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HPAE-PAD analysis of galactosyl-oligosaccharide-containing samples using dual eluent generation cartridges

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Keywords
Dionex ICS-6000, Dionex CarboPac PA200 column, Dionex EGC 400 MSA, Dionex EGC 400 KOH, complex carbohydrate, galactosyl oligosaccharides, GOS, infant formula

Goal

To demonstrate Dual Eluent Generation Cartridge (EGC) capability and performance for profiling galactosyl oligosaccharides using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)

Introduction

Thermo Scientific™ Dionex™ “Just Add Water” electrolytic eluent generation technology is an essential part of Reagent-Free™ Ion Chromatography (RFIC™) systems. This technology ensures excellent reproducibility and accuracy in eluent preparation.

Thermo Fisher Scientific offers a new operating mode for RFIC systems called Dual EGC mode. This option can replace the manual preparation of the sodium hydroxide/sodium acetate (NaOH/NaOAc) eluent gradients required for analyzing complex carbohydrates.

One of the most widely used methods for profiling oligosaccharides and quantification of known oligosaccharides is high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAE-PAD). HPAE-PAD allows complex carbohydrates analysis by direct detection and without sample derivatization.

The Dual EGC mode of operation is available on our recently introduced Thermo Scientific™ Dionex™ ICS-6000 Capillary HPIC™ system for the analysis of complex carbohydrates using RFIC-EG generated eluents (Figure 1). In Dual EGC mode, RFIC systems employ a methanesulfonic acid (MSA) and a potassium hydroxide (KOH) EGC cartridge, in series, to electrolytically generate potassium hydroxide/potassium methanesulfonate KOH/KMSA) eluents. This operating mode can be used with capillary (0.4 mm) and analytical column formats (1.0 mm).

This study assesses the performance of the Dual EGC mode for separating complex carbohydrates using the Thermo Scientific™ Dionex™ CarboPac™ PA200 1 mm column. We used the Dual EGC mode to profile galactosyl oligosaccharides (GOS) by HPAE-PAD in samples known to contain those oligosaccharides. GOS, also known as galactooligosaccharides, oligogalactosyllactose, oligogalactose, oligolactose, or trans-galactooligosaccharides (TOS), belong to the group of prebiotics. Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth and activity of beneficial bacteria in the colon. GOS occur in commercially available food products for both infants and adults. GOS consist of a chain of galactose units that arise through consecutive transgalactosylation reactions, with a terminal glucose unit (lactose is the starting material). The degree of polymerization (DP) of GOS can vary quite markedly, depending mainly on the type of the enzyme used and the degree of lactose conversion. Application Note 1151[1] described an HPAE-PAD profiling method for GOS in prebiotic dietary supplements using manually prepared sodium hydroxide/sodium acetate (NaOH/NaOAc) eluent gradients with a Thermo Scientific™ Dionex™ CarboPac™ PA200 3 mm column set on a Thermo Scientific Dionex ICS-5000+ HPIC system.

