Orient the cell assembly with the yoke knob on the bottom left and push the cell onto its mounting receptacles on the ED electronics case. If the detector is already installed, connect the reference electrode cable and the cell cable.

Calibrating the reference electrode

To calibrate the reference electrode, prepare two cups – one with pH 7 buffer and one with pH 10 buffer. Install the cell into the ED module and connect the yellow cable to the yellow port. Install the reference electrode (blue cable) into the black port. Immerse the reference electrode in the pH 7 buffer to completely cover the electrode's active surface. Select the "pH Calibration" button on the ED panel in the Chromeleon Panel "Tabset" and follow the instructions to calibrate the electrode at pH 7. When finished, continue the calibration with pH 10 buffer. The results appear in the audit trail and on the ED Calibration panel window. Install the reference electrode in the ED cell body and reconnect the reference electrode lead (blue) to the black ED receptacle.

Completing the plumbing

Complete the installation by installing the components in the HPAE-PAD/MS kit in their respective positions in the plumbing sequence as indicated in Figure 1 and Table 1. Ensure that all tubing is bottomed into the fittings and all ferrules are immovably seated.

Determining flow to the mass spectrometer

The IC/MS connection kit contains three lengths of 0.005" ID (red) tubing to apply backpressure to the ED cell. These are 90, 100, and 110 cm long. Attach the 100 cm length to the ED cell outlet.

Measure the suppressor eluent flow

1. Set the pump flow to the initial eluent conditions at 0.5 mL/min, and measure the flow through the suppressor by collecting the suppressor eluent channel elution over a 1 min period into a tared vial.
2. Subtract the tare weight (mg) from the collected weight (mg) to obtain the flow in µL/min.
3. If the flow is less than 50% of the total flow (i.e., <250 µL/min), stop the pump, and exchange the 100 mm tubing for the 110 mm tubing at the ED cell outlet. If the flow is more than 60% of the total flow (>300 µL/min), stop the pump and exchange the 100 mm tubing for the 90 mm tubing at the ED cell outlet.
4. Repeat steps 1 and 2 to verify the eluent flow through the suppressor is between 250 and 300 µL/min

Creating an instrument method

To create a new instrument method using the Chromeleon Wizard, select Create, Instrument Method and select Instrument. Enter the values from the Chromatographic Conditions section and add those in Table 3. Save the instrument method.

Table 3. Additional conditions to create a program.[3]

Page Title	Mode	Action
Sampler Options	Injection Mode	"Partial" or "µL Pickup" (µL Pickup requires provision of a transport fluid vial and related commands in the program. Please see the Dionex WPS autosampler operators manual)
	Loop Overfill	3
	Injection Wash Property	After Injection
	Wait for Temperature	Click the checkbox
	Reference	pH-Ag/AgCl
EDet Options	Select Waveform	Au, 4-Potential Carbohydrate
	pH	Lower limit = 10 Upper limit = 14
	Data Collection Rate	2.00 Hz
	Autozero	Yes

Continuous pH and column temperature monitoring

pH is an important parameter in electrochemical detection. Unexpected changes in pH can indicate an unstable reference electrode or improperly prepared eluents. To implement continuous monitoring of the pH, open the script section of the method, create an empty row after EDet. Autozero, insert “VirtualChannel” as the command line, and enter the commands as shown in Figure 3. The pH readings will be visible as one of the channels with each data file. Save the instrument method. Similarly, add the commands to implement column temperature monitoring (Figure 3).

0.00	**Inject**	
	EDet1.Autozero Wait	 Sampler.Ready
0.00	**Start Run**	
	EDet1.ED_1.AcqOn EDet1.ED_1_Total. AcqOn Pump_1.Pump_1_ Pressure.AcqOn	
	VirtualChannel	“pH”, EDet1.pH, Type=Analog Unit=”pH’ Evaluate=Yes
	VirtualChannel	“Column_Temp”, DC.Column_ TC.Temperature Type=Analog Unit=“°C”, Evaluate=Yes

Figure 3. Script commands for continuous pH and column temperature monitoring.

Results and discussion

High-performance anion-exchange chromatography is able to resolve native *N*-linked glycans released from glycoproteins with complex glycosylation patterns.[6] Figure 4 highlights that after efficient salt removal and using the set up described here, even minor glycans of the total glycome released from hu-AGP can be reliably annotated. For example, peak 3 is a very minor glycan peak observed in both the PAD and base peak MS chromatograms. Mass accuracy of the precursor ion is below 2 ppm, allowing analysts to look up glycans from different databases (Figure 4). If the requirement is to obtain detailed structural information, highly informative MS/MS spectra generated by HCD fragmentation can be entered in different data search engines, such as SimGlycan, and structures are rapidly annotated.

