APPLICATION NOTE 74042

Structural characterization of mucin O-linked glycans using a novel anion exchange column in HPAE-PAD-MS

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Goal

To introduce the system configuration hyphenating HPAE-PAD analyses to the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer for O-glycan analysis. Analysis was performed using a newly introduced Thermo Scientific™ Dionex™ CarboPac™ PA300-4µm column.

Introduction

Glycosylation, one of the most prevalent and diverse types of post-translational modifications (PTM), is known to be important for myriad cellular and developmental events, including cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis.[1] Two major forms of protein glycosylation exist: N-linked glycans are attached to asparagine (Asn) residues, and O-linked glycans are predominantly attached to serine (Ser) or threonine (Thr) residues. Mucins are a family of large glycoproteins that harbor high levels of O-linked glycans that are often clustered in central domains composed of amino acids rich in Ser and Thr. Overexpression of, or alterations to, mucin O-glycans has been correlated with the development of numerous malignancies. Therefore, understanding the O-glycan profiles of mucin proteins can provide not only valuable information about pathologies of many diseases, but can also serve as an important model to discover the functional mechanisms of O-glycans on other glycoproteins.[2]

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O-linked glycosylation is often quite challenging to study, not only because of the lack of a universal enzyme for O-glycan release from proteins, but also because the PTM is non-template driven. The PTM is not genetically encoded, and there is no consensus sequence dictating the site of glycan attachment on proteins. Furthermore, the heterogeneity of glycans, both in composition and in linkages, complicates their analysis and structural elucidation. Some proteins, like mucins, are particularly difficult to analyze due to a high density of clustered, heterogeneous glycans in a specific domain of the protein. Therefore, high-resolution chromatographic separations are important for subsequent mass spectrometric characterization.

There is an increasing demand for reproducible, fast, and simple methods for glycan profiling in the biologics and food industries. A variety of HPLC-based solutions have been proposed, most of which require complex sample preparation yet prove inadequate for separation. High-performance anion-exchange (HPAE) chromatography is a type of ion chromatography uniquely positioned for the structural analysis of various native glycans. The separations of linkage and positional isomers are based on charge and size of glycans.

The newly introduced Dionex CarboPac PA300-4µm column is packed with a unique resin composed of a highly crosslinked core and a nanobead anion-exchange layer attached to the surface. The latex nanobead anion-exchange layer has a controlled thickness, which results in excellent mass transfer characteristics and, consequently, very high efficiency. This column is designed for efficient separations of complex, heterogeneous samples with good chromatographic resolution of neutral and charged glycans alike. For applications requiring a range of glycan separations with varying sizes and charge states, the Dionex CarboPac PA300-4µm column is the recommended column with which to begin analysis. When a Thermo Scientific™ Dionex™ ICS-6000 system is coupled with a Q Exactive mass spectrometer, the platform enables the identification of glycan structures with no need for derivatization.

In this application note, we demonstrate a workflow combining the chemical release of O-linked glycans, followed by sample cleanup and analysis by HPAE-PAD hyphenated to a Q Exactive mass spectrometer. The Dionex CarboPac PA300-4µm column, a new member to the Dionex CarboPac suite of columns, uses a novel column technology that enables the simultaneous separation of neutral and charged glycans without the need of derivatization. High resolution MS data and tandem MS/MS spectra with diagnostic fragment ions provide highly reliable structural annotations of heterogenous glycans. The unique HPAE-PAD/MS workflow presented here provides confirmatory, complementary information for O-glycan analysis to that of conventional RPLC-MS based approaches.

Experimental

Sample preparation

- Sigma-Aldrich, Mucin from porcine stomach, Type III (P/N M1778-10G)
- Milli-Q™ water, Type I reagent grade, 18 MΩ·cm resistivity
- Fisher Scientific™, Sodium hydroxide, 50% w/w (P/N SS254-500)
- Fisher Scientific™, Sodium borohydride (P/N S678-25)
- ACROS Organics™, Formic acid, 97%, ACS reagent (P/N AC423755000)
- Fisher Chemical™, Methanol, Optima™ for HPLC (P/N A454-4)
- Fisher Chemical™, Acetonitrile, Optima™ LC/MS Grade (P/N A955-4)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid (TFA), LC-MS Grade (P/N PI28901)
- Thermo Scientific™ HyperSep™ Hypercarb™ filter plate, 40 µL bed volume (P/N 60110-504)
- Fisherbrand™, 96 well PCR plate (P/N 14230237)
- Thermo Scientific™ Dionex™ AS-AP autosampler vial kits (P/N 055428)

Thermo Scientific™ Dionex™ CarboPac™ PA300-4µm columns

- Dionex CarboPac PA300-4µm guard column (2 × 50 mm) (P/N 303347)
- Dionex CarboPac PA300-4µm analytical column (2 × 250 mm) (P/N 303346)

Inline trap column

- Thermo Scientific™ Dionex™ BorateTrap inline trap column (P/N 047078)

Electrochemical cells and electrodes

- Electrochemical detector (ED) without cell (P/N 072042)
- ED with reference electrode and spacer block (no working electrode) (P/N 072044)
- Gold on PTFE disposable electrode, 6/pk, including 6 gaskets (0.002 in) (P/N 066480)
- Palladium hydrogen reference electrode (PdH) (P/N 072075)
- Thermo Scientific™ Dionex™ IC PEEK Viper™ fittings kit for 2 mm Dionex ICS-6000 system with ED* (P/N 302966)

*To order the item # ask your local sales representative for a quote

Electrolytically regenerated desalter

- Thermo Scientific™ Dionex™ ERD™ 500 Electrolytically Regenerated Desalter, 2 mm (P/N 085089)
- 4 L bottle for desalter external regeneration solution (P/N 063292)

Equipment

- Thermo Scientific™ Dionex™ ICS-6000 HPIC system with
  - Dual pump
  - Detector/chromatography module
  - Thermo Scientific™ UltiMate™ WPS TBPL Thermostatted Biocompatible Standard Well Plate Autosampler (P/N 5823.0020)
- Mass spectrometer components
  - Thermo Scientific™ Q Exactive™ Hybrid Quadrupole Orbitrap™ mass spectrometer (P/N 0726042)
  - Thermo Scientific™ Dionex™ AXP auxiliary pump (P/N 063973)

HPAE conditions

Autosampler	
Injection volume	20 µL
Injection mode	Microliter pickup option
Flush volume	15 µL
Temperature	4 °C
Pump	
Eluent A	Milli-Q water
Eluent B	200 mM NaOH
Eluent C	25 mM Sodium acetate in 50 mM NaOH
Eluent D	250 mM Sodium acetate in 100 mM NaOH
Flow rate	0.25 mL/min
Column temperature	30 °C

	Time	% A	% B	% C	% D	Comments
Eluent program	-1.0	78.5	19.5	2	0	Equilibration
	0.0	78.5	19.5	2	0	Load/inject
	4.0	78.5	19.5	2	0	
	20.0	20	20	60	0	
	50.0	0	0	0	100	Regeneration
	59.9	0	0	0	100	
	60.0	78.5	19.5	2	0	Equilibration
	75.0	78.5	19.5	2	0	End

