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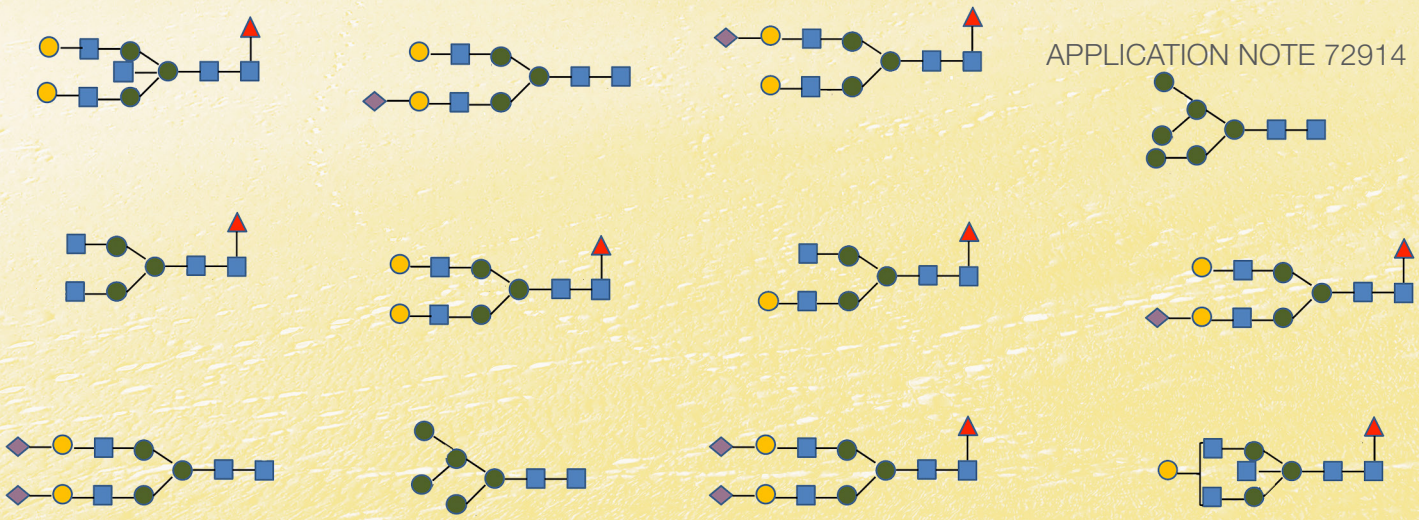

APPLICATION NOTE 72914

HPAE-PAD profiling of *N*-linked oligosaccharides from glycoproteins using dual eluent generation cartridges

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Keywords

Dionex ICS-6000, Dionex CarboPac PA200 column (1 mm i.d.), Dionex EGC 400 MSA, Dionex EGC 400 KOH, *N*-linked glycans, glycoprotein, immunoglobulin G (IgG), α_1-acid glycoprotein (AGP)

Goal

Demonstrate Dual EGC capability and performance for profiling *N*-linked oligosaccharides released from glycoproteins using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)

Introduction

Glycoprotein characterization and glycosylation profiling are important tasks in the development and production of biopharmaceutical proteins. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is an effective tool for separating the glycans present on glycoprotein therapeutics.

A new operating mode for HPAE-PAD systems, Dual Eluent Generation Cartridge (Dual EGC) mode, is available to support the analysis of complex carbohydrates. This mode of operation replaces the manual preparation of the sodium hydroxide/sodium acetate eluents required for analyzing complex carbohydrates by HPAE-PAD with electrolytic generation of potassium hydroxide (KOH)/potassium methanesulfonate acid (KMSA) eluent using MSA and KOH eluent generators installed in series. This enables analysts to run

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eluent gradient methods for oligosaccharide separations using an isocratic pump. Dual EGC guarantees generation of high purity eluents, eliminates potential system contamination related to sodium acetate quality, and improves method precision and productivity.

The Dual EGC mode of operation for the analysis of complex carbohydrates is supported only by the Thermo Scientific™ Dionex™ ICS-6000 chromatography system 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 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 Dual EGC when applied to the analysis of *N*-linked oligosaccharides released from glycoproteins using a Thermo Scientific™ Dionex™ CarboPac™ PA200 1 mm column for oligosaccharide separations. Oligosaccharides are identified based on comparison to known standards in combination with exoglycosidase digestions. Rohrer et al., reported an HPAE-PAD method to achieve superior resolution for IgG oligosaccharide separation using manually prepared sodium hydroxide/sodium acetate (NaOH/NaOAc) eluent gradients with a Thermo Scientific™ Dionex™ CarboPac™ PA200 3 mm column set on both a Thermo Scientific™ Dionex™ ICS-5000 system with an electrochemical detector and a Thermo Scientific™ Dionex™ ICS-3000 system with an electrochemical detector (second analyst and second system).[1]