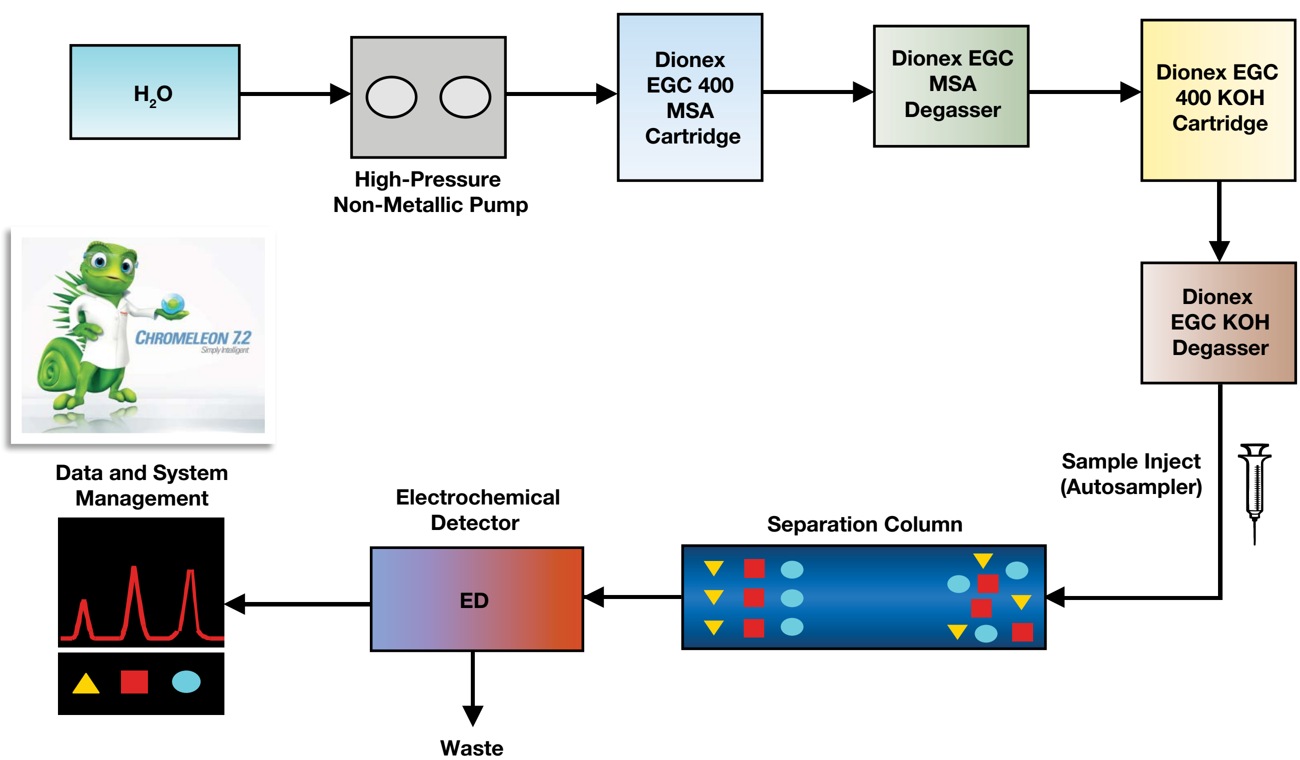

Figure 1. Ion chromatography system Dual EGC mode workflow

In this work, HPAE-PAD profiling for GOS samples was demonstrated using Thermo Scientific™ Dionex™ EGC 400 MSA and Thermo Scientific™ Dionex™ EGC 400 KOH Eluent Generator Cartridges with a Dionex CarboPac PA200 column set in the 1 mm format on a Dionex ICS-6000 HPIC system. Dual EGC mode offers improved reproducibility, and eluent gradient capabilities using an isocratic pump, eliminates manual preparation of eluents, maximizes instrument uptime, and minimizes pump maintenance. Eluent generation of MSA and KOH eliminates the potential risk of system contamination that can result from the use of lower quality sodium acetate.

