APPLICATION NOTE 73988

An improved method for galactosyl oligosaccharide characterization

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Introduction

Galactosyl oligosaccharides (GOS) are well known as prebiotics that improve digestive and immune health.[1] Prebiotics are non-digestible ingredients that beneficially affect human health by selectively stimulating the growth of one or a limited number of bacteria in the colon.[2] GOS are produced by the enzymatic transgalactosylation of lactose using β-galactosidase, giving oligomers with varied degrees of polymerization (DP) ranging from DP2 to DP10 with a terminal glucose[3] at the reducing end. Depending on the enzymatic source used for their synthesis, the chemical structure of these oligosaccharides varies.[4] Due to the beneficial effect of GOS on gut health, it is important to characterize the composition, enabling the study of structure-activity relationships.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is a well know technique that is routinely used to provide high-resolution separation of oligosaccharides.[5,6] Unlike HPLC and GC methods, HPAE-PAD does not require oligosaccharide derivatization. Oligosaccharides present in complex matrices can be analyzed directly without preprocessing and with high sensitivity.[7] HPAE-PAD has previously been used for GOS characterization.[8] Another great advantage of HPAE-PAD is that it can be coupled to a mass spectrometer (HPAE-PAD/MS). Such coupling provides for a powerful analysis approach as it not only allows collecting DP information but also monosaccharide composition and linkage information depending on the intended application. This greatly simplifies GOS characterization, as collecting fractions and analyzing them for DP information becomes unnecessary. For example, fraction collection was needed when some disaccharide peaks were poorly resolved.[9] Disaccharides are the most abundant components of GOS and should therefore be chromatographically well separated. While the combination of HPAE-PAD and mass spectrometry has been beneficial for oligosaccharide analysis, there was room for improvement in the HPAE columns used for GOS analysis, including column

parameters that were not ideal for mass spectrometry (i.e., too large column i.d. and therefore requiring higher flow rates than ideally suited for MS detection).

Here, we describe a HPAE-PAD/MS method for high-resolution profiling of GOS. Separation of individual oligosaccharides in GOS was achieved on a Thermo Scientific™ Dionex™ CarboPac™ PA300-4µm column. This anion exchange column is primarily designed for the analysis of complex oligosaccharides from heterogeneous biological and food samples. The Dionex CarboPac PA300-4µm column is packed with a new latex agglomerated supermacroporous resin to achieve high efficiency and high-resolution separations. It is stable over a pH range of 0–14. This column improves the separation of GOS compared to other HPAE columns. After separation, oligosaccharides are detected in parallel by PAD and mass spectrometry. Neither PAD nor mass spectrometry require sample derivatization for detection. For mass spectrometry, the oligosaccharides pass through a desalting device that replaces sodium present in the eluent for hydronium ions. Intact oligosaccharides are first analyzed without fragmentation. Then, oligosaccharide fragmentation in the negative ionization mode by high energy collisional dissociation (HCD) provides MS^2 spectra that include glycosidic as well as cross-ring fragments that were used to identify oligosaccharide DP. This approach is an effective way to achieve high-resolution separation and thorough analysis of a complex sample such as GOS.

Experimental

Equipment

- The Thermo Scientific™ Dionex™ ICS-5000+ Reagent-Free Ion Chromatography system* is an integrated ion chromatograph that includes:
  - DP Dual Pump (P/N 061712) with degas option
  - DC detector compartment (P/N 061767) with single-temperature zone
  - Electrochemical detector (P/N 061719) and cell (P/N 061757)
  - Conductivity detector (P/N 079829)
  - pH-Ag/AgCl reference electrode (P/N 061879)
  - Carbohydrate disposable Au working electrode, pack of 6 (two 2.0 mil gaskets included) (P/N 066480)
  - 2.5 µL sample loop
- Thermo Scientific™ Dionex™ AS-AP autosampler (P/N 074926) with cooling tray option (recommended)
- 1.5 mL polypropylene autosampler vials, with caps and split septa (P/N 079812)
- Thermo Scientific™ Nalgene™ Rapid-Flow 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (P/N 164-0020)
- Thermo Scientific™ Dionex™ BorateTrap™ column, 4 × 50 mm (P/N 047078)
- HPAE-PAD/MS Assembly Kit (P/N 302854)

* Note—A Thermo Scientific™ Dionex™ ICS-6000 Reagent-Free Ion Chromatography system can also be used for this work.

Columns

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

Software

- Thermo Scientific™ Xcalibur™ software with SII for Xcalibur software
- Simglycan 5 (Premier Biosoft, Palo Alto, CA)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software (data processing)

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)
- Bimuno GOS syrup (https://clasado.com/)
- Ultra high purity grade nitrogen (Airgas P/N NI UHP200)

Chromatography conditions

Parameter	Value
Flow rate	0.25 mL/min
Injection volume	2.5 µL
Column temperature	30 °C
Compartment temperature	25 °C
Tray temperature	4 °C
Eluents	A) DI water; B) 50 mM NaOH; C) 50 mM NaOH, 100 mM NaOAc
Elution conditions	Table 1
Detection	Pulsed amperometry
Working electrode	Gold on PTFE working electrode
Reference electrode	Ag/AgCl
PAD waveform	Four-potential waveform for carbohydrates (Table 2)

Table 1. Gradient program for elution

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)	Elution
-20	80	20	0	Equilibration
0	80	20	0	Initial condition
8	80	20	0	Maintain initial condition for 8 min
30	0	100	0	10 to 50 mM NaOH
55	0	30	70	0 to 70 mM NaOAc, isocratic 50 mM NaOH
65	0	30	70	Maintain 70 mM NaOAc, 50 mM NaOH for 10 min
65	80	20	0	Re-equilibrium

Table 2. Carbohydrate four-potential waveform for the ED

Time (s)	Potential (V)	Gain region*	Ramp*	Integration
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 Dionex systems.