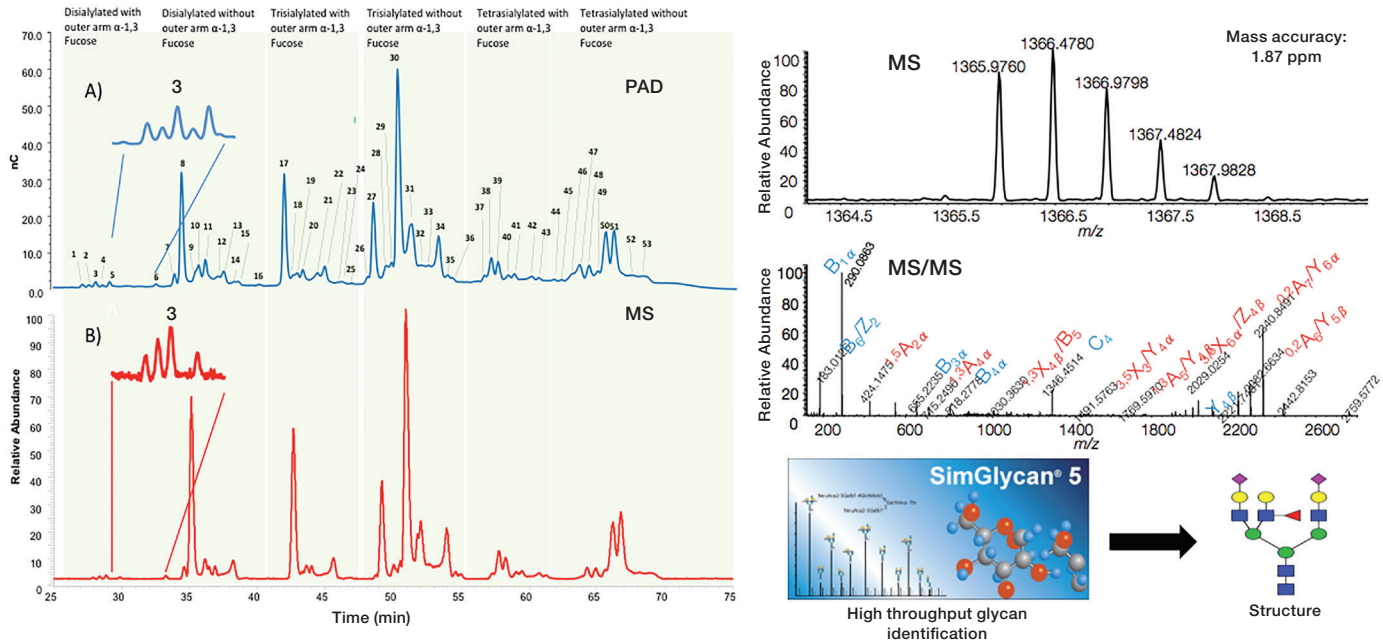


Figure 4. General workflow of high-performance anion-exchange chromatography coupled to mass spectrometry for *N*-linked glycan annotation. Glycan annotation is based on the Consortium of Functional Glycomics.[7]

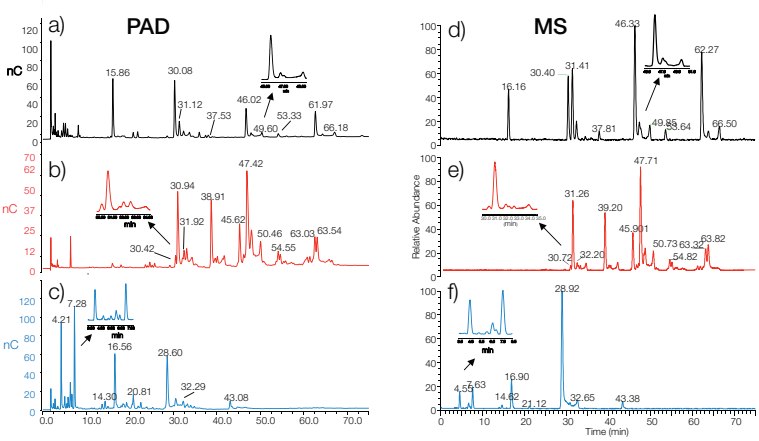


Figure 5 compares traces monitored simultaneously by the two different detection techniques. As shown, resolution obtained by electrochemical detection does not materially deteriorate in the MS base peak chromatograms.

The generated MS/MS spectra, which are rich in cross-ring fragmentations, allow analysts to identify known (Figure 6a) and unknown structures (Figure 6b).

Figure 5. Comparison of PAD and MS traces of N-linked glycans released from bovine fibrinogen (a and d), hu-AGP (b and c) and bovine fibrinogen (c and f).