PAD conditions

Channels	ED_1, ED_1_Total
Amperometry cell	On
Data collection rate	2 Hz
Autozero	No
Reference electrode	PdH

PAD detection	Carbohydrate, Quad Potential
Working electrode	Gold on PTFE disposable electrode
Detection temperature	30 °C
Waveform (PdH reference)	*Gold, Carbo, Quad (Carbohydrates, Quad Potential)

	Time(s)	Potential	Last step	Ramp	Gain region	Integration
EDet1.Waveform	0	+1.02	Off	On	Off	Off
EDet1.Waveform	0.2	+1.02	Off	On	On	On
EDet1.Waveform	0.4	+1.02	Off	On	Off	Off
EDet1.Waveform	0.41	-1.08	Off	On	Off	Off
EDet1.Waveform	0.42	-1.08	Off	On	Off	Off
EDet1.Waveform	0.43	+1.52	Off	On	Off	Off
EDet1.Waveform	0.44	+0.82	Off	On	Off	Off
EDet1.Waveform	0.5	+0.82	On	On	Off	Off

*The waveforms are adjusted in the script section of the instrument method for the eluent gradient in this specific application.

Electrochemically regenerated desalter conditions

AXP pump flow rate	4 mL/min
ERD current	250 mA
ERD regeneration solution	Milli-Q water

MS conditions

MS instrument	Q Exactive hybrid quadrupole-Orbitrap
Ion source	Thermo Scientific™ Heated Electrospray Ionization (HESI-II) probe
Ionization mode	Negative ionization
Spray voltage	3.2 kV
Capillary temperature	320 °C
Sheath gas flow rate	40 arbitrary units
Auxiliary gas flow rate	20 arbitrary units

Scan range	400–2000 *m/z*
Resolution	60,000
AGC target	3×10^6
Maximum IT	120 ms
dd-MS2 resolution	15,000
dd-MS2 AGC target	1×10^5
dd-MS2 Maximum IT	300 ms
TopN	5
Isolation window	3 *m/z*
(N)CE	28

Data processing & software

Mass data acquisition	Thermo Scientific™ Xcalibur™ 4.3.73.11
MS/MS annotation	UniCarb-DB Database[3]

Sample preparation

O-linked glycans were released from mucin glycoproteins by reductive β-elimination, whereafter they were purified with porous graphitized carbon resin (Thermo Scientific™ HyperSep™ Hypercarb™ filter plates, 40 µL). The detailed sample preparation steps are demonstrated in Figure 1. The purified O-glycan mix was stored at -30 °C and resuspended in 50 µL water for analysis.

Instrument configuration

The Dionex ICS-6000 HPIC system is configured for the hyphenation of electrochemical and MS detections. Figure 2 shows a schematic of a possible HPAE-PAD-MS platform configuration. Two pumps are used—one for the eluent system for IC separations, and another for water regeneration of the downstream desalter. Column effluent first passes through the PAD detector with a PdH reference electrode. The oxyanions of carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold working electrode. The cell effluent then passes through the Dionex ERD 500 electrolytically regenerated desalter, where sodium ions are exchanged for hydronium and eluting glycans are now in water (hydroxide-only eluent) or weak acetic acid. This effluent, containing glycans, is injected into the MS with a HESI-II probe.