Methods

Preparation of Bimuno GOS solution

Weigh 1 g of Bimuno GOS syrup into a 125 mL polypropylene bottle. Add DI water to 100 g to make a 10,000 mg/L stock standard. Store the stock standard at 4 °C. Using this stock standard, freshly prepare working standards (100–2,000 mg/L) before analysis to avoid degradation.

Eluent preparation

The use of high-quality deionized water (DI) of high resistivity (18 MΩ·cm) as free of dissolved carbon dioxide as possible is recommended for eluent preparation. It is essential to minimize contamination by carbonate, a divalent anion at high pH that binds strongly to the columns, causing a loss of chromatographic 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 required sodium hydroxide source. Fresh eluents should be prepared every week or when inert gas cover is lost. Do not shake the eluent bottles as this will introduce carbonate into the eluents.

Methods of preparation for two eluents used in this work are described here.

- 50 mM sodium hydroxide: To 900 mL of DI water, previously filtered through a 0.2 µm Nalgene, 1 L nylon filter and degassed, add 2.6 mL of 50% (w/w) sodium hydroxide solution, mix gently, and immediately install on the system under UHP grade nitrogen cover at 34 to 55 kPa (5 to 8 psi).

- 0.1 M sodium acetate in 0.05 M sodium hydroxide: Dissolve 8.2 g of anhydrous sodium acetate in 800 mL of previously degassed DI water. Vacuum filter this solution through a 0.2 µm Nalgene, 1 L nylon filter to remove particles from the sodium acetate that can damage parts of the pump. Transfer the solution to a 1 L volumetric flask, add 2.6 mL of 50% (w/w) NaOH and bring to volume, mix gently, and immediately install on the system under UHP grade nitrogen gas cover at 34 kPa (5 psi).

Use dedicated eluent bottles for hydroxide only and hydroxide eluents containing acetate to avoid the possibility of cross contamination. For additional details on mobile phase preparation refer to Thermo Scientific Technical Note 71.[10]

High performance anion exchange chromatography

The GOS were separated on a Dionex CarboPac PA300-4 µm analytical column (2 × 250 mm) installed in a Dionex ICS-5000⁺ HPIC dual IC system. The system was equipped with a Thermo Scientific™ Dionex™ ERD™ 500 electrolytically regenerated desalter. Desalting is accomplished using a current of 250 mA and 3.5 mL/min regenerant water flow. The column flow is split immediately post column and ~125–130 µL/min (50–60% of the total flow) is diverted to the desalter and into the mass spectrometer with the other half going to the PAD.

Coupling HPAE to the mass spectrometer

The Dionex ICS-5000⁺ HPIC system is configured for electrochemical detection, operating under high-pressure conditions up to 5000 psi. Figure 1 shows the system configuration for this application. To install this application, first connect the Dionex AS-AP autosampler, Dionex ICS-5000⁺ HPIC system, Dionex ERD 500 desalter, and the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer modules using the HPAE-PAD/MS Assembly Kit. The IC/MS connection kit contains three lengths of 0.005" i.d. (red) tubing to apply backpressure to the ED cell. These are 90, 100, and 110 cm long. Start with the 100 cm length tubing attached to the ED cell outlet. If needed, test the other two to ensure 50–60% eluent flow to the desalter. For detailed instruction on system configuration see Technical Note 72478.[11]

