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# Towards a More Complete Glycome: Advances in Ion Chromatography-Mass Spectrometry (IC-MS) for Improved Separation and Analysis of Carbohydrates

Neil G. Rumachik,* Tian Tian,* Yuanxue Hou, Charanjit Saini, Zoltan Szabo, Yan Liu, Chris Pohl, Thermo Fisher Scientific, 1228 Titan Way, Sunnyvale, CA, 94086; *contributed equally to this work

## ABSTRACT

**Purpose:** Demonstrate the coupling of high performance anion exchange (HPAE) chromatography with Orbitrap mass spectrometry to enable more complete glycome characterization

**Methods:** Developed novel Thermo Scientific™ Dionex™ CarboPac™ PA300 column to use with a Thermo Scientific™ Dionex™ ICS-6000 HPIC™ coupled to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole Orbitrap™ mass spectrometer for structural characterization of neutral and charged glycans

**Results:** Novel anion exchange column technology improves resolution of neutral glycans while maintaining capabilities to separate charged species. Improved platform performance is demonstrated via analysis of complex oligosaccharides from biologics and food samples

## INTRODUCTION

Glycosylation is one of the most prevalent and diverse types of post-translational modification (PTM), and it is known to be important for myriad cellular and developmental processes, including cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis. This PTM is often very challenging to study, as glycosylation is non-template driven, heterogeneous, and substoichiometric. O-linked glycan sample preparation is particularly challenging, as there is no known enzyme for the universal release of O-glycans from biomolecules. Additionally, glycans are often complex both in structure and composition, often creating high-density, heterogenous clusters of glycans on regions of biomolecules like mucins. Therefore, high resolution chromatographic separations become important for subsequent mass spectrometric characterization.

To date, few tools are available for the analysis of the glycome without derivatization, a process which is known to introduce issues such as differential loss of sialic acid and incomplete labeling. We have previously reported the use of ion chromatography-mass spectrometry (IC-MS) to analyze native sialylated and sulfated glycans [1]. Here, we expand upon our previous reports, introducing the Dionex CarboPac PA300 column, a new introduction into the Dionex CarboPac suite of columns that uses novel column technology to enable the simultaneous separation of neutral and charged glycans without the need of derivatization. Using the column in an IC-MS workflow takes advantage of the improved chromatographic separation as well as the high resolution and mass accuracy of the Orbitrap mass analyzer. The MS data and tandem MS/MS spectra provide diagnostic fragment ions, allowing for the reliable structural annotation of heterogenous glycans. The unique HPAE-PAD/MS workflow presented here provides complementary often orthogonal information to that of other standards for O-glycan analysis.

## MATERIALS AND METHODS

### Sample Preparation

For glycan release, samples were incubated in 50 mM NaOH containing 1 M $NaBH_4$ at 50 ºC for 16 h. After incubation, samples were kept on ice and neutralized by careful addition of formic acid. The neutralized samples were evaporated to dryness in a speedvac. The samples were then washed three times with 0.5 mL methanol. Afterwards, samples were dissolved in 0.5 mL of 0.1 % TFA solution (in water). Free and released glycan samples were passed through porous graphitic carbon (Thermo Scientific™ HyperSep™ filter plates, 40 µL). After washing the resin twice with 0.5 mL of 0.1 % TFA solution, oligosaccharides were eluted in 400 µL of 40 % acetonitrile containing 0.1 % TFA and evaporated to dryness.

### High performance anion exchange chromatography (HPAEC)

A Dionex ICS-6000 HPIC dual channel IC system coupled with a Thermo Scientific™ Dionex™ AS-AP Autosampler was used for glycan separations. The system was equipped with a Thermo Scientific™ Dionex™ BorateTrap Inline Trap Column between the gradient mixer and injection valve. A Dionex CarboPac PA300 analytical column (2 x 250 mm) and guard column (2 x 50 mm) were used at 0.25 mL/min flow rate for separations. The column effluent was sent either to the PAD or MS for detection of glycans. In the system configuration with MS, a Thermo Scientific™ Dionex™ ERD 500 electrolytically regenerated desalter (anion, 2 mm) was placed prior to MS ionization.