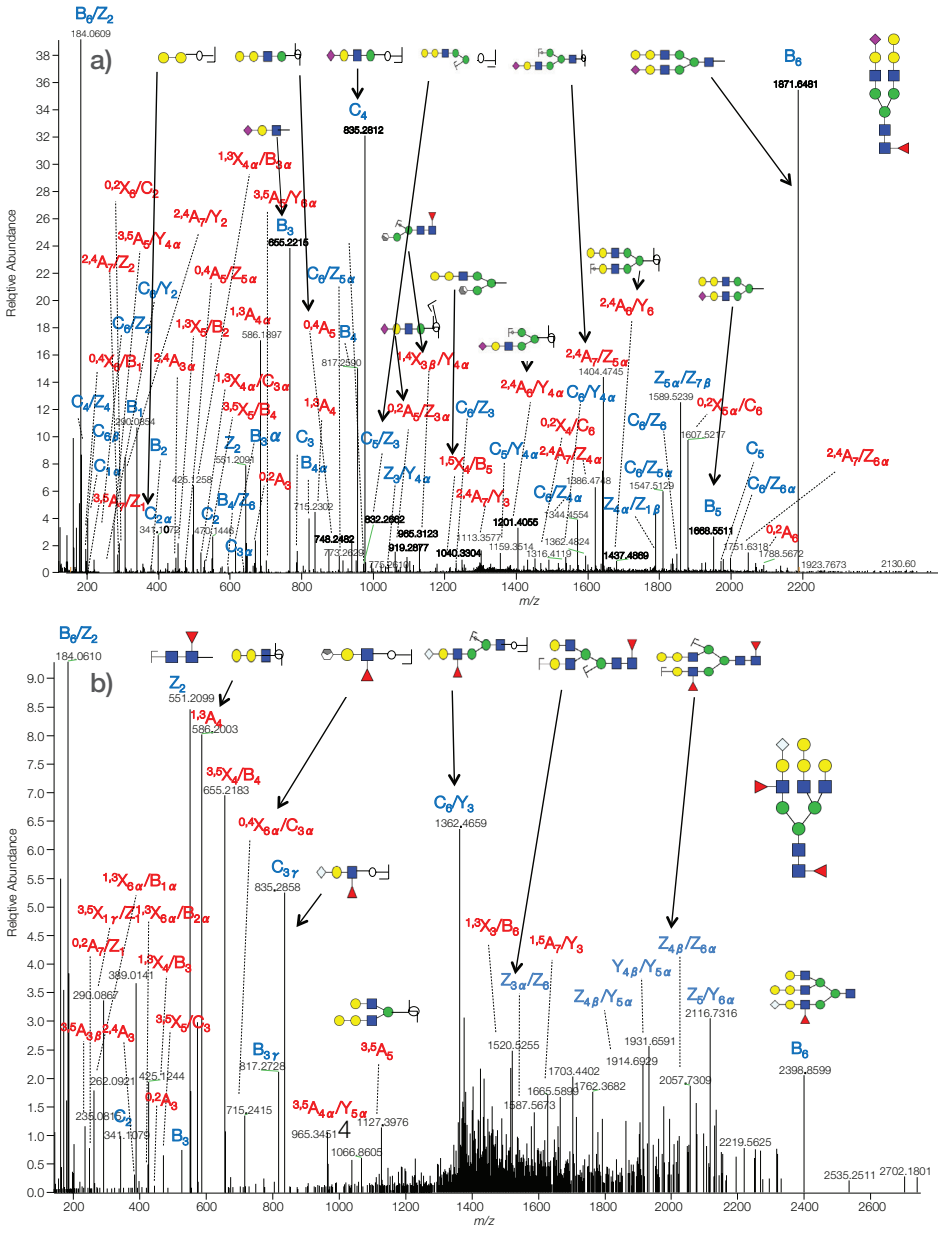


Figure 6. MS/MS spectra annotated from a previously described (a) and an unknown structure (b). Ions in blue are glycosidic and glycosidic/glycosidic fragments, ions annotated in red are cross-ring and glycosidic/cross-ring fragments.

The biopharma industry can take advantage of HPAE coupled to MS. While performing glycoprofiling based on data provided by electrochemical detection (Figure 7a, 7b, and 7d), simultaneous MS and MS/MS data at hand helps analysts annotate the structures. Isomer peaks can also be identified. For example, the analyst can assign sialic acid residues in monosialylated structures to antenna position.