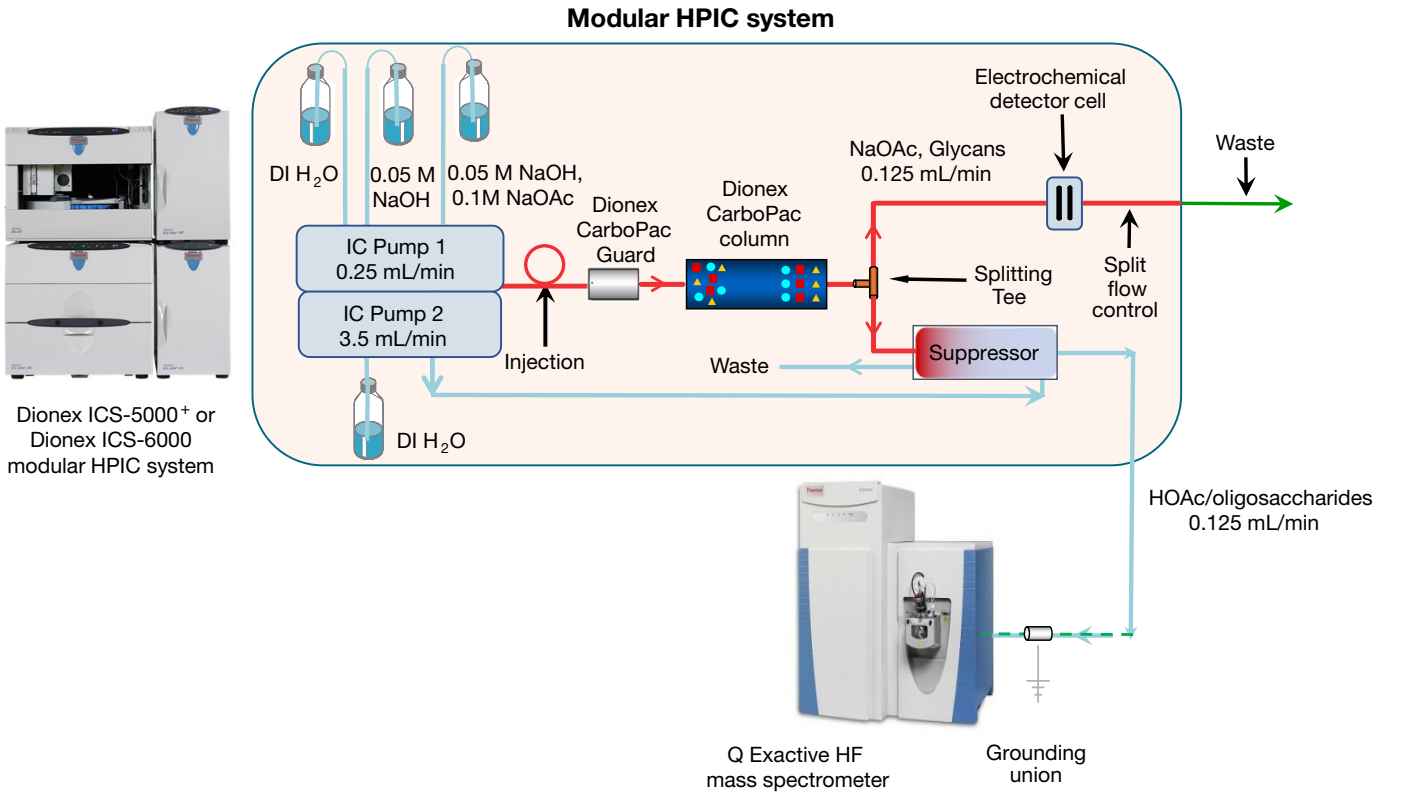

Figure 1. IC system configuration for coupling to the Q Exactive mass spectrometer for carbohydrate analysis

Configuring the Dionex ERD 500

The Dionex ERD 500 Electrolytically Regenerated Desalter is designed for removing sodium ions from column effluent before passing into the mass spectrometer. A properly operating Dionex ERD 500 will exchange ≥99.5% of sodium ions present (up to 0.35 M) under recommended conditions. When first installing, it is important to hydrate the desalter by running the regenerant until no bubbles are observed exiting the device. This could take up to 5 min. Then allow the Dionex ERD 500 to stand for 20 min without regenerant flow. This procedure is described in more detail in the Dionex ERD 500 manual (Thermo Scientific P/N 031761-05).[12] Before coupling HPAE to the mass spectrometer, it is important to ascertain reproducible desalting during a sample run. Reproducible operation can be ensured by running at least three DI water injections and measuring conductivity using the conductivity detector. After confirming reproducible desalting, HPAE can be coupled to the mass spectrometer.

The experimental conditions used for desalting in this work are as follows.

Dionex ERD 500 type	2 mm
Eluent flow	0.25 mL/min (provided by pump 1)
Regenerant DI water flow	3.5 mL/min (provided by pump 2)
Suppressor current	250 mA

Note—The Dionex ERD 500 is rated to 150 psi total post desalter backpressure. It is important not to exceed this pressure limit. If necessary, adjust tubing lengths accordingly to maintain the backpressure on the Dionex ERD 500 below 150 psi. Refer to the Dionex ERD 500 manual for more details.[12]

Mass spectrometry conditions

A Q Exactive mass spectrometer used in negative ion electrospray mode was coupled to the IC system.

Parameter	Value
Spray voltage	3.2 kV
Capillary temperature	320 °C
Probe heater temperature	200 °C
Sheath gas flow	30 arbitrary units
Auxiliary gas flow	10 arbitrary units
Acquisition range	*m/z* 150–2000
Resolution	120,000 FWHM (at *m/z* 200)
Microscans	1
Quad isolation window	4 *m/z*
MS experiments	
Automatic gain control (AGC) target	3×10^6
Maximum injection time	100 ms
Data-dependent MS^2 experiments	
Target	Top five ions
Normalized collision energy (NCE)	30 kV
AGC target	1×10^5
Maximum injection time	150 ms

Configuring the IC modules in SII for Xcalibur software

To configure the IC system, first close all MS data acquisition programs. Open the Configuration program (gear symbol), select the SII module, select configure, and then add the IC modules. Three modules are added to the instrument configuration of Dionex ICS-5000+ HPIC system: Dionex DP pump and Dionex DC detector modules, and Dionex AS-AP autosampler. Change the detector and linked pump settings for the DC detector module to reflect the correct pump-detector pairing (e.g., electrochemical detector). Select appropriate syringe (250 µL), buffer loop (1200 µL), and injection loop (2.5 µL) volumes for the Dionex AS-AP autosampler.

Creating an instrument method and running a sample sequence

To create a new instrument method using the Xcalibur Instrument Set Up wizard, enter the values from the Chromatographic and Mass Spectrometry conditions sections. Save the instrument method. For running the sequence, open the Sequence Setup wizard and then

choose the appropriate instrument method, vial position, data folder path (where data files will be saved), and injection volume. Select Thermo Scientific Xcalibur SII to be the start instrument program when running a single sample or entire sequence. This will ensure that sample injection by the Dionex AS-AP autosampler serves at the trigger for starting a run.

Sample analysis

Equilibrate the column with initial mobile phase conditions for a minimum of 20 min and make sure the column temperature has reached 30 °C. Inject a 2.5 µL DI water blank sample and run the gradient program at least twice to equilibrate the column and system before starting GOS sample analysis per the conditions listed earlier.