In this work, HPAE-PAD profiling for *N*-linked oligosaccharides released from human serum IgG was demonstrated using Thermo Scientific™ Dionex™ EGC 400 MSA and Thermo Scientific™ Dionex™ EGC 400 KOH Cartridges with a Dionex CarboPac PA200 column set in the 1 mm format on a Dionex an ICS-6000 HPIC system. Dual EGC uses a single isocratic pump to generate KOH/KMSA gradients of various concentrations and slopes, which offers comparable separation of oligosaccharides to that using manually prepared NaOAc/NaOH eluent and improved reproducibility. We also evaluated the separations of *N*-linked glycans (oligosaccharides) released from other glycoproteins as well as exoglycosidase treatments of those glycans. Overall Dual EGC offers a reproducible assay of *N*-linked glycans released from glycoproteins that requires no eluent preparation.

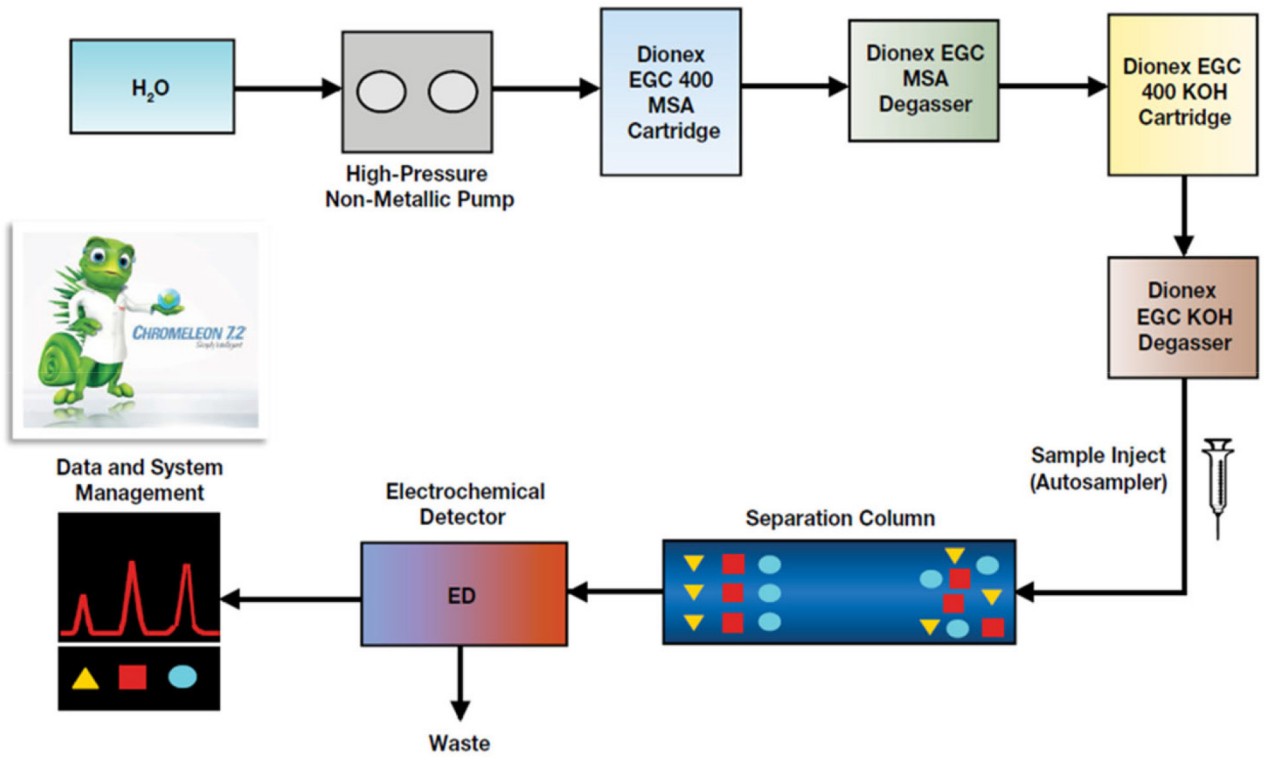

Figure 1. Ion chromatography system Dual EGC mode workflow

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 Tray Temperature Control (P/N 074926), 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)
- Thermo Scientific™ 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 072162)
- Gold on PTFE Disposable Electrode Including four 2 mil gaskets (P/N 066480) Note: The 2 mil gaskets are not used in this application.
- Gasket, (PTFE) for Disposable Electrode 0.001 in. (P/N 072161)
- 1 mm Dual EGC Viper Kit, Dionex ICS-6000 system with Electrochemical Detector (ED) (P/N 302793)
- Dionex AS-AP Autosampler Vial Kits 10 mL (P/N 074228), 1.5 mL (P/N 079812), and 0.3 mL (P/N 055428)
- 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)
- Thermo Scientific™ HyperSep™ Hypercarb™ SPE Cartridges (100 mg) (P/N 60106-302)