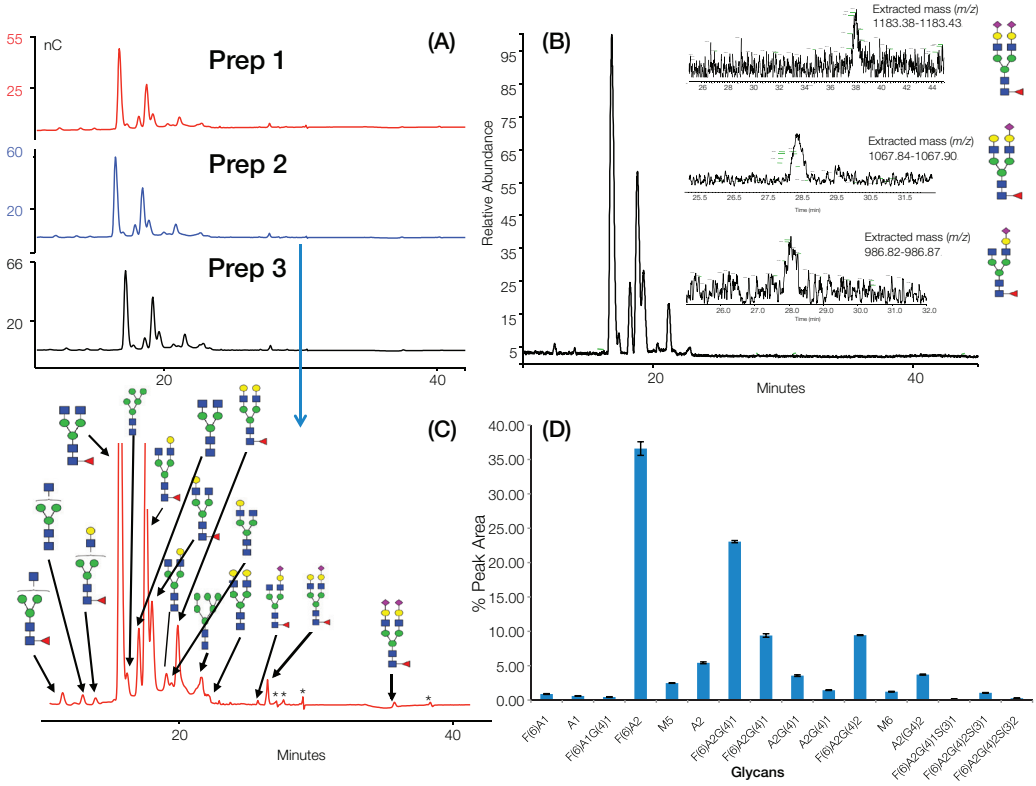
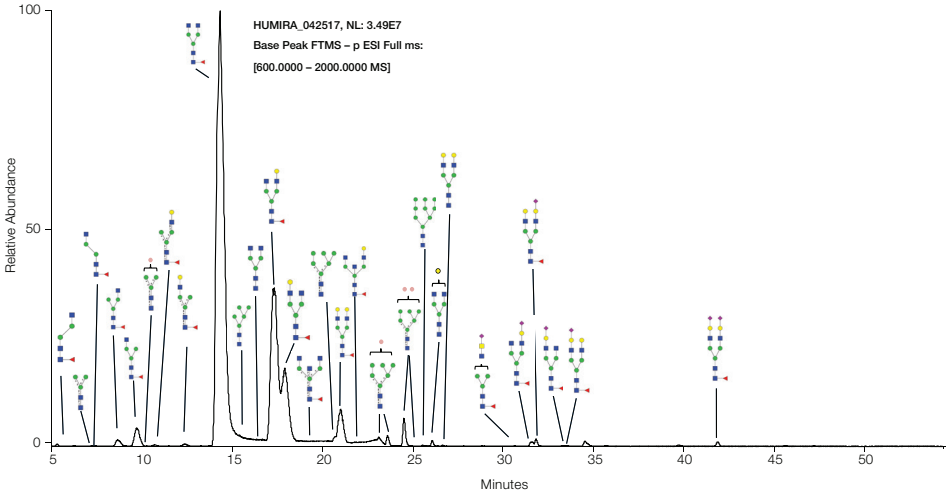


Figure 7. Glycoprofiling of glycans released from a mAb. Figures (a) and (d) show the reproducibility of sample preparation monitored by electrochemical detection and traces (b) and (c) show peak annotation based on mass spectrometry.