Results and discussion

Separation of GOS

The separation of GOS by HPAE was achieved on a Dionex CarboPac PA300-4µm column. We first evaluated the GOS separation using different hydroxide and acetate concentrations. Based on these results, a 70 min elution method that uses 50 mM sodium hydroxide and 100 mM sodium acetate was designed (Table 1). The column effluent containing separated oligosaccharides was then passed through a Dionex ERD 500 to electrolytically replace the sodium ions for hydronium ions in the effluent prior to mass spectrometry. We used HPAE-PAD coupled to an HRAM mass spectrometer with an Orbitrap mass analyzer in negative electrospray mode. Figure 2 shows the HPAE-PAD chromatogram and resultant extracted ion chromatogram (EIC) for the GOS sample. Starting with the glucose/galactose peak (peak 1) at ~14 min, 28 individual GOS peaks were detected. Resolution and peak shapes were generally preserved as peaks pass from the desalter into the mass spectrometer. Passage through the desalter can sometimes lead to poor monosaccharide recovery, as observed here for the glucose/galactose peak. The time lag between an eluting peak detected by PAD and mass spectra was about 0.8 min. This time accounted for the dead volume of the Dionex ERD 500 (~60 µL) and tubing used for connections.

Desalting before MS analysis

For coupling of HPAE separation to the mass spectrometer, sodium ions present in the eluent must be removed. Here, the Dionex ERD 500 Electrolytically Regenerated Desalter is used in the external water mode, with DI water as reagent, to electrolytically replace the sodium ions in the eluent for hydronium ions. The Dionex ERD 500 contains microporous cation-exchange screens to transfer the sodium ions from the eluent channel to the regenerant channel, replacing them with hydronium ions. The exchange of sodium ions for hydronium ions generates dilute acetic acid that assists in ionization of oligosaccharides in the MS^1 (and fragmentation in MS^2). We monitored the conductivity of the eluent after passage through the desalter. The gradient elution method to separate GOS uses an acetate concentration that increases from 0 to 70 mM from 30 to 55 min. Figure 3 shows reproducible sodium removal comparing three consecutive injections. The conductivity increases non-linearly with the increase in acetic acid concentration.

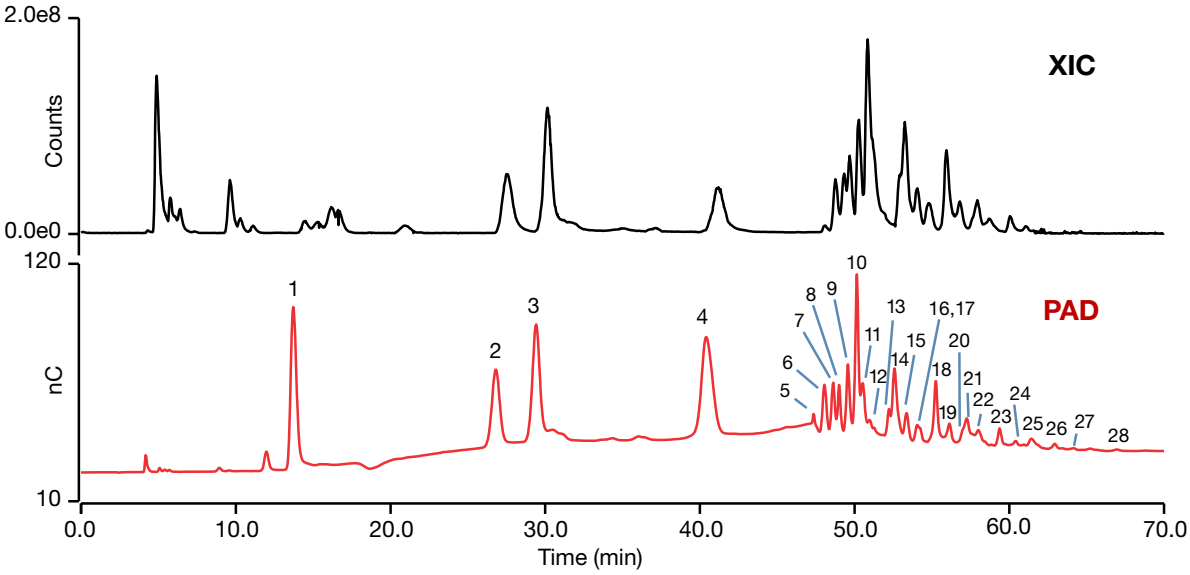

Figure 2. Comparison of PAD and MS traces for GOS separated on a Dionex CarboPac PA300 column

The passage through the Dionex ERD 500 impacts peak resolution. It is important to minimize peak dispersion upon passage through the desalter. To verify this, oligosaccharide peak resolution before and after passage through the Dionex ERD 500 was measured. (Figure 4). To accomplish this, we allowed the Dionex ERD 500 to run without power and regenerant, which soon stopped the removal of sodium and thus the eluent remained alkaline so that oligosaccharides could be detected by PAD. Oligosaccharide resolution before and after passage through the desalter was measured and detected by PAD only. The peak labels indicate the resolution values as measured by Chromeleon CDS software using the statistical moments method. For the GOS, the average change in resolution observed after passage through the 2 mm Dionex ERD 500 was 14.5%. This demonstrates that passage through the Dionex ERD 500 does not lead to a significant loss of peak resolution.