Experimental

Equipment and consumables

- Dionex ICS-6000 HPIC system including:
  - Dionex ICS-6000 DP Isocratic Pump or a single isocratic pump module with degassing option
  - Dionex ICS-6000 EG Eluent Generator module with Dionex RFIC EGC 400 MSA Degasser (P/N 22181-60210) and Dionex RFIC EGC 400 KOH Degasser (P/N 22181-60201)
  - Dionex ICS-6000 DC Detector/Chromatography module
  - 4-port Valve Rebuild Kit (P/N 074699), which includes a 0.4 µL injection loop
  - Tablet control
- Thermo Scientific™ Dionex™ AS-AP Autosampler with Sample Syringe, 250 µL (P/N 074306) and Buffer line, 1.2 mL (P/N 074989)
- Dionex EGC 400 KOH Eluent Generator Cartridge (P/N 302766)
- Dionex EGC 400 MSA Eluent Generator Cartridge (P/N 302767)
- Thermo Scientific™ Dionex™ ED Electrochemical Detector (P/N 079831)
- Dionex ICS-5000^{+}/4000 ED Electrochemical Cell (P/N 072044)
- pH, Ag/AgCl Reference Electrode (P/N 061879)
- Gasket for pH, Ag/AgCl Reference Electrode (P/N 172162)
- Gold on PTFE Disposable Electrode (P/N 066480)
- Gasket, (PTFE) for Disposable Electrode 0.001 in. (P/N 072161)
- Thermo Scientific™ Dionex™ IC PEEK Viper™ Fittings Kit, Dionex ICS-6000 Capillary system with Electrochemical Detector (ED) (P/N 088802)
- Dionex AS-AP Autosampler Vials 10 mL (P/N 074228)
- Thermo Scientific™ Nalgene™ Syringe Filters, PES, 0.2 µm (Fisher Scientific P/N 09-740-61A)
- AirTite™ All-Plastic Norm-Ject® Syringes, 5 mL, Sterile (Fisher Scientific P/N 14-817-28)
- Thermo Scientific Nalgene 1000 mL, 0.2 µm Nylon Filter Units (P/N 09-740-46)
- Amicon® Ultra-15 Centrifugal Filter Unit with Ultracel®-3 membrane (P/N UFC900396)

Software

- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) version 7.2 SR 7

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- Bimuno® GOS powder (www.Bimuno.com) containing 52% w/w galacto-oligosaccharide

Sample

- An organic infant formula (milk-based European infant formula)

Conditions

IC system:	Dionex ICS-6000 HPIC system (analytical format)
Tubing:	Dionex IC PEEK Viper Fittings Kit, Dionex ICS-6000 Capillary system with Electrochemical Detector (ED) (P/N 088802)
Columns:	Dionex CarboPac PA200 guard column (1 × 50 mm, P/N 302862) Dionex CarboPac PA200 analytical column (1 × 250 mm, P/N 302861)
Eluent source:	Dionex EGC 400 MSA Eluent Generator Cartridge in series with Dionex EGC 400 KOH Eluent Generator Cartridge
Eluent:	Potassium methanesulfonate/ Potassium hydroxide (KMSA/KOH)
Gradient:	20 mM KMSA/70 mM KOH to 70 mM KMSA/70 mM KOH, 0–45 min; 100 mM KMSA/70 mM KOH, 45–55 min; 20 mM KMSA/70 mM KOH, 55–70 min
Flow rate:	0.063 mL/min
Inj. volume:	0.4 µL (full loop)
Temperature:	30 ºC (column and detector compartments)
Backpressure:	~3310 psi
Detection:	Pulsed amperometric, Gold on PTFE Disposable Working Electrode, Ag/AgCl reference, 1 mil gasket
Background:	~30 nC
Noise:	60–80 pC
Run time:	70 min

Carbohydrate 4-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

Preparation of solutions and reagents

Eluent solutions

To generate eluents consisting of potassium methanesulfonate with potassium hydroxide, a Dionex EGC 400 MSA cartridge is paired with a Dionex EGC 400 KOH cartridge. Chromeleon CDS version 7.2 SR 7 provides the feature of Dual EGC Control, which will track the amount of MSA and KOH used and calculate their remaining lifetime. Dual EGC mode guarantees high purity eluents, eliminates potential system contamination related to sodium acetate quality, and improves method precision and productivity.

The Dual EGC mode of operation is available on a supported instrument such as the Dionex ICS-6000 system, facilitating the analysis of complex carbohydrates using RFIC-EG generated eluent. For 1 mm operation, the Dionex EGC 400 cartridge is designed to enable operation at flow rates between 20 µL/min and 200 µL/min. The Dionex EGC 400 MSA cartridge is connected in series to a Dionex EGC 400 KOH cartridge to generate KOH/KMSA up to 200 mM (combined concentration) at flow rates of 20–63 µL/min, and up to 63 mM at flow rates up to 200 µL/min.

Standard solutions

Bimuno GOS standard

Dissolve 0.96 g of Bimuno GOS (52% w/w) in 100 mL DI water to make a 5 mg/mL standard solution. Store the standard solution at 4 °C. Pass the liquid through a Nalgene syringe filter before analysis. GOS standards must be prepared fresh daily to avoid the degradation of GOS and thus inconsistent results.