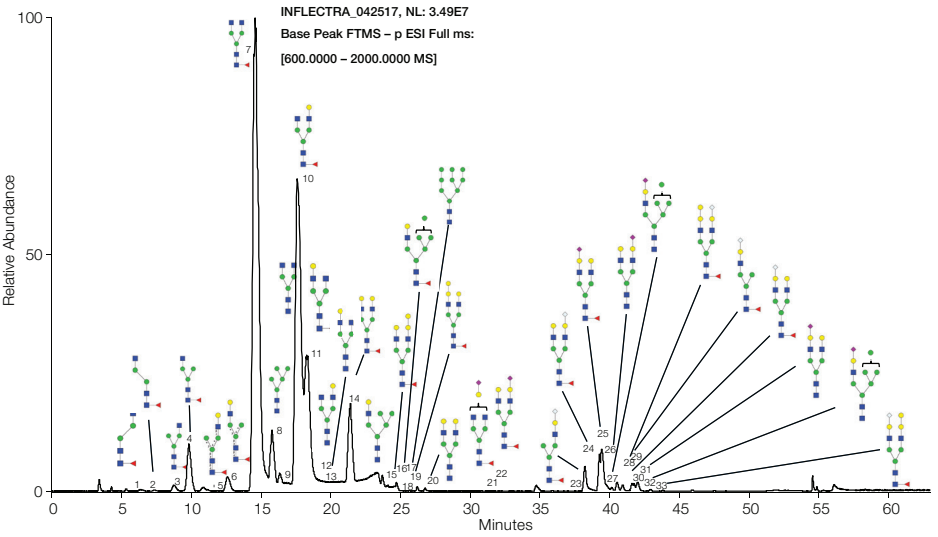
(A) IC-MS of Humira®



#	Designation	Observed m/z	Ion	Actual m/z	Retention (min)	Comments	Mass Error
1	M2N3F(6)	1096.4048	[M-1H]-1	1096.4050	5.31	Isomer1	0.2
2	M2N3F(3)	1156.426	M-H+HOac]-1	1156.4254	7.27	Isomer2	0.5
3	M3F+N(6)	1258.4576	[M-1H]-1	1258.4578	8.65	Isomer1	0.1
4	M3F+N(3)	1318.4791	M-H+Oac]-1	1318.4782	9.72	Isomer2	0.7
5	G1F-N(6)	709.7524	[M-2H]-2	709.7516	10.88	Isomer1	1.1
6	G1F-N(3)	739.7629	M-2H+Oac]-2	739.7616	12.63	Isomer2	1.7
7	GOF	730.2656	[M-2H]-2	730.2649	14.33	1 Isomer	0.9
		760.2769	M-2H+Oac]-2	760.2749			2.6
8	Man5	616.21	[M-2H]-2	616.2094	15.5	1 Isomer	1.0
		646.2206	M-2H+Oac]-2	646.2194			1.9
9	GO	687.2471	M-2H+Oac]-2	687.2460	16.2	1 Isomer	1.6
		1315.4786	[M-1H]-1	1315.4793			0.5
10	G1F(6)	811.2926	[M-2H]-2	811.2913	17.35	Isomer1	1.5
11	G1F(3)	841.3032	M-2H+Oac]-2	841.3013	17.92	Isomer2	2.2
12	GOFB	831.8042	[M-2H]-2	831.8046	19.3	1 Isomer	0.5
		861.8161	M-2H+Oac]-2	861.8146			1.7
13	Man6	727.247	M-2H+Oac]-2	727.2458	20.7	1 Isomer	1.7
		1395.4773	[M-1H]-1	1395.4789			1.1
14	G2F	922.3292	M-2H+Oac]-2	922.3277	21	1 Isomer	1.6
		1785.6415	[M-1H]-1	1785.643			0.7

#	Designation	Observed m/z	Ion	Actual m/z	Retention (min)	Comments	Mass Error
15	G1FB	942.8418	M-2H+Oac]-2	942.8410	21.7	1 Isomer	0.8
		912.8311	[M-2H]-2	912.831			0.1
16	Man7	808.2736	M-2H+Oac]-2	808.2722	23.1	Isomer1	1.8
17	Man7	778.263	[M-2H]-2	778.262	23.6	Isomer2	1.0
18	Man8	889.3002	M-2H+Oac]-2	889.2986	24.5	Isomer1	1.8
19	Man8	1719.5829	[M-1H]-1	1719.584	25.1	Isomer2	0.9
20	Man9	970.3264	M-2H+Oac]-2	970.3250	25.6		1.5
		940.3161	[M-2H]-2	940.315			1.2
21	G1	768.2741	M-2H+Oac]-2	768.2724	26.1	1 Isomer	2.3
		738.2625	[M-2H]-2	738.262			0.1
22	G2	849.3007	M-2H+Oac]-2	849.2988	26.7	1 Isomer	2.3
		819.2888	[M-2H]-2	819.289			0.0
23	G1FS-N	885.3119	M-2H+Oac]-2	885.3093	30.5	1 Isomer	2.9
		855.3007	[M-2H]-2	855.299			1.6
24	G1FS1(6)	986.8504	M-2H+Oac]-2	986.8490	31.4	Isomer1	1.4
26	G1FS1(3)	956.84	[M-2H]-2	956.839	33.2	Isomer2	1.0
25	A1F(6)	1067.8769	M-2H+Oac]-2	1067.8754	31.8	Isomer1	1.4
27	A1F(3)	1037.8663	[M-2H]-2	1037.865	33.5	Isomer2	0.8
28	A2F	1183.4134	[M-2H]-2	1183.413	41.9		0.2
					Avg. Error (ppm)		1.2