Assignment of GOS DPs

The mass accuracy of measurement was determined by taking experimental masses for representative oligosaccharides from DP2 to DP6 and comparing them to accurate masses. The results (Table 3) show accurate mass measurement with the highest measurement error of 1.69 ppm. Figure 5 shows extracted ion chromatograms (XIC) for *m/z* values corresponding to DP2 to DP6 values from GOS mass spectral data. The XICs show the distribution of DPs based on *m/z* of their molecular ion $[M-H]^-$ across the chromatogram. The retention times, DP, and proposed structures of GOS peaks identified in this work are summarized in Table 4. The DP and linkage information were determined by identifying characteristic glycosidic and cross-ring fragments in negative ion mode MS^2 data. Figure 6 A-E shows various peaks in the MS^2 spectra that were used for confirming GOS DP assignment. However, no information on the type of linkage (α or β) and monosaccharide composition could be obtained from the MS^2 data. The denoted monosaccharide composition assumes galactose residues connected to terminal glucose at the reducing end.

Table 3. Mass accuracy of representative GOS DPs

DP	Chemical formula	Exact mass $[M-H]^-$ (Da)	Measured mass $[M-H]^-$ (Da)	Error (ppm)
2	$C_{12}H_{22}O_{11}$	341.1089	341.1087	-0.59
3	$C_{18}H_{32}O_{16}$	503.1618	503.1616	-0.40
4	$C_{24}H_{42}O_{21}$	665.2146	665.2152	0.90
5	$C_{30}H_{52}O_{26}$	827.2674	827.2688	1.69
6	$C_{36}H_{62}O_{31}$	989.3202	989.3202	989.3214

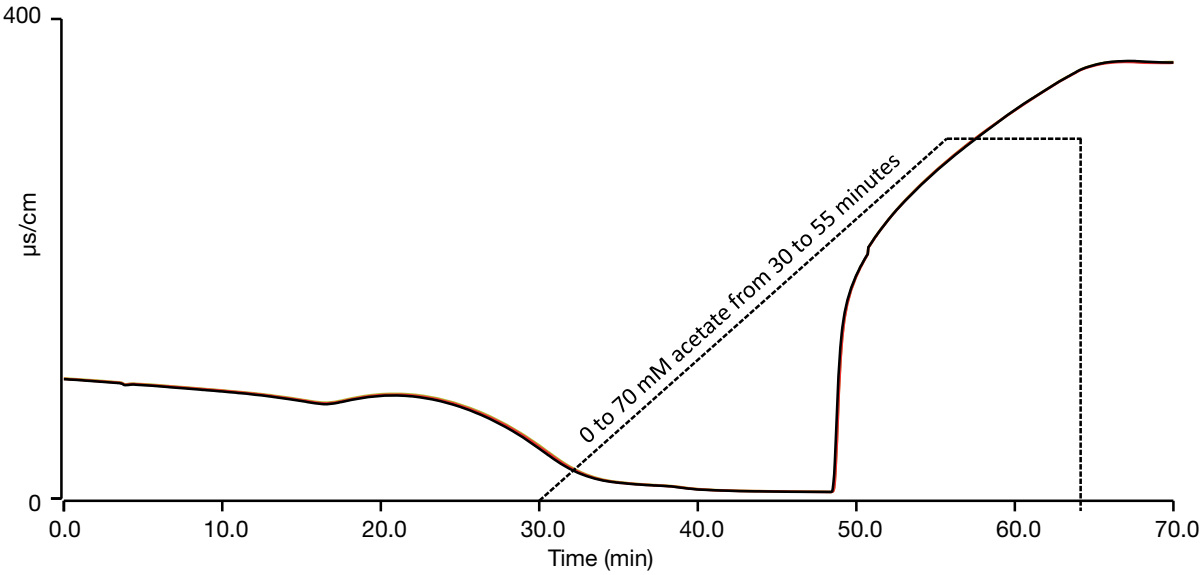

Figure 3. Reproducibility of Dionex ERD 500 desalter operation during GOS oligosaccharide separation (n=3)

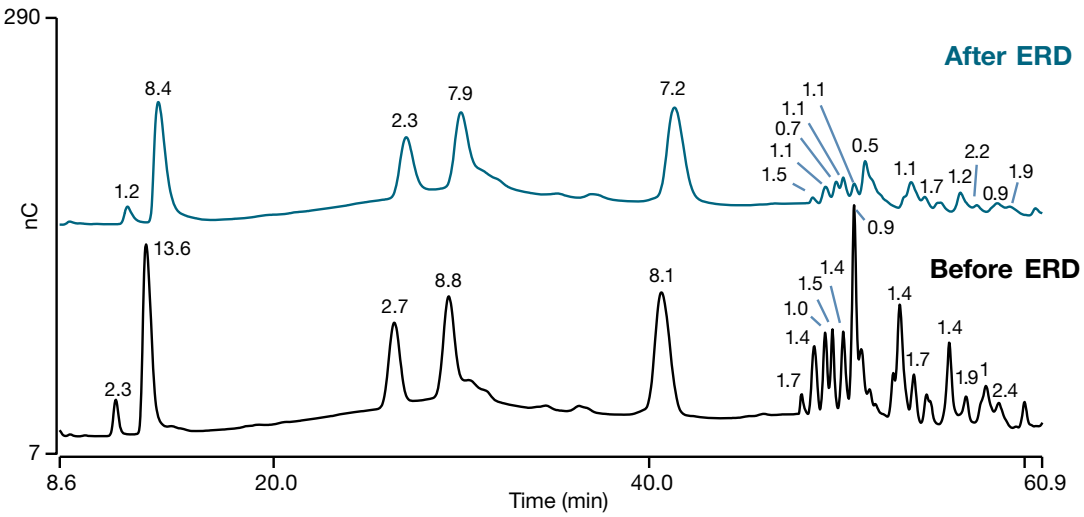

Figure 4. Effect of a 2 mm Dionex ERD 500 desalter on oligosaccharide resolution (the peak labels are resolution values)