### Mass spectrometry

All MS experiments were performed on a Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer in negative mode ESI with Labile Compound User License. The mass spectrometer was operated with full mass scan: *m/z* 400-2,000 at a resolution of 60,000 (FWHM at *m/z* 200). The AGC target was set to $3x10^6$ with maximum IT: 120 ms. Data dependent $MS^2$ were acquired using the Top 10 method. $MS^2$ spectra were interpreted using the UniCarb-DB database.

## RESULTS

Figure 1 shows the improved separation of neutral glycan species with the new Dionex CarboPac PA300 column. While maintaining the ability separate charged species towards the latter half of the run, the new column technology affords improved separation of early eluting glycans. This introduces the ability to tailor HPAE-PAD/MS workflows based on sample composition, expanding the toolbox of technologies to study and characterize the glycome.

**Figure 1. Chromatograms of released O-glycans from a porcine gastric mucin type III sample for the Dionex CarboPac PA200 (bottom trace) and Dionex CarboPac PA300 (top trace). The window in which neutral glycans elute from the columns is shown with dashed lines.**

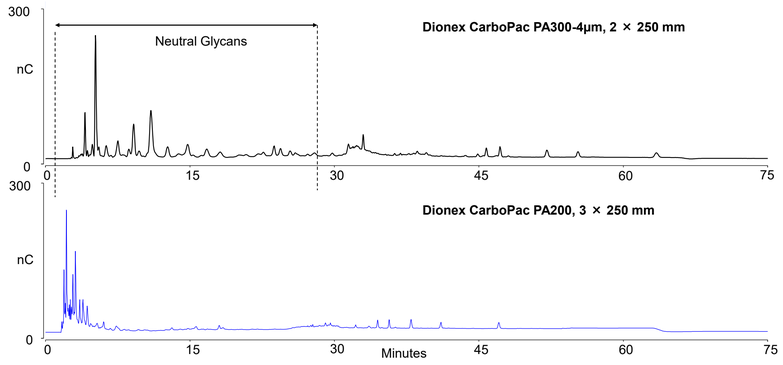
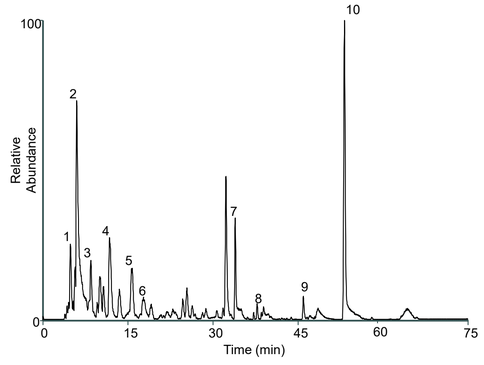



**Figure 2. Base peak chromatogram of porcine gastric mucin type III O-glycans injected into a Q Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer.**



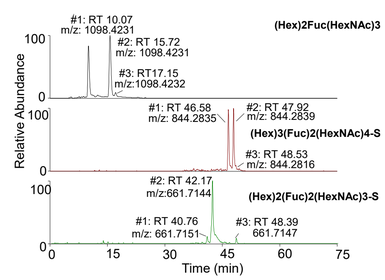

When the CarboPac PA300 is used in an IC-MS workflow, identification of peaks eluting throughout the chromatographic run is possible. Figure 2 shows the base peak chromatogram of a sample of released O-glycans from a porcine gastric mucin type III sample. A selection of peaks corresponding to an array of glycan compositions were identified and labeled. The peak identities are tabulated below in Table 1, showing remarkable mass accuracy. Compositions were confirmed with previously published data [2].