Software

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

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- *N*-Acetylneuraminic acid, >98%, MP Biomedicals™ (Fisher Scientific P/N ICN15385125)
- G0 (NGA2) (ProZyme P/N GKC-004300)
- G0F (GA2F) (ProZyme P/N GKC-004301)
- G1F (NA2G1F) (ProZyme P/N GKC-014301)
- G2SI (A1) (ProZyme P/N GKC-124300)
- G2FS1 (A1F) (ProZyme P/N GKC-124301)
- α2-3,6,8 Neuraminidase (New England Biolabs, P/N P0720S)
- Rapid™ PNGase F (New England Biolabs, P/N P0710S)
- α1-2,4,6 Fucosidase O (New England Biolabs, P/N P0749S)

Samples

- IgG from human serum, reagent grade, ≥95% (SDS-PAGE), essentially salt-free, lyophilized powder (Sigma, P/N I4506)
- Human IgG (Rockland Immunochemicals Inc, P/N 009-0102)
- α1-Acid Glycoprotein, human plasma (EMD Millipore, P/N 112251)
- α1-Acid Glycoprotein from human plasma (Sigma, P/N G9885)

Conditions

IC system:	Dionex ICS-6000 HPIC system (analytical format)
Tubing:	1 mm Dual EGC Viper Kit, Dionex ICS-6000 system with Electrochemical Detector (ED) (P/N 302793)
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 for the analysis of *N*-linked glycans from IgG

-5 min:	0.3 mM KMSA in 65 mM KOH
0–25 min:	0.3–1 mM KMSA in 65 mM KOH
25–44 min:	1–28 mM KMSA in 90 mM KOH
44–46 min:	28–42 mM KMSA in 90 mM KOH
46–55 min:	42 mM KMSA in 90 mM KOH
55–60 min:	100 mM KMSA in 100 mM KOH
60–75 min:	0.3 mM KMSA in 65 mM KOH

Gradient for the analysis of *N*-linked glycans from AGP

0–70 min:	5–80 mM KMSA in 70 mM KOH
70–75 min:	80 mM KMSA in 70 mM KOH
75–90 min:	5 mM KMSA in 70 mM KOH

Flow rate:	0.063 mL/min
Injection volume:	0.4 µL (full loop)
Temperature:	For IgG glycans, 30 °C (column and detector compartments) For AGP glycans, 30 °C (detector) 25 °C (column)
Backpressure:	~3300 psi
Detection:	Pulsed amperometric, Gold on PTFE Disposable Working Electrode, Ag/AgCl reference, 1 mil gasket
Background:	~30 nC
Noise:	60–80 pC

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	-0.2	Off	On	Off
0.42	-0.2	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

To generate eluents consisting of potassium methanesulfonate with potassium hydroxide, DI water is supplied to a Dionex EGC 400 MSA connected in series with a Dionex EGC 400 KOH. The Chromeleon CDS software version 7.2 SR 7 or higher provides the feature of Dual EGC Control, which will track the amounts of MSA and KOH used and calculate remaining cartridge lifetime for each.

Sample preparation

N-linked oligosaccharide release from glycoproteins

One-step rapid PNGase F digestion: Add 20 µL of 1:10 dilution of Rapid PNGase F enzyme to 200 µL protein (5 mg/mL for IgG, and 8 mg/mL for AGP) without adding buffer. Incubate the mixture at 50 °C for 2 h. After the reaction, cool the solution to ambient temperature. The released oligosaccharides are analyzed without further treatment.

Two-step protocol of deglycosylation (Rapid PNGase F digestion with denaturing): Some antibodies require a preheating step for efficient deglycosylation. Add 40 µL of Rapid PNGase F Buffer (5X) to 160 µL IgG protein (10 mg/mL) to make a 200 µL total reaction volume. Incubate at 80 °C for 2 min and then allow to cool to room temperature. Add 10 µL of Rapid PNGase F. Incubate 2 h at 50 °C. After the reaction, cool the solution to ambient temperature. The released oligosaccharides are analyzed after purification.