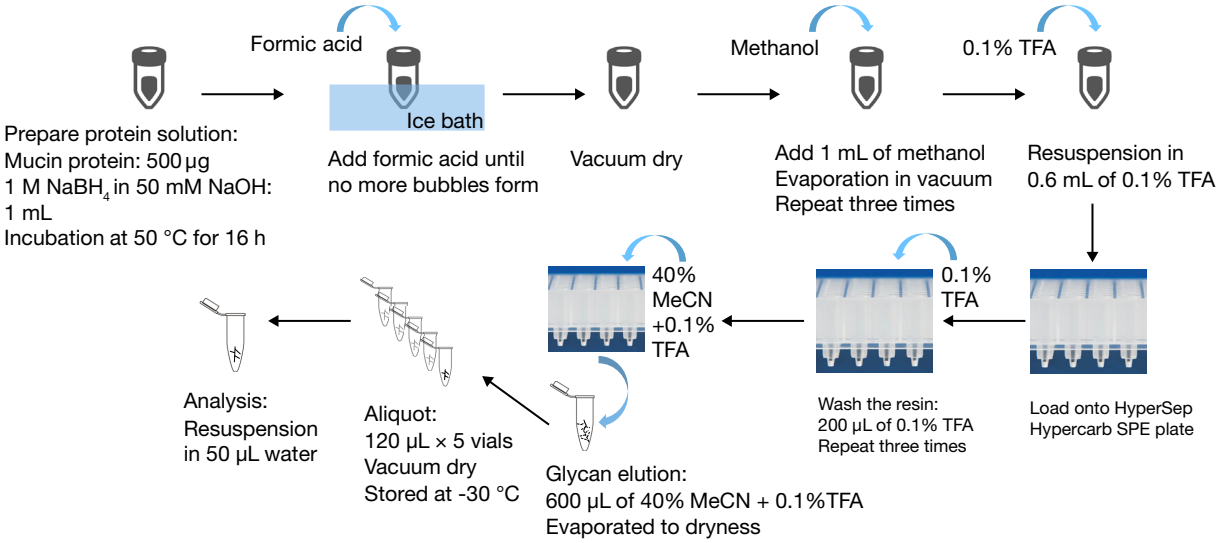

Figure 1. Sample preparation and purification workflow for O-glycans

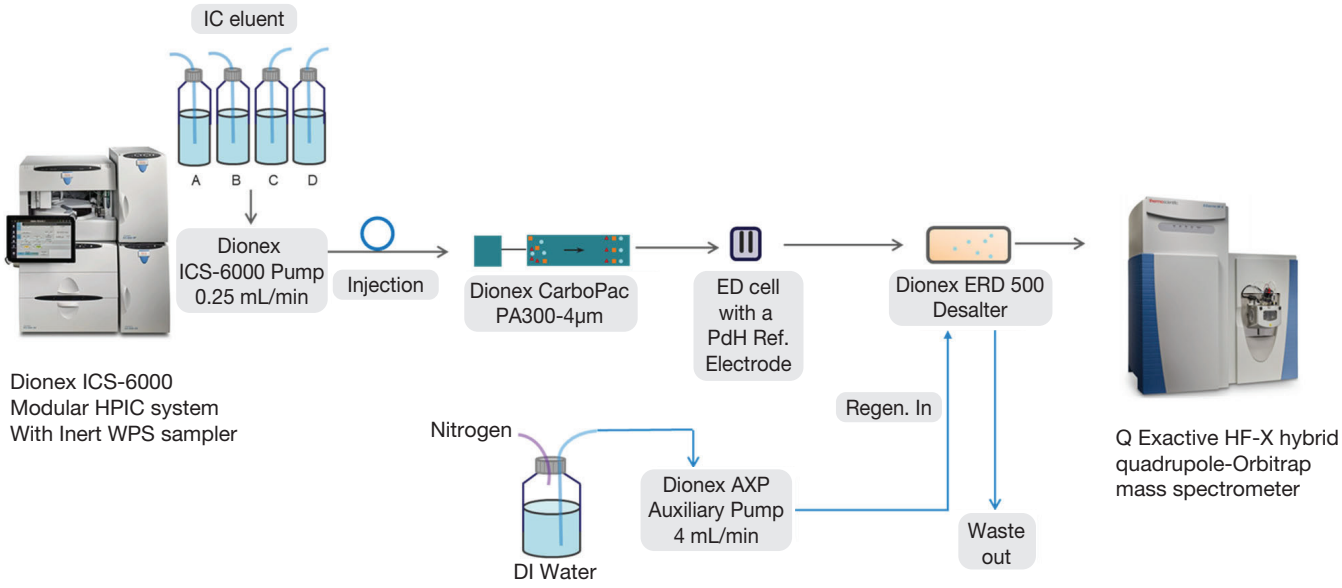

Figure 2. The system configuration of HPAE-PAD with a PdH reference electrode hyphenated to Q Exactive Orbitrap Mass Spectrometer

An alternative instrument configuration for glycan analysis was previously introduced to characterize native N-glycans in negative mode by HPAE-PAD/MS using a Dionex CarboPac PA200 column.[3] In this instrument setup, a post-column splitter was added to adjust eluent flow to the PAD detector and MS to accommodate the optimal conditions for detection with both HPAE-PAD and MS. This particular instrument configuration was well-suited for high flow applications (0.5 mL/min), but its use for lower-flow applications can be somewhat problematic because of the difficulty in controlling the flow and sample splitting between PAD and MS with high precision. Therefore, the system configuration with the sequential detection of PAD and MS was developed for Dionex CarboPac PA300-4µm applications. To eliminate the risk of electrolyte leakage and post-column diffusion introduced by ED cell volume, the PdH reference electrode is used in this system.

Palladium hydrogen reference electrode

The ED cell is a miniature flow-through amperometric detection cell that includes three different electrodes: a titanium cell body (the counter electrode), a working electrode, and a reference electrode. For carbohydrate applications, pulsed amperometric detection is typically performed at a gold working electrode with a series of potentials where the analytes are detected. A reference electrode is required to ensure application of the proper working electrode potential, and the reference electrode should have a constant electrochemical potential. Inaccurate or shifting reference potential can lead to an unusually high background response from the working electrode as well as reduced signal response.

The two most commonly used reference electrodes for electrochemical detection in liquid chromatography are the silver/silver chloride (Ag/AgCl) electrode and the palladium type solid state reference electrode (for example, palladium hydrogen electrode, PdH). The Ag/AgCl electrode is an aqueous electrode and is susceptible to electrolyte leakage to the test solution, creating potential problems when the MS is configured downstream of the ED. A PdH reference electrode, on the other hand, is a solid state reference electrode, which is more robust and does not require calibration. More importantly, it is easier to minimize the dead volume for the ED cell, thus reducing any peak broadening induced by the post-column dispersion that might be observed in the MS base peak chromatogram.

A brief overview of the installation of the PdH reference electrode is shown in Figure 3. First, insert a gasket into the reference electrode well. Be careful not to deform the gasket cutout (thin layer cell flow path) with the tweezer tip (Step 1). Align the PdH gasket to form a free flow path between the inlet and outlet of the reference electrode well (Step 2). Next, align the two knobs with the two alignment grooves of the cell body (Step 3) and insert the electrode into the well. Finger tighten the electrode clockwise into the reference electrode well and tighten the bolt an extra ~1/8 turn with a wrench (Step 4). Do not overtighten the bolt because it could damage the electrode. For detailed installation and troubleshooting instructions, please refer to the Dionex ICS-6000 Ion Chromatography System Operator's Manual (Document No. 22181-97002).

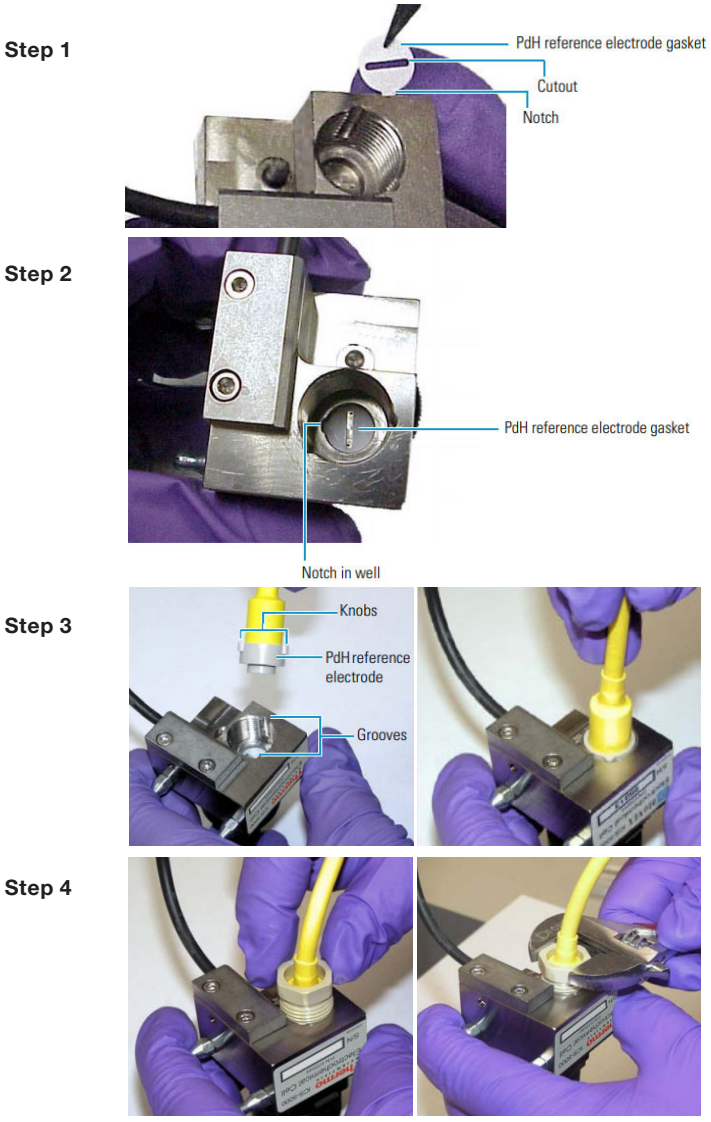

Figure 3. Overview of the installation of a palladium hydrogen (PdH) reference electrode

To initialize a new PdH reference electrode, set up the system with the 100 mM hydroxide eluent concentration and the recommended flow rate for the column. For the Dionex CarboPac PA300-4µm column in 2 mm × 250 mm format, the recommended flow rate is 0.25 mL/min. After turning on the pump flow, set the ED reference electrode type to "PdH." The power control of the PdH electrode will automatically switch to "On." Keep in mind that the ED cell should be kept as "OFF" for now. Otherwise, it can damage the working electrode due to the use of an incorrect potential set by an unconditioned PdH reference electrode. Condition the electrode for 2 hours (overnight for the best performance) at these settings.