Sample preparation

Organic infant formula (a milk-based European infant formula)

- Weigh 1 g infant formula powder and dissolve in 50 mL DI water. Shake for 2–3 min.
- Transfer 12 mL to a 50 mL Amicon Ultra-15 centrifugal filter device and cap. Centrifuge for 60 min at 5000 rpm at 20 °C.
- Filter through a 0.2 µm filter and dilute the filtrate 20-fold with DI water before injection.

System preparation and configuration

To achieve the best chromatography, we recommend clean-up of the entire system (without installing the columns) for at least 2 h with 100 mM KOH at 0.1 mL/min.

To ensure a stable baseline and low background noise, it is crucial to have sufficient removal of the hydrogen and oxygen gases formed with the production of the EGC generated eluents. For a 1-mm system, connect the vents of the Dionex RFIC EGC 400 MSA Degasser and the Dionex RFIC EGC 400 KOH Degasser to the Vacuum Port located at the back of the Dionex DP module. Make sure all connections and fittings for the vacuum degas are vacuum tight before using the system. Keep the eluent water blanketed under 34–55 kPa (5–8 psi) of nitrogen at all times to reduce carbonate and microorganism contamination.

Only turn on the EGC power when the system pressure is above 2600 psi. This step is important to ensure best system performance. In a properly working system, the electrochemical detection (ED) background for the Dionex CarboPac PA200 QAR and most applications is typically 25–45 nC.

Follow the flow diagram (Figure 2) to plumb the consumables and modules of the Dionex ICS-6000 system for Dual EGC mode. Note: Plumbing has to be with narrow bore tubing [Dionex IC PEEK Viper Fittings Kit for Dionex ICS-6000 Capillary system with Electrochemical Detector (ED)]. Deionized water is pumped first into the EGC 400 MSA cartridge, and then the MSA is passed into the EGC KOH cartridge to titrate the potassium hydroxide to potassium methanesulfonate. By balancing the concentration of the two cartridges, pure KMSA can be generated. By generating an excess of KOH compared to MSA, a basic solution of KMSA plus KOH can be generated (Basic Eluent mode). By generating an excess of MSA compared to KOH, an acidic solution of KMSA plus MSA can be generated (Acidic Eluent mode). The system can be switched between the two modes as needed. For this application, we are only using the Basic Eluent mode.

Dionex ICS-6000 Dual EGC start-up procedure for CarboPac PA200 column (1 mm)

Install the Dionex CarboPac PA200 Guard (1 × 50 mm) and the Dionex CarboPac PA200 Analytical (1 × 250 mm) columns in the lower compartment of the DC module according to the Dual EGC Installation Guide.[2]

Dionex ICS-6000 Dual EGC system re-start procedure

Note: The systems under Dual EGC mode are designed to be operated non-stop for months at a time. If the system has been shut down for more than 1–2 days, the system should be re-started following the steps as described in Section 1.2 ICS-6000 Dual EGC System Re-start of the Dionex ICS-6000 in the Dual EGC Installation Guide.[2]