Glycan purification

After deglycosylation, the released glycans are purified from the salts and detergents used in the deglycosylation, and isolated from the IgG protein, by the use of a HyperSep Hypercarb SPE Cartridge (50 or 100 mg). The SPE cartridge is preconditioned with 1 mL of acetonitrile (MeCN), followed by 0.5 mL of 40% MeCN/0.1% TFA, 0.5 mL of 5% MeCN/0.1% TFA, and 0.5 mL of 0.1% TFA. After the sample is slowly loaded onto the SPE cartridge, the cartridge is washed two times with 0.5 mL of 0.1% TFA, followed by 0.5 mL of 5% MeCN/0.1% TFA. Released glycans are eluted off the cartridge using 1 mL of 40% MeCN/0.1% TFA and collected into a 2 mL microcentrifuge tube. The eluted glycans are dried in a Thermo Scientific™ Savant™ SpeedVac™ concentrator and reconstituted in 100 µL of DI water before injection.

Neuraminidase digestion

Ten microliters of α2-3,6,8 neuraminidase and 90 µL of the PNGase F released oligosaccharides without added buffer are incubated at 37 °C for 1 h. The resulting neuraminidase digestion sample is injected without further purification.

α1-2,4,6 Fucosidase O digestion

α1-2,4,6 Fucosidase O cleaves α1-6 fucose residues more efficiently than other linkages. Ten microliters of α1-2,4,6 Fucosidase O enzyme preparation and 100 µL of the PNGase F released oligosaccharides from IgG without added buffer are incubated at 37 °C for 8, 24, 48, and 60 h. The resulting fucosidase digestion samples are injected without further purification.

System preparation and configuration

To achieve the best chromatography, we recommend clean-up of the entire system (before 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 the possibility of 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 shown in Figure 2 to plumb the consumables and modules of the Dionex ICS-6000 Dual EGC system. Deionized water is first pumped into the Dionex EGC 400 MSA cartridge, and then the MSA is passed into the Dionex 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 described in Section 1.2 ICS-6000 Dual EGC System Re-start of the Dionex ICS-6000 Dual EGC Installation Guide.[2]

User Guide of Chromeleon 7 (Version 7.2.7 or higher) for Dual EGC Mode

Follow "User Guide of Chromeleon 7 (Version 7.2.7 or higher) for Dual EGC Mode"[3] to set up the configuration and instrument method in Chromeleon 7 CDS using a 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 being trapped in the cell, increase the backpressure on the cell by connecting backpressure tubing to the cell outlet. Do not exceed the backpressure limit for the ED cell of 690 kPa (100 psi). 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. 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]

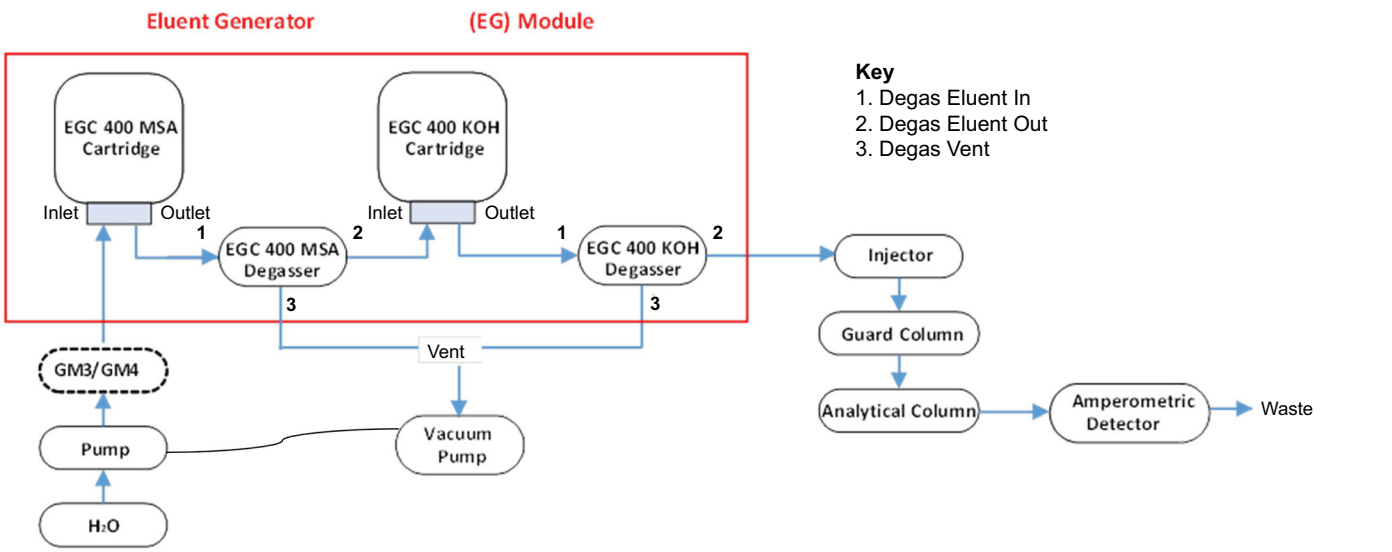

Figure 2. Plumbing schematic for electrolytic generation of potassium methanesulfonate eluents using a Dionex EGC 400 KOH cartridge and Dionex EGC 400 MSA cartridge in an RFIC-EG system. Note: Vacuum pump is integrated with pump

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.