Once the conditioning step is complete, correct waveforms need to be selected to operate with the PdH reference electrode. While keeping the cell control "OFF," click the "Download Waveform" button and select "Gold, PdH RE. Carbo. Quad" from the dropdown manual, as shown in Figure 4. After selecting the correct waveform, the cell control for ED can be turned "ON."

To create an instrument method using a PdH reference electrode for carbohydrate applications, both reference electrode type (PdH) and Eluent Type (Base) need to be selected in the method file. When the sequence finishes running, the system is switched to standby, the pump is turned off, and the PdH reference electrode power is set to "Off." The waveforms are customized in the script section of the instrument method for the eluent gradient in this specific application. For detailed method parameters, please refer to the "PAD conditions" table in the "Experimental details" section. Additional guidance in creating an instrument method using a PdH reference electrode and applications to demonstrate the performance of a PdH reference electrode in comparison to a silver/silver chloride reference electrode can be found in Technical Note 73348.[4]

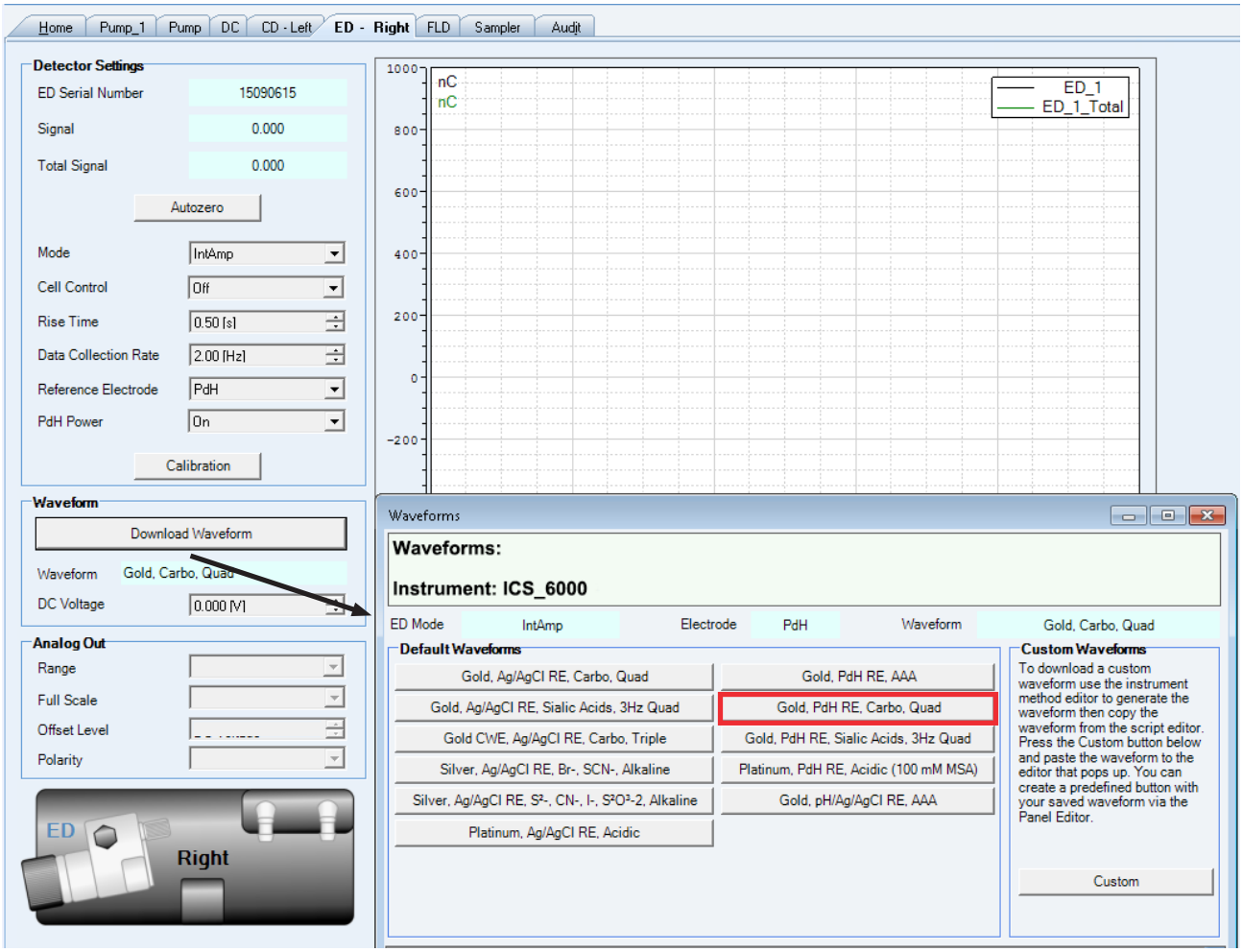

Figure 4. The choice of a correct waveform for carbohydrate analysis using a PdH reference electrode

O-glycan structural annotation

MS data were processed with Xcalibur software. All proposed glycan structures were identified using the monoisotopic *m/z* of the precursor mass and accompanying MS/MS product ion spectra that were generated with higher-energy collision dissociation (HCD). Annotation of fragmentation spectra was achieved de novo, where glycan fragments were assigned manually and verified using available structures in UniCarb-DB, an online database of LC-MS mucin O-glycan fragmentation data.[5] The graphical representations of identified glycans and their MS/MS fragment ions were generated using GlycoWorkbench.[6] The nomenclature of the glycan structures and their MS/MS fragment ions was based on Domon and Costello nomenclature.[7] The common O-GalNAc glycan core structures, types of N-acetyllactosamine extensions, and the blood group related epitopes were described according to previous publications.[8,9]

Results and discussion

The protocol outlined in this technical note details a sensitive and informative workflow for identifying and characterizing the O-glycans from porcine gastric mucin glycoproteins. The workflow starts with a simple sample preparation void of derivatization. O-glycans are released from porcine gastric mucin using reductive β-elimination under alkaline borohydride conditions. This process converts the N-acetyl-galactosamine (GalNAc) at the reducing end into its alditol form. The obtained glycan alditols are subsequently purified by porous graphitized carbon, and the samples are then ready for analysis by HPAE-PAD-MS. The workflow presented here does not require specific enrichment or derivatization steps prior to analysis. The label-free analysis not only eliminates the extra reaction and cleanup steps, but it also retains the original glycan profile without losing structures or adding ambiguous information with extraneous labeling reactions. The analytical platform is demonstrated with mucin O-glycan samples, but it can be applied with ease to released O-glycans or N-glycans from other glycoproteins or free oligosaccharides.

O-glycans from porcine gastric mucin were separated on the Dionex CarboPac PA300-4µm column. The eluent gradient starts with a low concentration of sodium hydroxide and sodium acetate, facilitating the separation of small, neutral glycans. With the elevated concentration of hydroxide and acetate, larger neutral glycans started to elute from the column, followed by charged glycans, such as sialylated and sulfated glycans.