(B) IC-MS of Inflectra®



#	Designation	Observed m/z	Ion	Actual m/z	Retention (min)	Comments	Mass Error (PPM)
1	M2N3F(6)	1096.4053	[M-1H]-1	1096.405	5.36	Isomer 1	0.3
2	M2N3F(3)	1156.4262	[M-H+HOac]-1	1156.4254	7.42	Isomer 2	0.7
3	M3F+N(6)	658.7364	[M-2H+HOac]-2	658.7352	8.8	Isomer 1	1.8
4	M3F+N(3)	1258.4583	[M-1H]-1	1258.458	9.8	Isomer 2	0.2
5	G1F-N(6)	709.7516	[M-2H]-2	709.752	10.87	Isomer 1	0.6
6	G1F-N(3)	1420.5094	[M-1H]-1	1420.511	12.64	Isomer 2	1.1
7	GOF	760.2765	[M-2H+HOac]-2	760.2749	14.61		2.1
		1461.5367	[M-H]-1	1461.537			0.2
8	Man5	646.2203	[M-2H+HOac]-2	646.2194	15.5		1.4
		1233.4259	[M-H]-1	1233.426			0.1
9	GO	687.2468	[M-2H+HOac]-2	687.246	16.2		1.2
		1315.4775	[M-H]-1	1315.479	16.35		1.1
10	G1F (6)	841.303	[M-2H+HOac]-2	841.3013	17.56	Isomer 1	2.0
11	G1F (3)	1623.5892	[M-H]-1	1623.59	18.31	Isomer 2	0.5
12	G1 (6)	738.2633	[M-H]-1	738.262	19.7		1.8
13	G1(3)	738.2625	[M-H]-1	738.262	26.2		0.7
		768.274	[M-2H+HOac]-2	768.2724			2.1
		768.2736	[M-2H+HOac]-2	768.2724			1.6
14	G2F	892.3189	[M-1H]-1	892.318	21.4		1.0
		922.3295	[M-2H+HOac]-2	922.3277			2.0
15	M5N3G1	798.7768	[M-2H]-2	798.775	24.1		2.3
		828.7874	[M-2H+HOac]-2	828.7855			2.3

#	Designation	Observed m/z	Ion	Actual m/z	Retention (min)	Comments	Mass Error (PPM)
16	G2F+aGal (6)	973.3447	[M-2H]-2	973.344	24.7	isomers	0.7
	M7F+N or	952.8347	[M-2H]-2	952.831	25.2	isomers	3.9
17	M6F1N3G1						
		982.8427	[M-2H+HOac]-2	982.8408			1.9
18	Man9	940.3175	[M-2H]-2	940.315	25.6		2.7
		970.3263	[M-2H+HOac]-2	970.325			1.3
19	G2F+aGal (3)	1003.3557	[M-2H+HOac]-2	1003.3541	25.9		1.6
20	G2	819.2883	[M-2H]-2	819.289	26.8		0.9
		849.3006	[M-2H+HOac]-2	849.2988			2.1
21	G1FS1	956.8425	[M-2H]-2	956.839	31.3		3.7
		986.8506	[M-2H+HOac]-2	986.849			1.6
22	A1F	1037.8676	[M-2H]-2	1037.865	31.6	isomers	2.5
25		1067.8797	[M-2H+HOac]-2	1067.8754	39.25		4.0
23	G1FS1-N(NGNA)	863.2983	[M-2H]-2	863.297	38.2	isomers	1.5
29		893.3088	[M-2H+HOac]-2	893.3068	40.9		2.2
24	A1F(NGNA)	1045.8641	[M-2H]-2	1045.863	39.1	isomers	1.1
30		1075.8739	[M-2H+HOac]-2	1075.8729	41.8		0.9
26	A1	964.8376	[M-2H]-2	964.836	39.6	isomers	1.7
31		994.8484	[M-2H+HOac]-2	994.8465	42		1.9
27	M6N3G1S1	1025.3486	[M-2H]-2	1025.35	40.1	isomers	1.4
32		1055.3608	[M-2H+HOac]-2	1055.3596	42.7		1.1
28	A1F +aGal(NGNA)	1126.8899	[M-2H]-2	1126.889	40.5	isomers	0.8
33		1156.9008	[M-2H+HOac]-2	1156.8993	42.9		1.3
					Avg. Error (ppm)		1.5

Figure 8. In depth profiling of glycans released from mAbs.