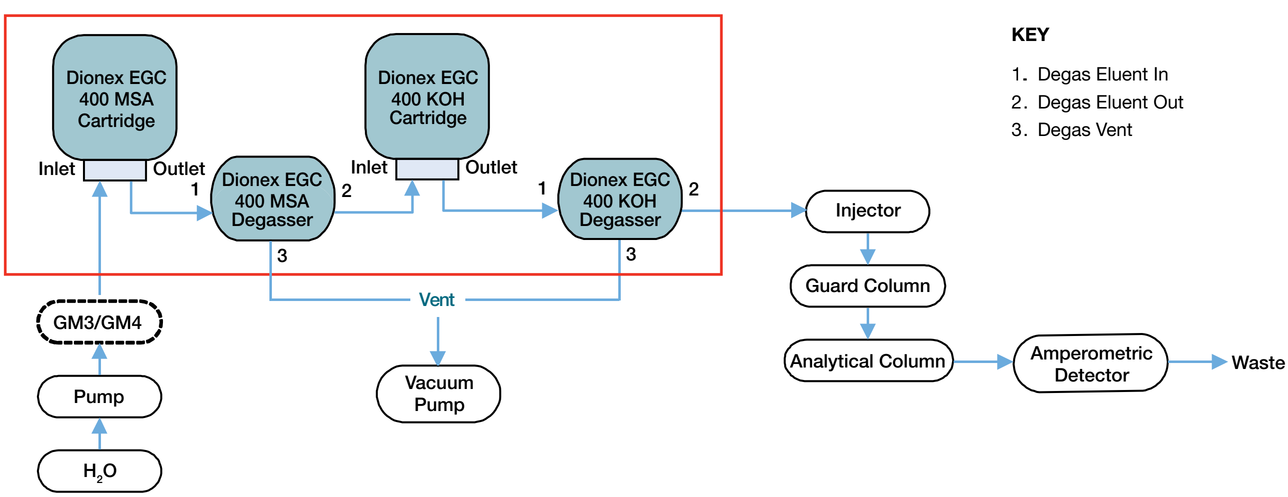

Figure 2. Plumbing schematic for electrolytic generation of potassium methanesulfonate eluents using a Dionex EGC 400 KOH cartridge and a Dionex EGC 400 MSA cartridge in an RFIC-EG system

Chromeleon CDS 7 (Version 7.2.7) for Dual EGC mode

Follow the User Guide of Chromeleon CDS 7 (Version 7.2.7) for Dual EGC mode[2] to set up the configuration and instrument method in Chromeleon CDS 7 while using the Dionex ICS-6000 system Dual EGC mode.

ED cell, reference electrode, and gasket

While running at the low flow rate in this work, bubbles are more likely to be trapped in the ED cell. Air bubbles in the cell can cause pulsations of the baseline, random noise, and low readings. To prevent air from becoming trapped in the cell, increase the backpressure on the cell by connecting backpressure tubing to the cell outlet. The backpressure limit for the ED cell is 690 kPa (100 psi). Do not exceed this limit. In this study, three to four inches of blue (0.0025" i.d.) PEEK tubing at the cell outlet was found to prevent bubble formation and not exceed the backpressure limit.

Keep an additional pH-Ag/AgCl reference electrode at all times. For best results, replace the reference electrode every six months of use, or when the pH of the eluent, monitoring the pH readout of a solution with a known composition has shifted by more than 0.5 pH units.[3] As a result of exposure to alkaline solutions—such as flowing sodium or potassium hydroxide—the 3 M KCl electrolyte inside the reference electrode gradually becomes alkaline, and the silver chloride layer on the Ag wire in the electrode dissolves or converts to a mixture of silver oxide and silver hydroxide. When this happens, the reference potential shifts and becomes increasingly unstable.[4]

Reference potential shifting can lead to unusually high background response from the working electrode, reduced signal response, or a combination of both effects. If the reference electrode fails while the electrochemical detection cell is on, replace the disposable working electrode as well as the reference electrode. A large shift in reference potential can damage the disposable working electrode.

Replace the gasket if there is a leak between the gasket and electrode, or between the gasket and cell body. Note: The standard gasket that comes with the Gold on PTFE electrodes (P/N 066480) is 0.002 in. The 0.001 in. gasket was used in our study.

Results and discussion

Gradient methods using an isocratic pump

Uncharged oligosaccharides are typically separated on a Dionex CarboPac PA200 3 mm column set using a sodium acetate gradient in an eluent containing either 100 mM or 150 mM sodium hydroxide. In Dual EGC mode, eluent generators methanesulfonic acid (MSA) and potassium hydroxide (KOH) cartridges, are installed in series to generate KOH/KMSA eluents required for analyzing complex carbohydrates by HPAE-PAD. This extension of RFIC technology now enables analysts to run gradient methods using an isocratic pump. The user now has the flexibility to modify or change the eluent concentrations anytime through the Chromeleon CDS software.