**Table 1. Glycan identities of the labeled peaks in Figure 3.**

| Peak | RT (min) | Observed *m/z* | Theoretical *m/z* | Mass accuracy (ppm) | Composition | Ion |
|---|---|---|---|---|---|---|
| 1 | 4.85 | 733.2896 | 733. 2884 | 1.6465 | HexFuc(HexNAc)2 | $[M-H]^-$ |
| 2 | 6.08 | 530.2080 | 530.2090 | 1.8860 | HexFucHexNAc | $[M-H]^-$ |
| 3 | 8.46 | 733.2892 | 733. 2884 | 1.0910 | HexFuc(HexNAc)2 | $[M-H]^-$ |
| 4 | 11.97 | 1041.4006 | 1041.3992 | 1.4403 | (Hex)2(Fuc)2(HexNAc)2 | $[M-H]^-$ |
| 5 | 15.58 | 1098.4233 | 1098.4206 | 2.4581 | (Hex)2Fuc(HexNAc)3 | $[M-H]^-$ |
| 6 | 17.66 | 1203.4506 | 1203.4520 | 1.1633 | (Hex)3(Fuc)2(HexNAc)2 | $[M-H]^-$ |
| 7 | 34.01 | 821.3063 | 821.3045 | 2.1916 | NeuAcHexFucHexNAc | $[M-H]^-$ |
| 8 | 38.19 | 852.2811 | 852.2848 | 4.3413 | N-glycan, hybrid, sulfated | $[M-2H]^{2-}$ |
| 9 | 45.92 | 1121.3582 | 1121.3560 | 1.9619 | (Hex)2(Fuc)2(HexNAc)2-S | $[M-H]^-$ |
| 10 | 53.19 | 813.2474 | 813.2452 | 2.7052 | HexFuc(HexNAc)2-S | $[M-H]^-$ |

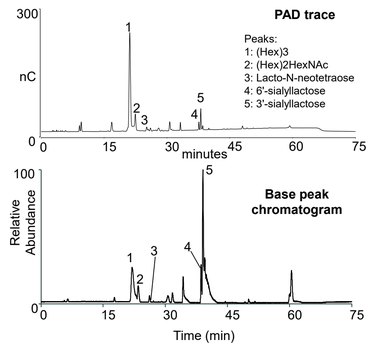
Hex: Hexose; Fuc: Fucose; HexNAc: N-Acetylhexosamine; NeuAc: N-Acetylneuraminic acid; S: Sulfate

**Figure 3. The Dionex CarboPac PA300 column provides isomer resolution of both neutral and sulfated O-glycans from a porcine gastric mucin type III sample.**




The excellent resolution achieved by the Dionex CarboPac PA300 column is especially important for compositionally identical but chemically distinct isomeric species. Coupled with the high mass accuracy of the Orbitrap mass analyzer, separation by HPAE chromatography affords the identification of otherwise coeluting isomers, as in the three examples shown in Figure 3 for $(Hex)_2Fuc(HexNAc)_3$, $(Hex)_3(Fuc)_2(HexNAc)_4$-S, and $(Hex)_2(Fuc)_2(HexNAc)_3$-S.

**Figure 4. Free oligosaccharides from commercial lactose-free milk with the Dionex CarboPac PA300 column.**




Structural annotation of oligosaccharides in foods has become increasingly important, as these glycans are often used as prebiotics to promote healthy gut microbiomes. Figure 4 shows the ability of CarboPac™ PA300 column to separate neutral, non-sialylated glycans from charged, sialylated structures. A selection of peaks are numbered, and the corresponding peak identity is shown in the legend of the upper panel.

## CONCLUSIONS

- High performance anion exchange (HPAE) offers highly efficient chromatographic O-glycan separations
- HPAE shows outstanding separation of sulfated O-Glycan alditols and their isomers
- The coupling of HPAE to an Orbitrap mass spectrometer enables the collection of highly informative $MS^2$ spectra, allowing reliable structural annotation
- The Dionex CarboPac suite of columns affords workflow solutions to tailor analysis based on specific sample composition
- IC-MS provides access to the acidic subset of the glycome and offers complementary data to RPLC-MS-based approaches

## REFERENCES

1. Anal. Bioanal Chem. 2017;409(12):3089-3101.
2. Mol. Cell Proteomics. 2017;16(5):743-758.

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## TRADEMARKS/LICENSING



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