The Dionex CarboPac PA300-4µm column introduces a novel column technology with unique selectivity that provides high-resolution separations of neutral, sialylated, and sulfated glycans. The column improves the separation of neutral glycans that elute in the beginning of the gradient program relative to previous Dionex CarboPac columns. The advantage of the Dionex CarboPac PA300-4µm column in separating neutral glycans is evident when it is used to analyze complex O-glycan mixtures released from protein samples. As shown in Figure 5, the neutral O-glycans released from porcine gastric mucin glycoprotein were separated in an extended elution window. It should be noted that, while the Dionex CarboPac PA300-4µm column provides excellent separation of neutral and charged species alike, the resolution of chromatographic peaks in the charged region might be slightly worse than that of the Dionex CarboPac PA200 column. Therefore, the two columns provide complementary data, expanding the toolkit available to analyze glycans and allowing for tailored implementation to ion chromatography workflows based on the sample of interest.

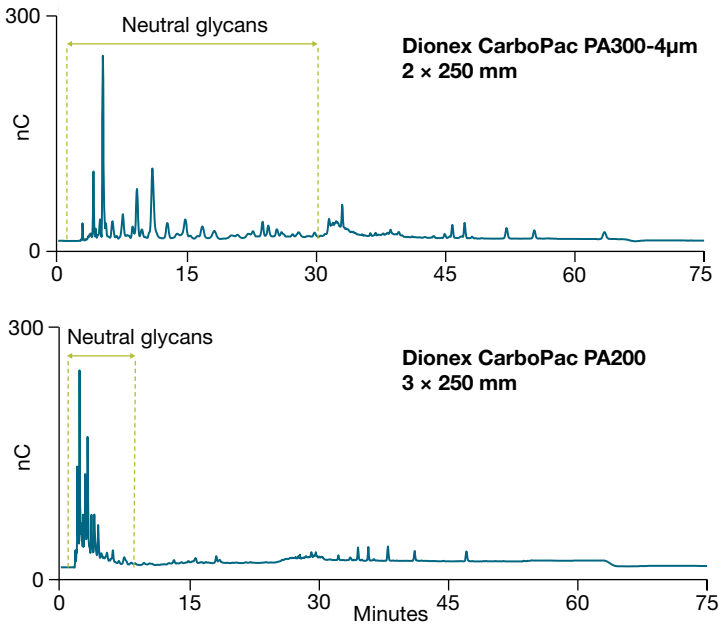

Figure 5. Comparison of chromatographic traces of porcine gastric mucin type III O-glycans on the Dionex CarboPac PA200 (bottom) and Dionex CarboPac PA300-4µm (top) columns. The approximate window during which neutral glycans elute is highlighted in the arrowed region.

The O-glycan profile was analyzed sequentially by the two different detection techniques, PAD, and Q Exactive Orbitrap mass spectrometry.

Figure 6 shows the chromatograms from MS (base peak chromatogram; BPC) and PAD acquired from the same injection. MS detection occurs downstream of PAD, so the BPC shows an expected minor delay in retention time (approximately 0.5 min), which is independent of the glycan type. The resolution of glycans in the BPC remains similar to the PAD chromatogram, yet the presence of minor peak broadening/tailing indicates the potential of post-column peak dispersion caused by the electrolytically regenerated desalter. Comparison of the PAD chromatogram and the BPC also shows differences in response factors. Response factors provided by the two different detection methods for each glycan species is largely dependent on the glycan structures.

Glycans were not modified prior to analysis and were observed as deprotonated anions, primarily singly charged with some doubly charged ions observed for higher molecular weight structures. The mass accuracy of all the detected structures was less than 5 ppm, ensuring high confidence peak annotations. Detailed information of the proposed glycan compositions, their observed and theoretical *m/z*, and the mass errors are shown in Table 1. The average mass error was 1.5 ppm.

The set of released O-linked glycans from porcine mucin type III protein includes a wide range of diverse structures, varying in their sizes, linkage positions and α- or β-stereochemistries, monosaccharide composition, and further post-translational modification with sulfate groups. As annotated in Figure 6 and Table 1, peaks eluting from 4.2 min to 32.4 min are small, neutral glycans, from 33.1 to 35 min are sialylated glycans, and charged sulfated glycans elute afterwards.

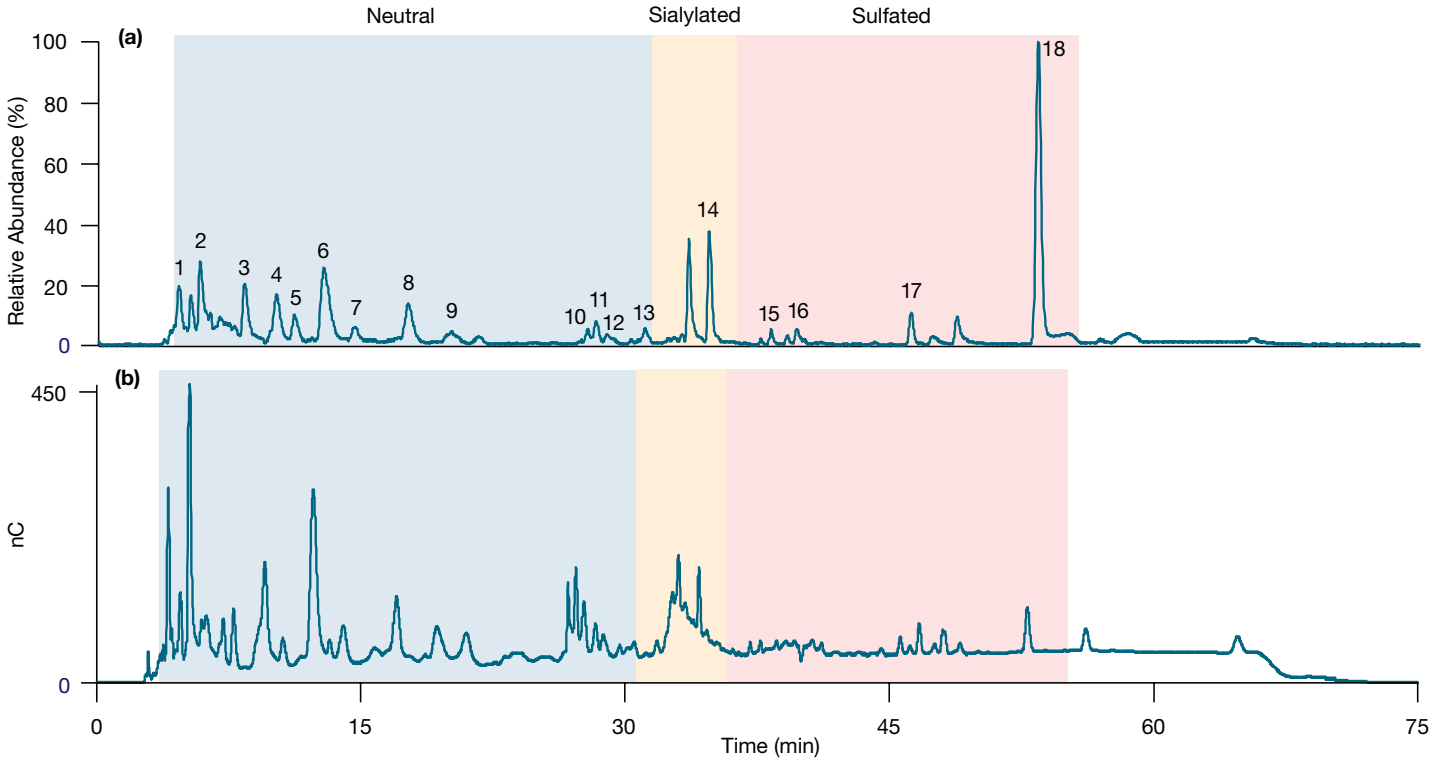

Figure 6. Comparison of MS base peak chromatogram (a) and PAD chromatogram (b) of O-linked glycans released from a porcine gastric mucin type III