The hydroxide is present to ionize the saccharide hydroxyl groups to oxyanions so that the saccharide binds to the Dionex CarboPac column, a strong anion exchanger. In NaOH/NaOAc eluent gradients, the acetate ion is a stronger eluent than the hydroxide ion and is used to elute the bound saccharide. In KOH/KMSA eluents, methanesulfonate (MSA^-) ion replaces the acetate ion for eluting the bound saccharide. The eluting strength of MSA^- is approximately 3-fold stronger than acetate ion.

Note: Oligosaccharide analysis applications typically require an excess of potassium hydroxide for operation; this is achieved by operating in Basic Eluent mode. Column cleaning processes may require an excess of methanesulfonic acid; this is achieved by operating in Acidic Eluent mode.

When operated in Basic Eluent mode pure KMSA can be generated, or an excess of KOH to create a basic eluent. In this mode the concentrations of generated eluent species are governed by the formulas:

$$MSA_{act} = KMSA_{prog}$$
$$KOH_{act} = KOH_{prog} + KMSA_{prog}$$

When operated in Acid Eluent mode, pure KMSA can be generated, or an excess of MSA to create an acidic eluent. In this mode the concentrations of generated eluent species are governed by the formulas:

$$KOH_{act} = KMSA_{prog}$$
$$MSA_{act} = MSA_{prog} + KMSA_{prog}$$

Where:

- $KMSA_{prog}$ is the concentration of potassium methanesulfonate that has been programmed by the operator
- KOH_{prog} is the concentration of potassium hydroxide that has been programmed by the operator; this is zero in Acidic Eluent mode
- MSA_{prog} is the concentration of methanesulfonic acid that has been programmed by the operator; this is zero in Basic Eluent mode
- MSA_{act} is the concentration of methanesulfonic acid generated by the EGC MSA cartridge
- KOH_{act} is the concentration of potassium hydroxide generated by the EGC KOH cartridge.

The Chromeleon CDS or IC system front panel will automatically calculate the MSA_{act} and KOH_{act} values based on input $KMSA_{prog}$, MSA_{prog}, and KOH_{prog} values. By setting the $KMSA_{prog}$ value to zero, it is possible to control each cartridge individually and generate pure KOH or MSA.

Separation on the Dionex CarboPac PA200 1 mm column

Oligosaccharides were separated on a DionexCarboPac PA200 1 mm column set using a Dionex EGC 400 MSA cartridge connected in series to a Dionex EGC 400 KOH cartridge. A solution of Bimuno GOS was prepared, and an aliquot (0.4 µL) of the solution was injected onto the column and eluted at 0.063 mL/min with a linear gradient of KMSA (20–70 mM in 45 min) in KOH (70 mM KOH). Figure 3 shows the profile of Bimuno GOS sample using Dual EGC mode eluent preparation compared to chromatography from AN1151. We can observe DP1-13 as reported in AN1151.[1] Moreover, the individual peaks were sharp and well resolved, similar to published separations.[1] The assignment of the chromatographic peaks was based on AN1151 and the accepted assumption that the retention time of a homologous series of carbohydrates increases as DP increases, and thus each successive peak represents a GOS that has one galactose more than the previous peak.

Columns: Dionex CarboPac PA200, 3 mm (guard + separator)
DionexCarboPac PA200, 1 mm (guard + separator)

Gradient: Dionex CarboPac PA200, 3 × 250 mm
0-5 min: 50 mM NaOAc in 100 mM NaOH
5-45 min: 50-150 mM NaOAc in 100 mM NaOH
45-55 min 500 mM NaOAc in 100 mM NaOH
55-60 min: 50 mM NaOAc in 100 mM NaOH

Dionex CarboPac PA200, 1 × 250 mm
0-45 min: 20-70 mM KMSA in 70 mM KOH
45-55 min: 100 mM KMSA in 70 mM KOH
55-70 min: 20 mM KMSA in 70 mM KOH

Flow Rate: Dionex CarboPac PA200 3 mm: 0.5 mL/min
Dionex CarboPac PA200 1 mm: 0.063 mL/min

Detection: Dionex CarboPac PA200, 3 × 250 mm:
PAD, Au on PTFE, 2 mil gasket, Ag/AgCl ref.
Dionex CarboPac PA200, 1 × 250 mm:
PAD, Au on PTFE, 1 mil gasket, Ag/AgCl ref.