For the Dual EGC application, we use a 1 mil gasket rather than the 2 mil gasket provided with the disposable electrode. Reducing the gasket thickness increases the linear flow rate at the electrode, further enhancing the sensitivity. Replace the gasket if there is a leak between the gasket and electrode, or between the gasket and cell body.

Results and discussion

IgG oligosaccharide separation on the Dionex CarboPac PA200 1 mm column

Using the dual electrolytic eluent generation platform, the most abundant uncharged (neutral) *N*-linked oligosaccharides and the major sialic acid-containing (charged) oligosaccharides released from human serum IgG were separated on a Dionex CarboPac PA200 1 mm column (Figure 3). A solution of *N*-linked oligosaccharides 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 shallow KMSA gradient from 0.3 to 1 mM in a constant 65 mM KOH in the first 25 min. From 25 to 44 min, a simultaneous linear KMSA gradient from 1 to 28 mM and linear KOH gradient from 65 to 90 mM is introduced to separate the charged glycans. The pH change caused by the KOH gradient results in a very slight increase of baseline. With the KOH concentration held at 90 mM, from 44 to 46 min, the KMSA concentration is raised to 42 mM and maintained at 42 mM between 46 and 55 min to elute the sialylated glycans. From 55 to 60 min, 100 mM KMSA/100 mM KOH is used to wash the column before the eluent concentration is returned to the initial condition for 15 min prior to the next injection. The Dionex CarboPac PA200 column is a strong anion exchanger; therefore, the presence of high salt concentrations and anionic detergents must be avoided. Human serum IgG was treated with Rapid PNGase F with and without denaturation. No buffers were added in the Rapid PNGase F digestions without denaturation. The glycan profile without denaturation shown in Figure 3 matches the separation in previously published work.[1] This method delivers good resolution of uncharged IgG oligosaccharides (glycans) comparable with traditional HPAE-PAD separations using sodium hydroxide/sodium acetate eluents. The neutral glycans are well resolved, including separation of high-mannose species from typical human IgG glycans. The more common human IgG *N*-linked glycan structures are shown in Figure 4. *N*-acetylneuraminic acid (Neu5Ac) elutes between G0 and G1F(1,6). Oligosaccharide identification was performed by comparison to known standards, exoglycosidase digestion of both standards and released glycans, and comparison with previous work.[1]

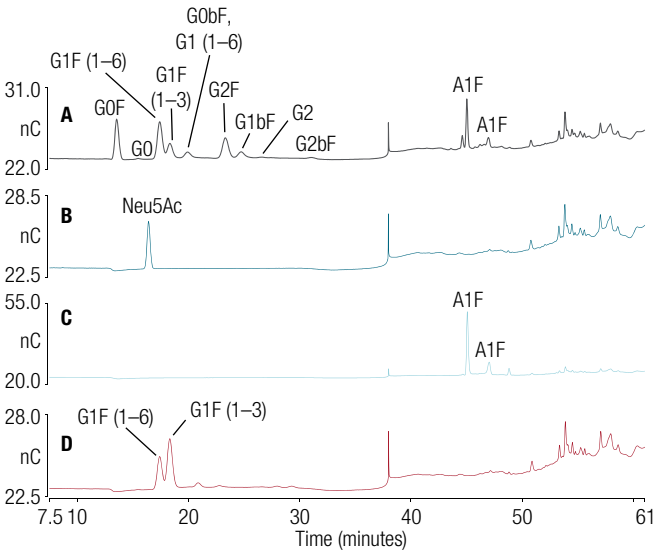

Figure 3. Separation of human serum IgG *N*-linked oligosaccharides using Dual EGC. Chromatographic conditions are shown in the *Conditions* section. Chromatograms: (A) PNGase F digest of human serum IgG; (B) *N*-acetylneuraminic acid (Neu5Ac) standard (9.68 µm); (C) A1F standard; (D) G1F standard