Table 4. Identification of GOS DPs (peak numbers from Figure 2)

No.	RT (Min)	Observed base peak *m/z*	DP	Tentative structure assignment*
1	14.0	179.1	1	Glucose/Galactose
2	27.2	683.2	2	Allolactose
3	30.0	683.2	2	Lactose
4	41.3*	683.2	2	Gal-(1-x**)-Glc + Gal-(1-x)-Glc
5	48.8*	503.2	3	Gal-(1-4)-Gal-(1-4)-Glc
6	49.5	503.2	3	Gal-(1-4)-Gal-(1-4)-Glc
7	50.0	503.2	3	Gal-(1-4)-Gal-(1-4)-Glc
8	50.4	503.2	3	Gal-(1-4)-Gal-(1-4)-Glc
9	51.0	563.2***	3	Gal-(1-x)-Gal-(1-6)-Glc
10	51.6	503.2	3	Gal-(1-x)-Gal-(1-x)-Glc
11	52.0	503.2	3	Gal-(1-x)-Gal-(1-x)-Glc
12	52.7	563.2***	3	Gal-(1-x)-Gal-(1-x)-Glc
13	53.1	503.2	3	Gal-(1-4)-Gal-(1-6)-Glc
14	53.7	503.2	3	Gal-(1-3)-Gal-(1-6)-Glc
15	54.0	503.2	3	Gal-(1-x)-Gal-(1-4)-Glc
16	54.8	665.2	4	Gal-(1-x)-Gal-(1-4)-Gal-(1-4)-Glc
17	55.5	665.2	4	Gal-(1-x)-Gal-(1-4)-Gal-(1-4)-Glc
18	55.6	665.2	4	Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Glc
19	56.6	665.2	4	Gal-(1-x)-Gal-(1-x)-Gal-(1-4)-Glc
20	57.5	665.2	4	Gal-(1-x)-Gal-(1-x)-Gal-(1-4)-Glc
21	58.5	665.2	4	Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Glc
22	58.6	665.2	4	Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Glc
23	59.3	827.3	5	Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Glc
24	60.7	827.3	5	Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Glc
25	61.7	827.3	5	Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Glc
26	62.6	827.3	5	Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Glc
27	64.1	989.3	6	Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Glc
28	65.2*	989.3	6	Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Gal-(1-x)-Glc

* Assignment of the monosaccharide composition assumes a reducing terminal glucose connected to galactose residues consistent with DP. It is not based on experimental data.
** x denotes an unknown bond.
*** A [M+Acetate-H]- adduct ion was used for DP assignment

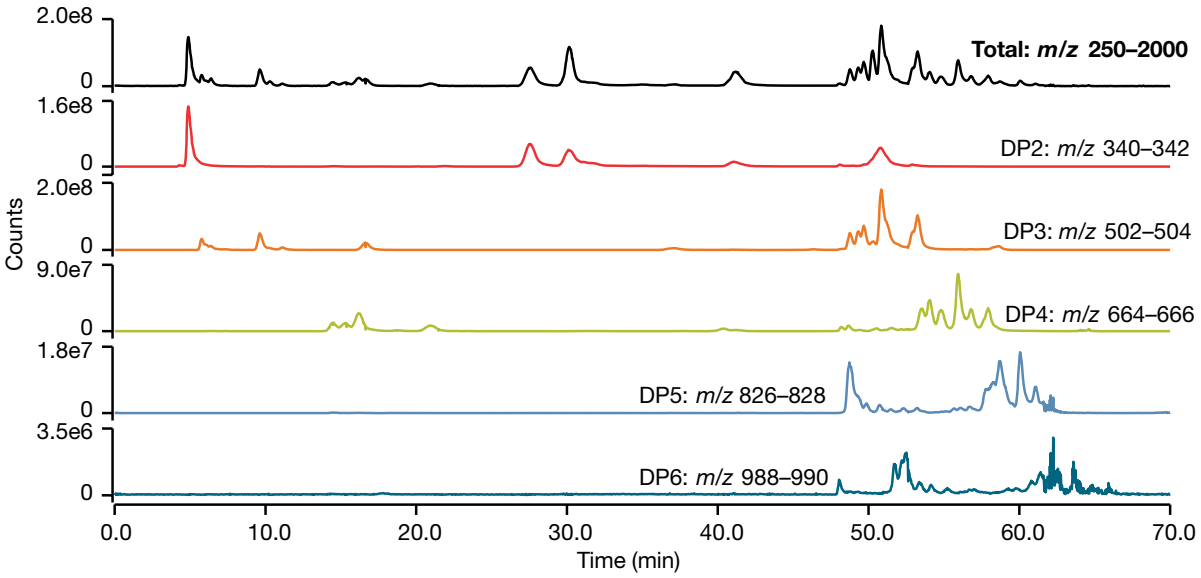

Figure 5. Extracted ion chromatograms showing profiles of GOS and DP2-DP6 peaks in negative mode