Inj. Volume: Dionex CarboPac PA200, 3 × 250 mm: 10 µL
Dionex CarboPac PA200, 1 × 250 mm: 0.4 µL

Samples: Dionex CarboPac PA200, 3 × 250 mm:
50 mg/L GOS (Bimuno GOS powder)
Dionex CarboPac PA200, 1 × 250 mm:
5 mg/mL GOS (Bimuno GOS powder)

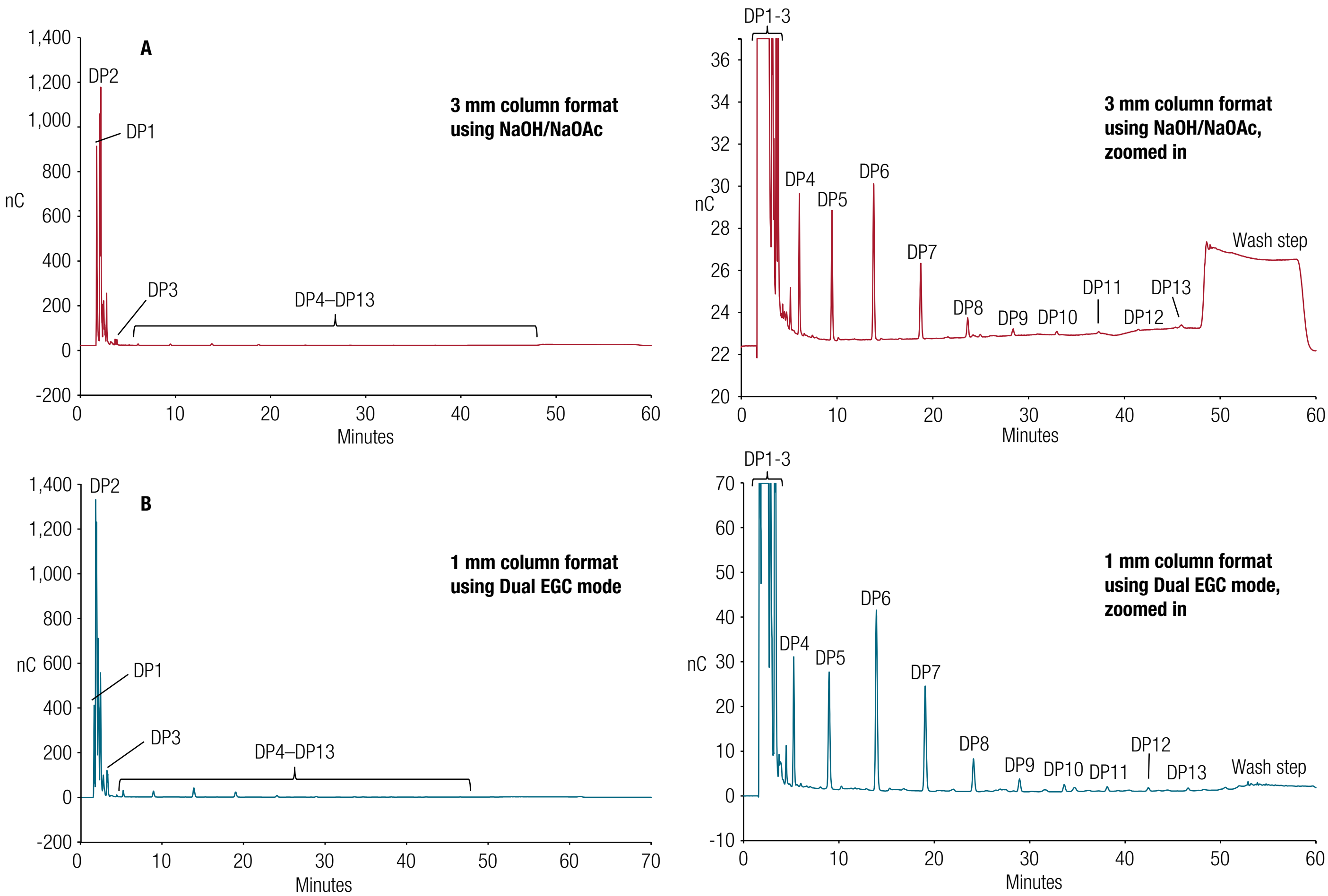

Figure 3. Bimuno GOS analysis using (A) manually prepared eluents (NaOH/NaOAc) (B) Dual EGC mode (KOH/KMSA)

Column wash and equilibration between injections

For this method, perform a wash step after every injection to maintain column performance. The wash step consists of 10 min of 100 mM KMSA in 70 mM KOH. This wash will ensure stable retention times and assist in maintaining a clean electrode. For good retention time reproducibility, the column must be equilibrated to the starting gradient conditions prior to each injection, and the re-equilibration period should be tightly controlled. In all separations shown in this application note, the column set was re-equilibrated at initial conditions for 15 min before the next injection.

Sample profiling

A solution of infant formula was prepared, and an aliquot (0.4 µL) was injected on the same HPAE-PAD system using the same method as that for the Bimuno GOS. Figure 4 shows the profile of GOS in infant formula on a Dionex CarboPac PA200 1 mm column using Dual EGC mode. The total separation time is 45 min with an additional 10 min wash and 15 min equilibration before the next injection. The separation of GOS in the infant formula sample demonstrated good peak resolution.