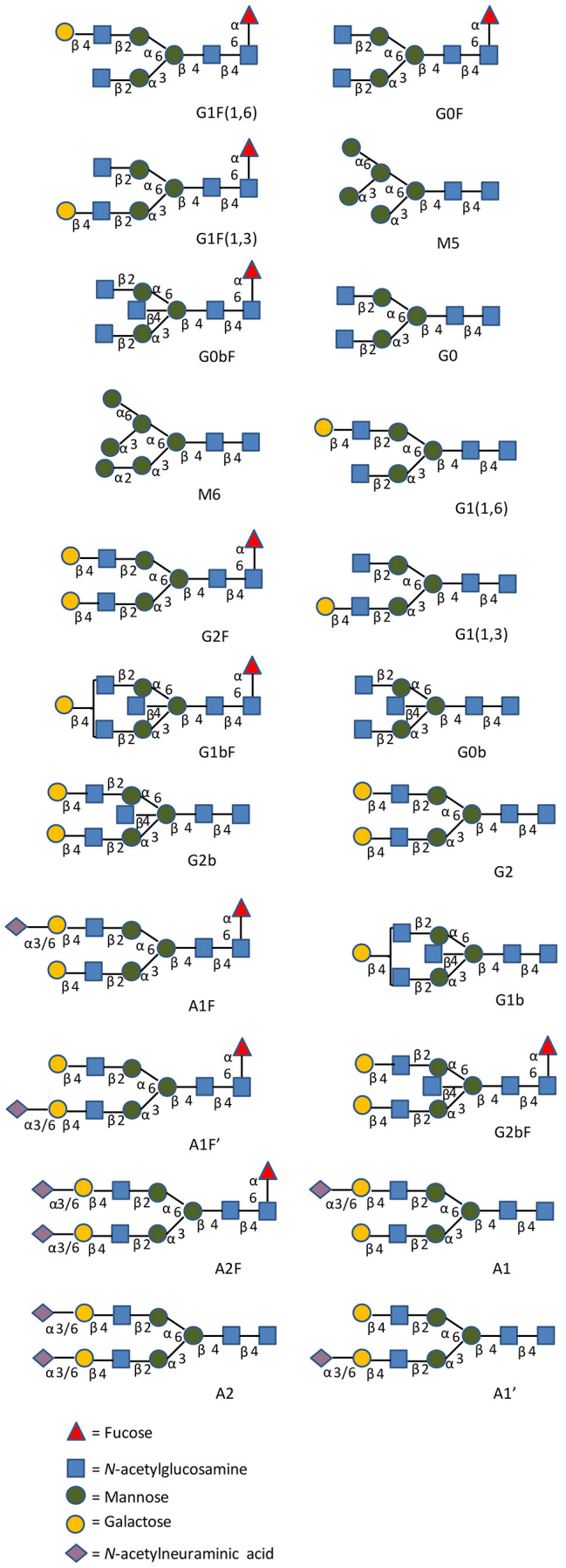

Figure 4. IgG oligosaccharide structures

Neuraminidase digestions of the *N*-linked oligosaccharides released from human serum IgG

As shown in Figure 3, neutral and charged glycans were present in the human serum IgG sample. Peak identities could be confirmed either by the appearance of new peak or disappearance of a peak after enzyme treatment. Neu5Ac presence can be confirmed by neuraminidase treatment showing disappearance of the putative Neu5Ac-containing oligosaccharides and the appearance of uncharged oligosaccharides as well as a Neu5Ac peak. α2-3,6,8 Neuraminidase catalyzes the hydrolysis of α2-3, α2-6, and α2-8 linked sialic acid residues from glycoproteins and oligosaccharides. After rapid PNGase F digest of human serum IgG, the released glycans were treated with α2-3,6,8 neuraminidase, Neu5Ac was released, and all the major peaks in the charged glycan region of the chromatogram were removed with a concomitant increase in neutral glycans (Figure 5). A water blank chromatogram shows the artifact peaks associated with the gradient. This suggests that all of the charged glycans are sialylated.

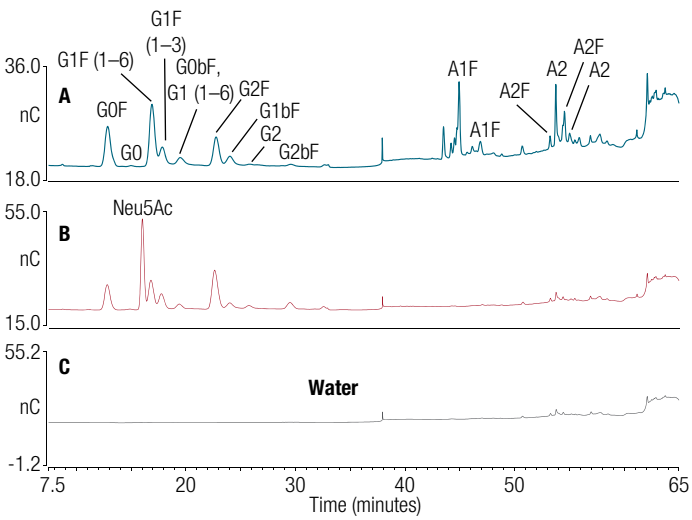

Figure 5. Separation of human serum IgG *N*-linked oligosaccharides with neuraminidase treatment using Dual EGC. Chromatographic conditions are shown in the Conditions section. Chromatograms: (A) Rapid PNGase F digest of human serum IgG; (B) IgG oligosaccharides with neuraminidase treatment; (C) DI water