Table 1. Compositional identification of selected O-glycans released from a porcine gastric mucin type III

Peak #	RT (min)	Composition*	Observed *m/z*	Theoretical *m/z*	Mass accuracy (ppm)	Charge
1	5.13	Hex Fuc $[HexNAc]_2$	733.2896	733.2884	1.64	1
2	5.83	Hex Fuc HexNAc	530.2080	530.2090	1.89	1
3	8.34	Hex Fuc $[HexNAc]_2$	733.2891	733.2884	0.95	1
4	10.10	$[Hex]_2$ Fuc $[HexNAc]_3$	1098.4210	1098.4206	0.36	1
5	11.14	$[Hex]_2$ Fuc $[HexNAc]_2$	895.3425	895.3412	1.45	1
6	12.83	$[Hex]_2$ $[Fuc]_2$ $[HexNAc]_2$	1041.3987	1041.3992	0.48	1
7	13.41	$[Hex]_2$ $[HexNAc]_4$	1155.4424	1155.4421	0.26	1
8	17.59	$[Hex]_2$ Fuc$[HexNAc]_3$	1098.4213	1098.4206	0.64	1
9	18.32	$[Hex]_3$ $[Fuc]_2$ $[HexNAc]_2$	1203.4508	1203.4520	1.00	1
10	27.78	$[Hex]_4$ $[Fuc]_3$ $[HexNAc]_4$	958.3604	958.3571	3.44	2
11	28.54	$[Hex]_3$ $[HexNAc]_4$	1317.4910	1317.4949	2.96	1
12	28.88	$[Hex]_4$ $[Fuc]_2$ $[HexNAc]_5$	986.8695	986.8678	1.72	2
13	31.05	Neu5Ac $[Hex]_2$ $[HexNAc]_2$	1040.3793	1040.3788	0.48	1
14	34.65	Neu5Ac Hex Fuc HexNAc	821.3060	821.3045	1.83	1
15	38.22	N-glycan, hybrid, sulfated	852.2811	852.2848	4.34	2
16	39.63	$[Hex]_3$ Fuc $[HexNAc]_3$-SO_3^-	669.7120	669.7115	0.79	2
17	46.13	$[Hex]_2$ $[Fuc]_2$ $[HexNAc]_2$-SO_3^-	1121.3568	1121.3559	0.78	1
18	53.34	Hex Fuc $[HexNAc]_2$-SO_3^-	813.2469	813.2452	2.09	1

*Hex: Hexose; Fuc: Fucose; HexNAc: N-Acetylhexosamine; Neu5Ac: N-Acetylneuraminic acid

The Q Exactive Orbitrap mass spectrometer has the capacity to generate HCD fragment ions with high resolution and mass accuracy. This allows for identification and differentiation of fragments ions with similar *m/z* ratios and is useful for branching annotation and linkage assignment. Figure 7 shows two examples of annotated MS/MS spectra, one of a fucosylated, and one of a sialylated glycan.

Many of the glycan structures identified are isomers. The different forms of glycan isomers are relevant to their diverse biological functions, and thus, it is crucial to characterize a glycan's structure to understand its impact on a biological system. The enormous heterogeneity of glycans in terms of composition and connectivity results in a highly complex, diverse set of glycan structures within a single sample. Therefore, it is essential to optimize the chromatographic separation of the isomeric structures and utilize high resolution mass spectrometry to achieve comprehensive characterization of all glycans within a sample. Figure 8 demonstrates three examples of isomeric separation with a Dionex CarboPac PA300-4µm column, including a neutral glycan (Figure 8a), and a sialylated glycan (Figure 8b), and a sulfated glycan (Figure 8c). Some isomer forms are present in low abundance but can still be identified using extracted ion chromatograms.