Column:	Dionex CarboPac PA200, Analytical, 1 × 250 mm Dionex CarboPac PA200, Guard, 1 × 50 mm
Eluent Source:	Dionex EGC 400 MSA Eluent Generator in series with Dionex EGC 400 KOH Eluent Generator
Eluent:	0–45 min: 20–70 mM KMSA in 70 mM KOH 45–55 min: 100 mM KMSA in 70 mM KOH 55–70 min: 20 mM KMSA in 70 mM KOH
Temperature:	30 ˚C
Flow Rate:	0.063 mL/min
Inj. Volume:	0.4 µL
Detection:	PAD, Au on PTFE (Disposable), 1 mil gasket, Ag/AgCl ref. electrode

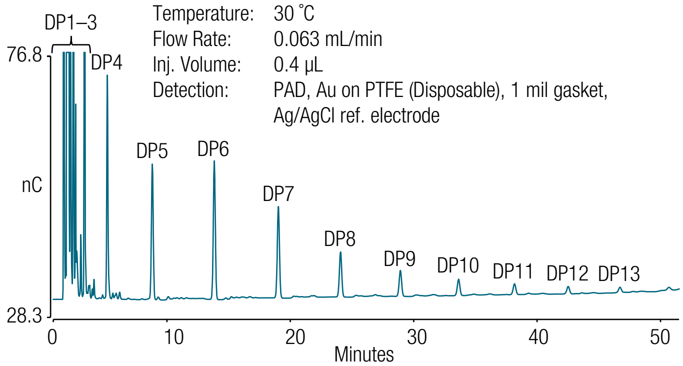

Figure 4. The profile of GOS in infant formula using Dual EGC mode

Conclusion

Our study demonstrated excellent performance of Dual EG cartridge technology when applied to the HPAE-PAD analysis of galactosyloligosaccharide-containing samples using a Dionex CarboPac PA200 1 mm column. The method achieved great separation using an eluent gradient generated using Dual EGC mode on a Thermo Scientific Dionex ICS-6000 system.

Dual EGC mode enables the analyst to run gradient methods using an isocratic pump, offering improved reproducibility, eliminating manual preparation of eluents, and minimizing pump maintenance because the pump only needs to pump water, thus maximizing instrument uptime. Dual EGC mode shows excellent performance for HPAE-PAD of complex carbohydrates.

Refrences

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