Figure 8A shows the annotated base peak profile of the Humira® glycans. Because modifications to the 6-arm of the trimannosyl core elute earlier in HPAE chromatography than the same modification on the 3-arm, these linkages can be differentiated and confidently annotated. This figure also tabulates both the molecular and acetate adduct ions observed for all but two of the 28 glycans observed in this profile, increasing confidence in the structural annotation. Note that some of these glycans are present at very low levels, but can be identified by displaying extracted-ion chromatograms. For this set of glycans, the average mass error delivered by the Q Exactive Orbitrap MS was 1.2 ppm. Some ions (e.g. G0Fb) are present at very low levels (<0.1%) so may not require regulatory quantification, but are readily observed in extracted ion chromatograms.

Figure 8B shows the comparable results for the glycans released from the biosimilar Inflectra® (infliximab-dyyb, Pfizer). In this case, 33 separate glycans were identified, and again both molecular and acetate-adducted ions are observed for nearly all the glycans annotated. This sample is different because it includes glycans with *N*-glycolyl sialylation, and glycans harboring α-galactose linkages. In the tabulation of the Inflectra glycans, the average mass error was 1.5 ppm.

In order to couple the highly selective anion-exchange separation to mass spectrometers, the sodium ions must be removed from the eluting glycans. In the IC-MS approach, this is performed with the Dionex ERD 500 Electrolytically Regenerated Desalter. This device employs microporous cation-exchange screens to transfer the sodium ions from the eluent channel to the regenerant channel, replacing them with protons. In this manner, the sodium ion exchange from protons generates dilute acetic acid that assists ionization of neutral glycans in the MS (and fragmentation in MS/MS, see Figure 6). Figure 9 examines the *conductivity* of the eluent after passage through the desalter.

The gradient used to resolve *N*-glycans found on IgG includes an initial curved gradient segment followed by a linear increase in sodium acetate and hydroxide. In order to verify consistent sodium removal by the desalter, this method was applied in sequence for several cycles including blanks, water samples and glycan injections, with conductivity detection. This test reveals that conductivity increases non-linearly with a linear increase in acetic acid concentration, as is expected for weak acids.

Another important aspect of desalting is the impact of passage through the Dionex ERD 500 Electrolytically Regenerated Desalter on peak resolution. Typically anionic (sialylated or sulfated) oligosaccharides are repelled by the anionic membranes in the desalter, and this helps maintain peak shape. For these glycans, peak resolution before the desalter is very similar to that after passing through the Dionex ERD 500 Electrolytically Regenerated Desalter. Neutral glycans are not repelled by the desalter membranes, so resolution of these glycans may deteriorate during desalting. In order to characterize this possibility, we examined peak resolution of neutral *N*-linked glycans before and after passage through the Dionex ERD 500 Electrolytically Regenerated Desalter after allowing the membranes to be fully exhausted (Figure 10).

In the two pairs of traces in Figure 10, glycan resolution before and after passage through the desalter is compared. Neutral *N*-linked glycans from Bovine RNAse-B (Ribo-B) (top two traces) and Porcine gamma globulin (PGG) (bottom two traces) were each separated using the same gradient and detected by PAD before (bottom of each set) or after (top of each set) passage through the 2 mm Dionex ERD 500 Electrolytically Regenerated Desalter. The peak labels indicate the resolution values as measured by the statistical moments method, which is available in the Chromeleon CDS

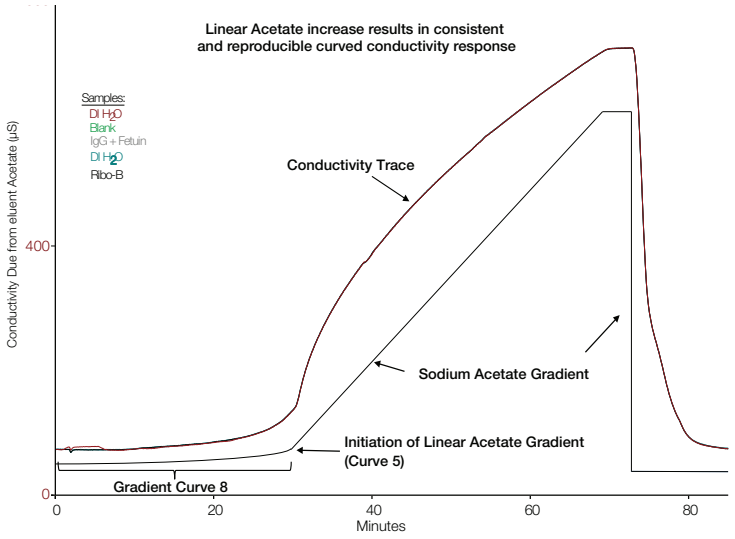


Figure 9. Reproducibility of desalter function during IgG-glycan separation.