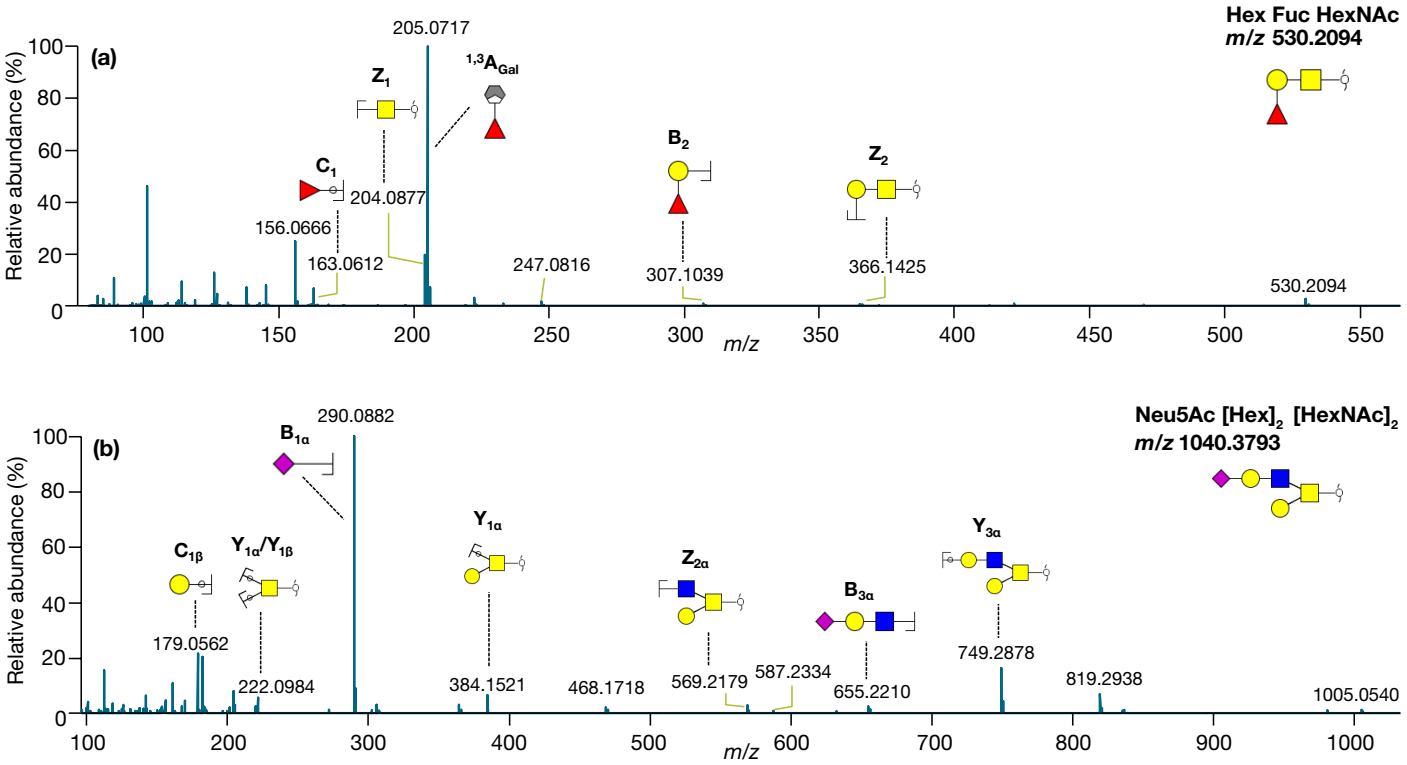

Figure 7. MS/MS spectra of two O-glycans observed in the negative ion mode: (a) Hex Fuc HexNAc, and (b) Neu5Ac $[Hex]_2$ $[HexNAc]_2$

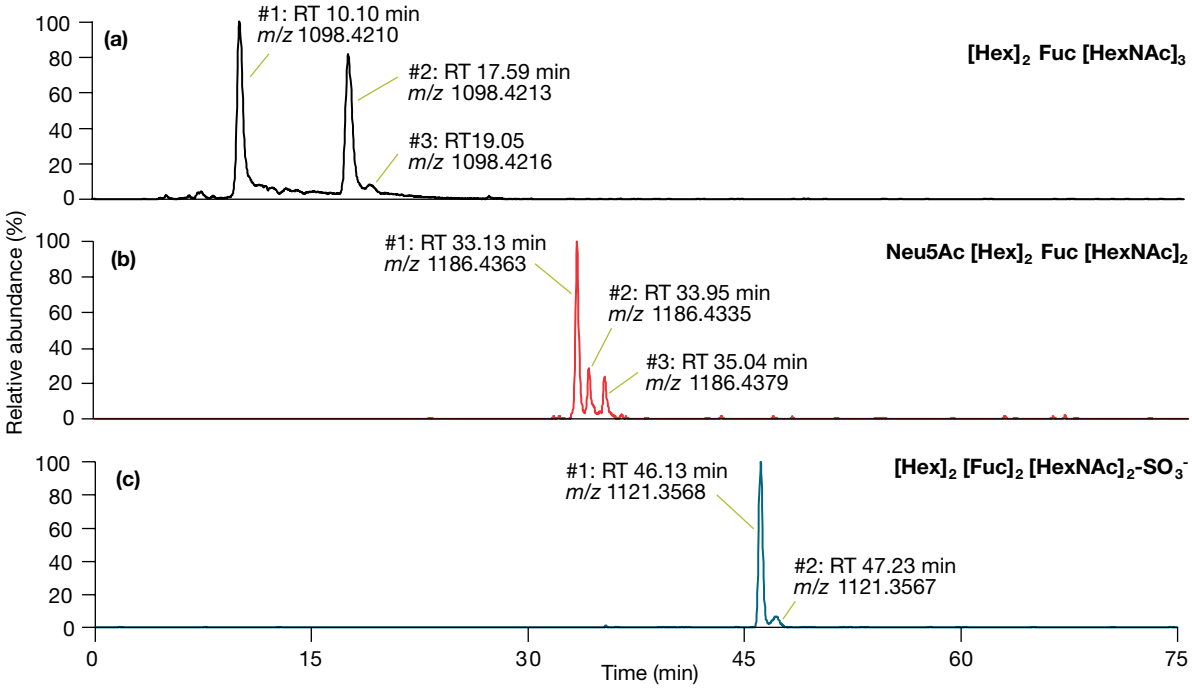

Figure 8. Extracted ion chromatograms of structural isomers: (a) $[Hex]_2$ Fuc $[HexNAc]_3$, (b) Neu5Ac $[Hex]_2$ Fuc $[HexNAc]_2$, and (c) $[Hex]_2$ $[Fuc]_2$ $[HexNAc]_2$-SO_3^-

The structural interpretation of the isomeric glycans is only possible based on their highly informative MS^2 spectra. Mass spectrometry with electrospray ionization in negative mode facilitates both the sequence and linkage characterization of O-glycans.[9] Diagnostic fragments from tandem MS enable the assignment of particular glycan epitopes, such as O-glycan core types, N-acetyllactosamine configurations, and blood group-related epitopes.[9,10] In Figure 9, MS/MS spectra and the annotation of the proposed fragment ions of two glycan isomers illustrate that different fragmentation patterns can be obtained for the same composition, which in this case is $[Hex]_2$ Fuc $[HexNAc]_3$. Figure 9 (a) shows a core 2 structure with a blood group H epitope attached to GalNAc alditol, while its isomer in Figure 9 (b) shows a core 4 structure also with a blood group H epitope type 1 or 2.

Differentiation of the structural isomers and glycosidic linkages was achieved by observation of characteristic ions, especially the A-type cleavages in the negative ion mode. The presence of $^{1,3}A_{Gal}$ at *m/z* 205.0719 indicates the presence of Fuc (α1-2) Gal in both isomers. In Figure 9a, the presence of $Y_{1\alpha}/Z_{2\beta}$ at *m/z* 366.1416 and $Z_{2\alpha}/Z_{1\beta}$ at *m/z* 389.1547 suggests a core 2 structure. In Figure 9b, the presence of $B_{1\beta}$ at *m/z* 161.0457, $B_{2\beta}$ at *m/z* 364.1263, and $^{0,4}A_{GalNAc}$ at *m/z* 424.1469 reveal the substituent on C-6 with the composition of Gal-GlcNAc.[10] The fragment ions of $Z_{2\alpha}/Z_{1\beta}$ at *m/z* 389.1571, $Z_{1\alpha}$ at *m/z* 569.2222, and $Z_{2\alpha}$ at *m/z* 772.3041 together suggests a core 4 structure.[9]

HPAE enables such high-resolution separation using high-pH eluents. Therefore, an electrochemically regenerated desalter was used to remove the high concentration of eluent salts prior to MS analysis. The Dionex ERD 500 employs a countercurrent cation exchange mechanism, where sodium ions are exchanged by hydronium ions. The ion exchange process is driven by both electrolytically generated and pneumatically supplied regenerant. A properly operated Dionex ERD500 has the capacity to exchange the sodium ions up to a concentration of 0.35 M. Oligosaccharides elute from the Dionex ERD 500 in dilute acetic acid, which assists the ionization of neutral glycans in the MS.