Fucosidase digestions of the *N*-linked oligosaccharides released from human serum IgG

Further analysis of the *N*-linked oligosaccharides released from a glycoprotein was achieved by sequential digestion with specific exoglycosidases. HPAE-PAD analysis is highly selective for linkage positions and component monosaccharides, which is particularly useful in the resolution of mixtures of complex structures. Additionally, retention times are dependent upon the presence or absence of peripheral monosaccharides: the presence of fucosylation causes a chain to elute earlier than its non-fucosylated counterpart while the addition of a bisecting GlcNAc increases its retention.[5] The use of α1-2,4,6 Fucosidase O cleaves core α1-6 fucosylation more efficiently than other linkages.

Figure 6 shows the time course of defucosylation of *N*-linked oligosaccharides released from human serum IgG using α1-2,4,6 Fucosidase O. The time course of increase of non-fucosylated oligosaccharides and disappearance of fucosylated forms can be directly measured by HPAE-PAD using Dual EGC mode. With the release of fucose, non-fucosylated oligosaccharides started to appear, and their fucosylated forms gradually disappear. We observed complete release after 60 h of incubation. Removal of fucose causes the oligosaccharide to be more strongly retained on the Dionex CarboPac column.[6] Peak identities could be confirmed either by the appearance of a new peak after enzyme treatment (e.g. G0b) or disappearance of a peak after enzyme treatment (e.g. the disappearance of all fucose-containing peaks).

AGP oligosaccharide separation on the Dionex CarboPac PA200 1 mm column

AGP is an important example of a naturally occurring plasma glycoprotein. Using 5–80 mM KMSA gradient in a constant 70 mM KOH eluent, followed by 5 min of isocratic column wash with 80 mM KMSA in 70 mM KOH, and 15 min at initial conditions prior to the next injection, the human AGP glycans were eluted at 25 °C column temperature based on their increasing negative charge, in the order of increasing retention time. Within each charge group, structures are further separated on the basis of size and sequence of monosaccharides that form the chain and the linkages between them. The structures with the same monosaccharides units but differing in one linkage are