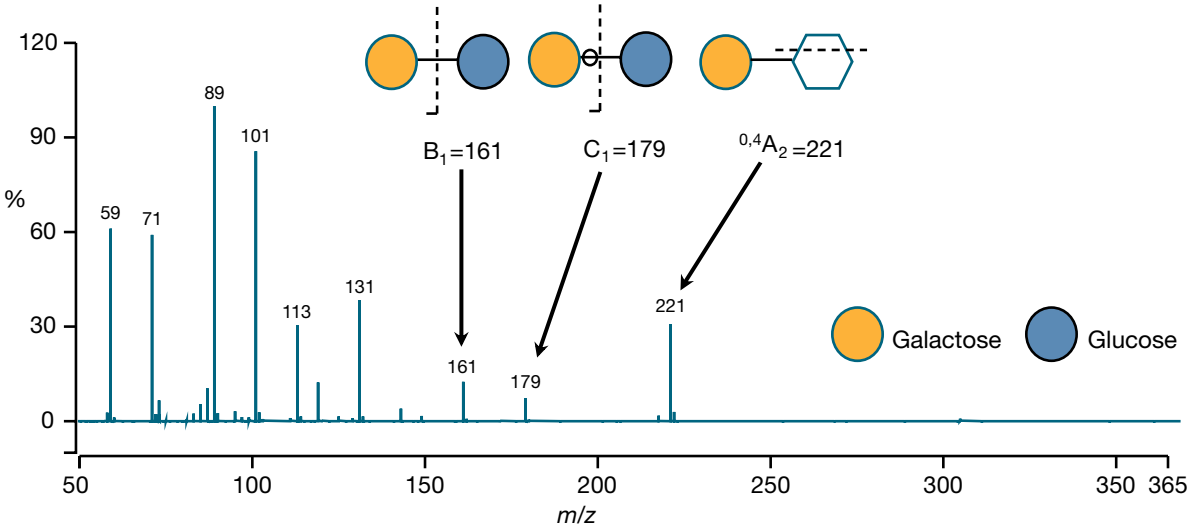

Figure 6. (A) Fragment ions indicating the presence of a DP2 oligosaccharide

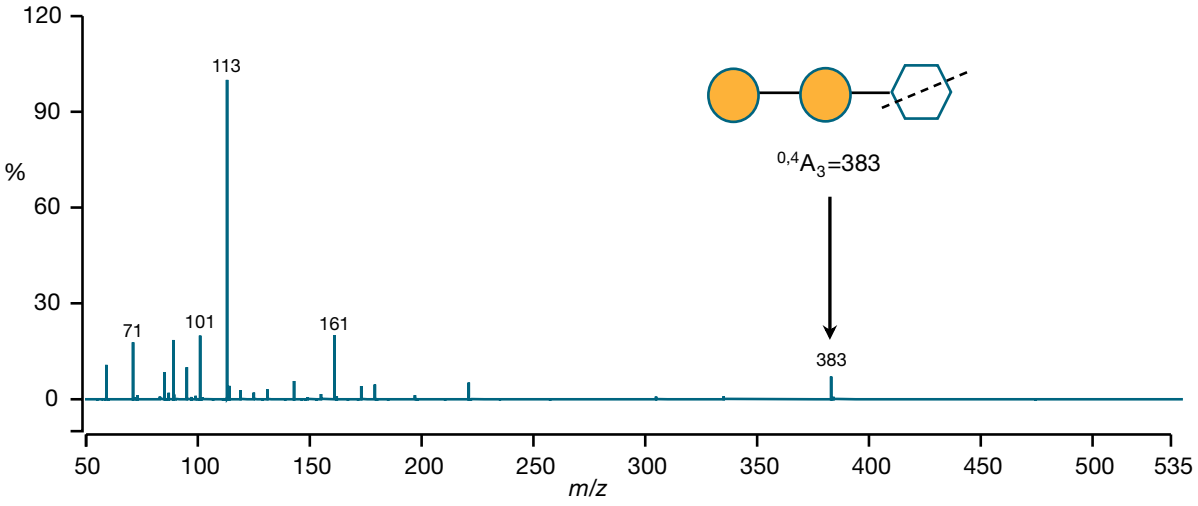

Figure 6. (B) Fragment ions indicating the presence of a DP3 oligosaccharide

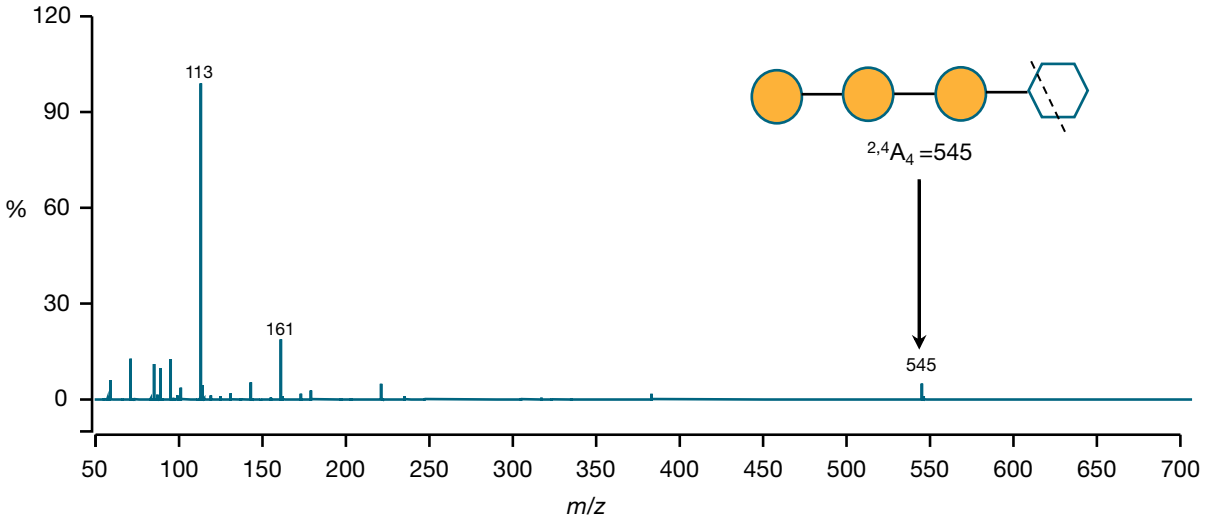

Figure 6. (C) Fragment ions indicating the presence of a DP4 oligosaccharide

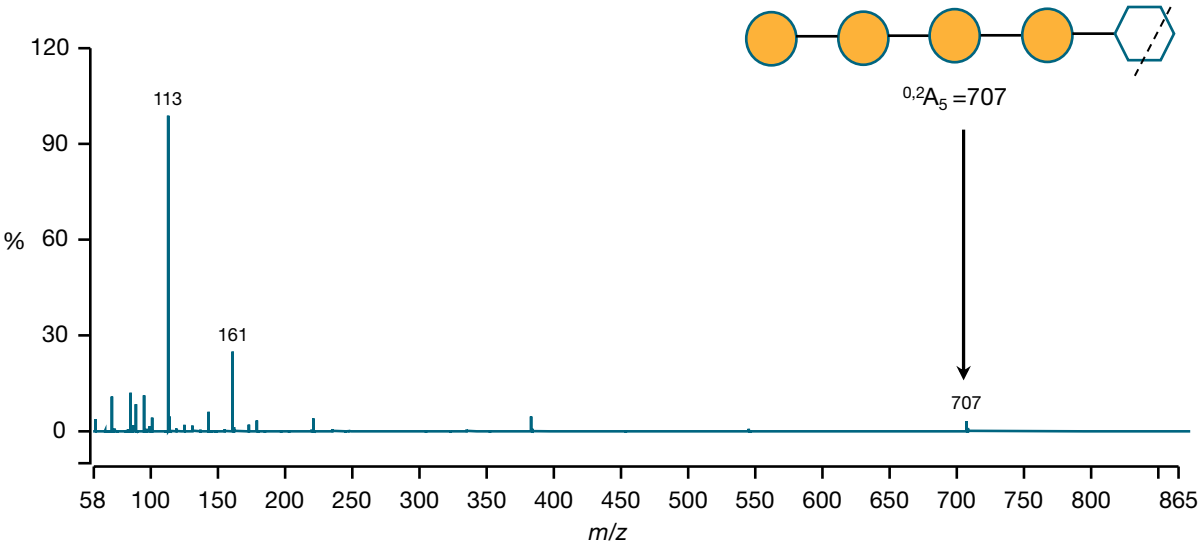

Figure 6. (D) Fragment ions indicating the presence of a DP5 oligosaccharide

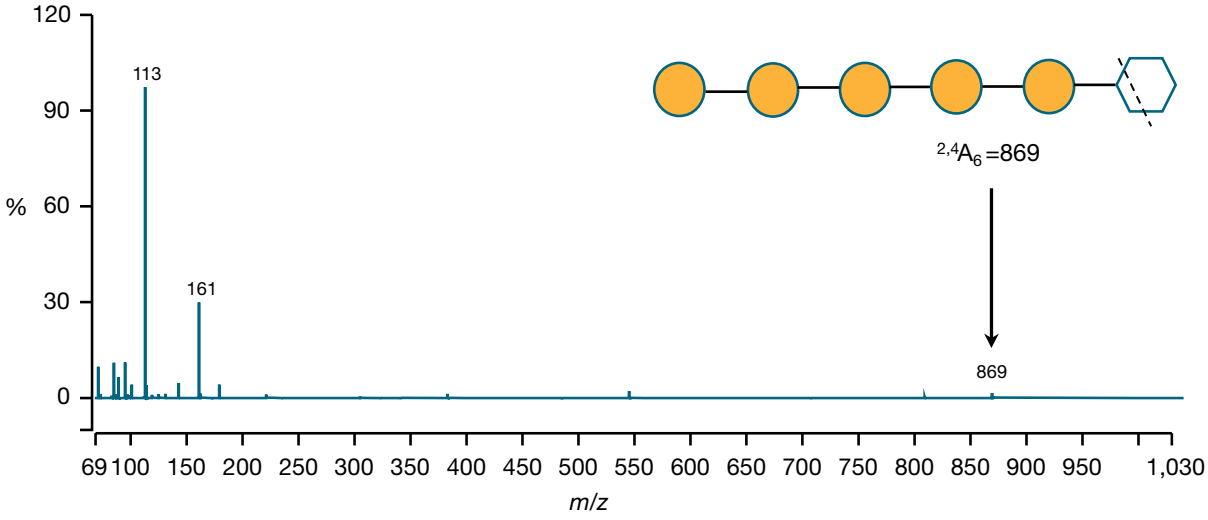

Figure 6. (E) Fragment ions indicating the presence of a DP6 oligosaccharide

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Conclusion

HPAE-PAD/MS analysis of GOS was conducted using a Dionex CarboPac PA300-4µm column. This approach offers a high-resolution separation method for GOS, eliminating the potential complexity of mass spectra and making their interpretation more efficient. Desalting to remove sodium using a Dionex ERD 500 was reproducible and had minimal impact on oligosaccharide peak resolution. Using the methods described here, DPs for 28 oligosaccharides were assigned based on MS^2 data. The synergy between resolving power of HPAE and accuracy of an Orbitrap mass spectrometer produces an excellent tool for GOS characterization.

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