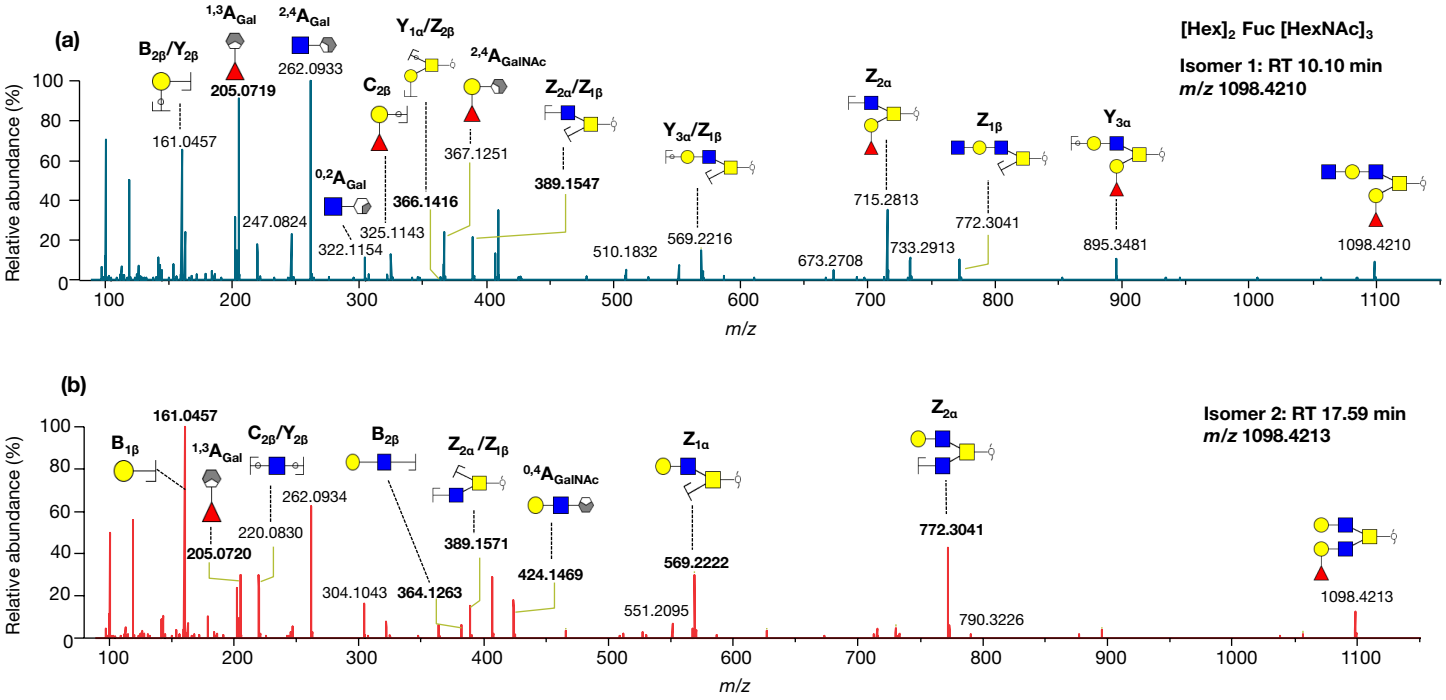

Figure 9. MS/MS spectra and proposed fragment ions from two isomeric structures of O-glycans with the composition $[Hex]_2$ Fuc $[HexNAc]_3$. (a) Isomer #1 eluting at 10.10 min. (b) Isomer #2 eluting at 17.59 min.

In order to characterize the impact of the ERD 500 on glycan peak resolution, we examined the peak shape of two free oligosaccharide standards before and after passing through the Dionex ERD 500. Towards this end, a fully exhausted ERD 500 was placed after the Dionex CarboPac PA300-4µm column and prior to the PAD detector with a PdH reference electrode. Figure 10 shows the PAD chromatograms of 2'-fucosyllactose (2'-FL) and 6'-sialyllactose (6'-SL) standards at 50 mg/L level. The peak characteristics are shown in Table 2.

The retention time shift caused by the Dionex ERD 500 was around 0.4 min, which was independent from glycan type. All peaks observe losses of peak asymmetry varying between 20 and 50% due to the post-column dispersion. The 6'-SL standard material also contained a small amount of 3'-sialyllactose (3'-SL). The peak resolution was therefore examined for those two peaks. In both cases, the Dionex ERD 500 caused a slight decrease in resolution. However, the Dionex ERD 500 Electrolytically Regenerated Desalter did not significantly degrade the resolution or affect the detection for the downstream MS analyses of the tested glycan standards.

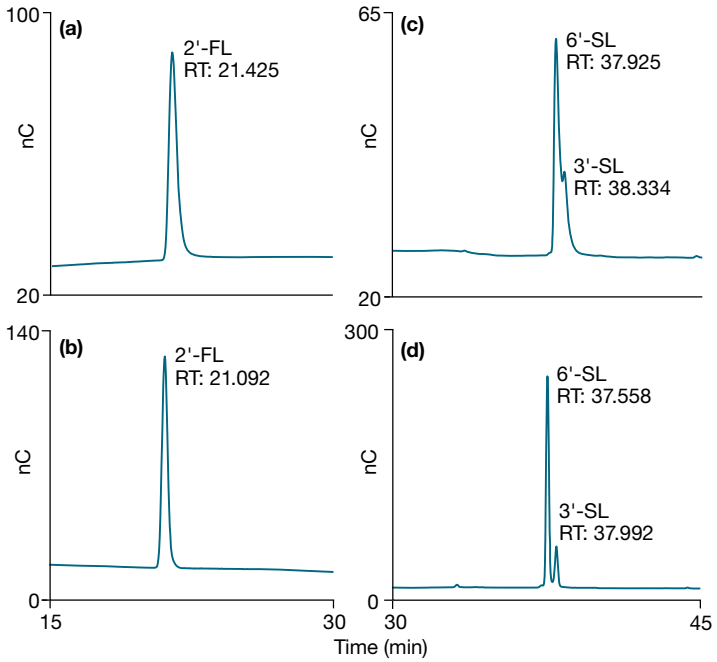

Figure 10. PAD chromatograms of three milk oligosaccharides: (a) and (c) with an exhausted a Dionex ERD 500, (b) and (d) without a Dionex ERD 500

Table 2. Peak properties of the selected free glycan standards with/without a Dionex ERD 500

Glycan standard (50 mg/L)	With/without an exhausted ERD 500	Retention time (min)	Peak asymmetry	Peak resolution
2'-FL	Without	21.092	1.12	n/a
	With	21.425	1.54	n/a
6'-SL	Without	37.558	1.05	1.79
	With	37.925	1.28	1.35
3'-SL	Without	37.992	1.01	5.45
	With	38.334	1.54	5.35

Conclusion

- Demonstrated the use of a novel Dionex CarboPac PA300-4µm column for the separation of a wide range of neutral and charged O-glycans, facilitating the sensitive detection by PAD and Q Exactive Orbitrap mass spectrometry.
- Introduced a new IC-MS system configuration with the sequential detection of PAD and MS for Dionex CarboPac PA300-4µm applications.
- HAPE-PAD-MS platform presents a powerful tool for the structural characterization of label-free O-glycans released from mucin glycoproteins. While PAD provides incredibly sensitive detection of the glycan profile, simultaneous MS and MS/MS data provides information for glycan structural annotation.

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