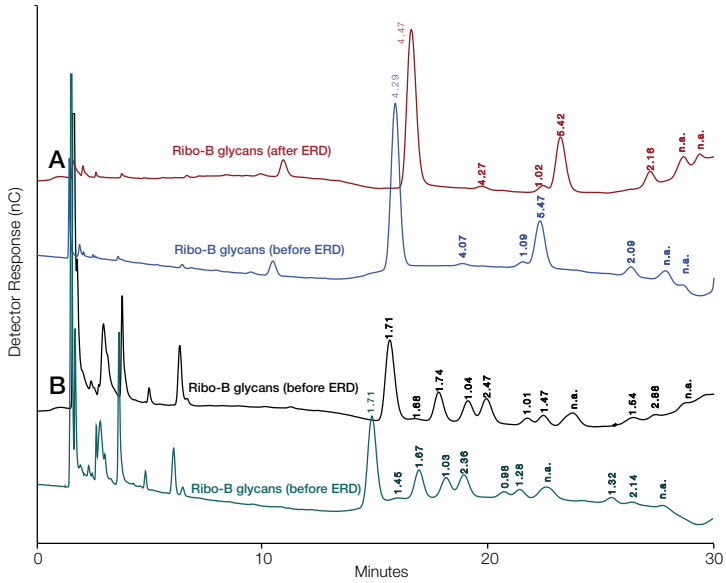


Figure 10. Effect of a 2 mm Dionex ERD 500 Electrolytically Regenerated Desalter on neutral glycan resolution.

software. This measurement employs every data point in each peak, therefore representing the most thorough chromatographic resolution measurement. For the Ribo-B glycans, the average change in neutral glycan resolution after passage through the 2 mm Dionex ERD 500 Electrolytically Regenerated Desalter was + 2.07%. For the porcine gamma globulin glycans, the average change after passage through the 2 mm Dionex ERD 500 Electrolytically Regenerated Desalter was + 10%. Not all peaks exhibit greater resolution after passage through the desalter, so these values are likely within the variability of the measurements. This demonstrates that the Dionex ERD 500 Electrolytically Regenerated Desalter does not significantly degrade neutral peak resolution for the downstream (MS) analyses.

Conclusions

Separation by HPAE is based on charge, linkage and positional isomerism, and fucosylation, resulting in excellent separation of many different glycan isomers. Using this new workflow, 53 unique *N*-glycans were identified from hu-AGP, and *N*-glycans from three different relevant mAbs were annotated, showing many common and several individual features. Adduct formation with acetate enhances MS fragmentation, producing diagnostic ions that support structural elucidation of neutral *N*-glycans. The conditions employed for desalter regeneration deliver reproducible removal of sodium ions. The desalter does not materially degrade glycan resolution between the column and the downstream MS probe. HPAE-PAD/MS is an excellent tool to profile and annotate complex mixtures of native *N*-glycans released from different glycoproteins and provides a different selectivity than other glycan separation techniques that have been coupled to MS.

References

1. ICH Q6B Guidelines (1999) [Online] https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf (accessed Jul. 27, 2017).
2. Thermo Scientific Technical Note 71. Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection. [Online] http://tools.thermofisher.com/content/sfs/brochures/TN-71-Eluent-Preparation-for-High-Performance-Anion-Exchange-Chromatogarphy-with%20APD-TN-70669.pdf (accessed Jul. 27, 2017.
3. Thermo Scientific Technical Note 21. Optimal Settings for Pulsed Amperometric Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector. [Online] https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/TN-21-Optimal-Settings-Pulsed-Amperometric-Detection-Carbohydrates-ED40-TN70670-EN.pdf (accessed Aug. 15, 2017).
4. Thermo Scientific Electrochemical Detection User's Compendium. [Online] https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Man-065340-Electrochemical-Detection-Man065340-EN.pdf (accessed Aug. 15, 2017).
5. Thermo Scientific Technical Note 110. Carbohydrate Determination of HPAE-PAD with Disposable Au on PTFE Working Electrodes [Online] http://tools.thermofisher.com/content/sfs/brochures/111176-TN110-IC-Carb-HPAEPADdisposAuPTFE-12Oct2011-LPN2952.pdf (accessed Aug. 15, 2017).
6. Szabo et. al., In-depth analyses of native *N*-linked glycans facilitated by high-performance anion exchange chromatography-pulsed amperometric detection coupled to mass spectrometry. *Anal. & Bioanal. Chem.* **2017**. 409(12): 3089-3101.
7. Eavenson, M., Kochut, KJ., Miller, JA., Ranzinger, R., Tiemeyer, M., Aoki, K., York, WS. Qrator: a web-based curation tool for glycan structures. *Glycobiology.* **2015**. 25(1): 66–73.

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