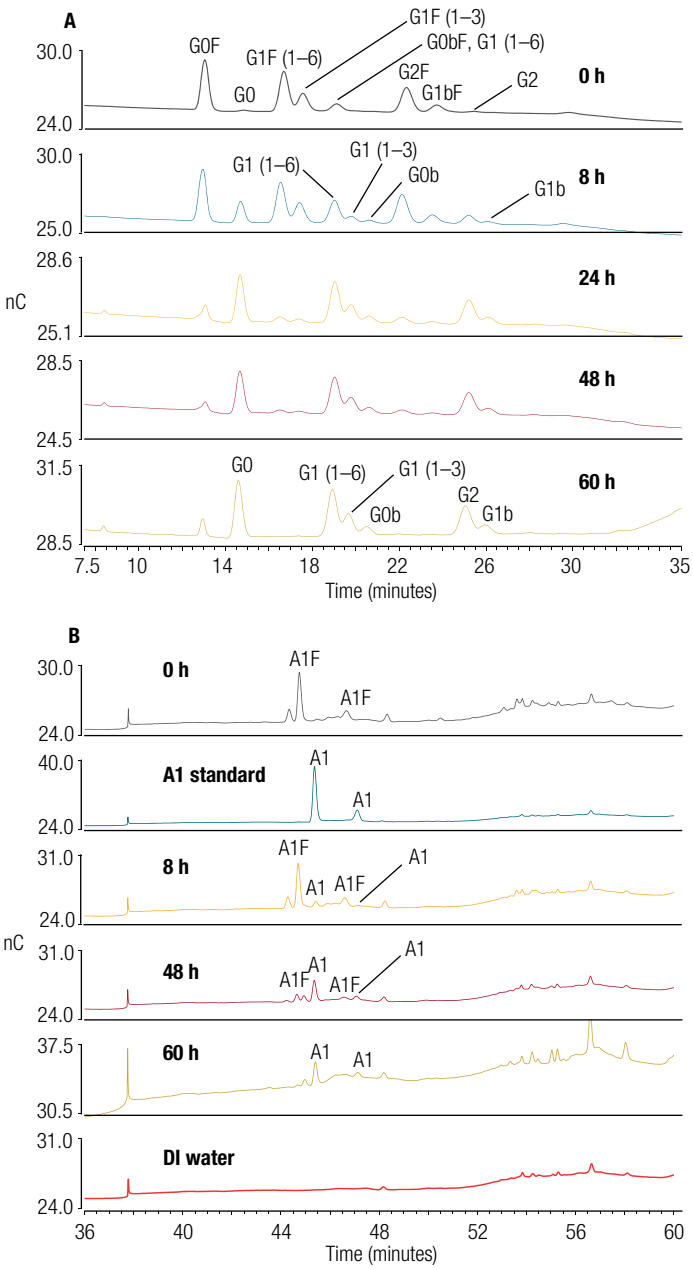

Figure 6. The time course of the released IgG oligosaccharides with α1-2,4,6 Fucosidase O measured by HPAE-PAD using Dual EGC. Chromatograms: (A) Neutral *N*-linked oligosaccharides; (B) Charged *N*-linked oligosaccharides compared with A1 standard (10 µg/mL) and DI water

separated with good resolution, similar to that obtained with NaOH/NaOAc eluent.[7] Figure 7 shows the separation of *N*-linked oligosaccharides released from human plasma AGP using Dual EGC. Various forms of di-, tri-, and tetra-antennary carbohydrate chains contribute to the human AGP glycan complexity. The variation in the glycoforms resulted in significant heterogeneity of the oligosaccharides. The results revealed that the samples from various vendor sources have different profiles, which indicated our method can be used for quality control of AGP or any other glycoprotein.

Column:	Dionex CarboPac PA200, Analytical, 1 × 250 mm
	Dionex CarboPac PA200, Guard, 1 50 mm
Eluent Source:	Dionex EGC 400 MSA in series with Dionex EGC 400 KOH
Eluent:	0-70 min: 5-80 mM KMSA in 70 mM KOH
	70-75 min: 80 mM KMSA in 70 mM KOH
	75-90 min: 5 mM KMSA in 70 mM KOH
Temperature:	30 °C (detector) 25 °C (column)
Flow Rate:	0.063 mL/min
Inj. Volume:	0.4 µL
Detection:	PAD, Au on PTFE (Disposable), 1 mil gasket, Ag/AgCl ref. electrode

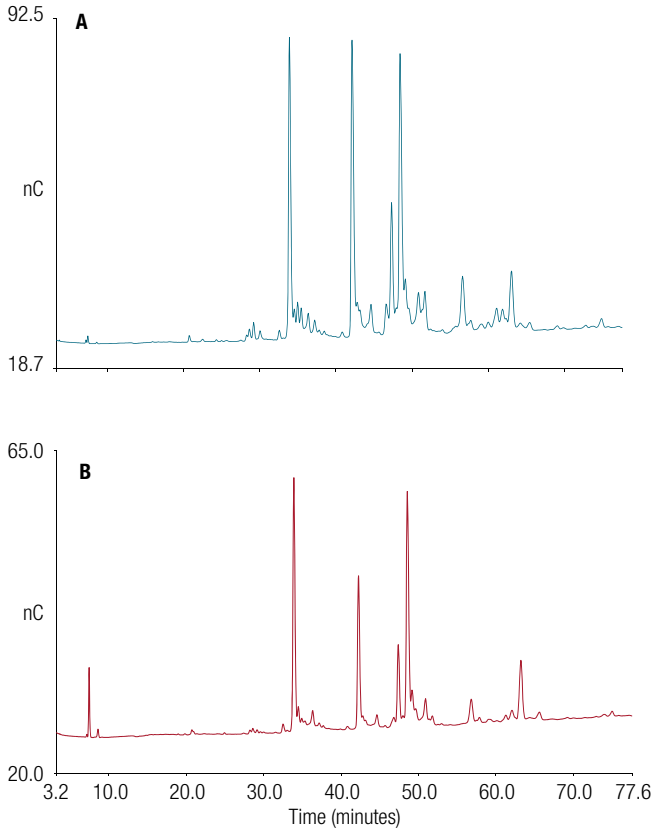

Figure 7. Separation of *N*-linked oligosaccharides released from human plasma AGP using Dual EGC. Chromatograms: (A) PNGase F digest of human plasma AGP from EMD Millipore; (B) PNGase F digest of human plasma AGP from Sigma

Neuraminidase digestions of the *N*-linked oligosaccharides released from human plasma AGP

Human AGP is a highly negatively charged acidic plasma glycoprotein, containing complex bi-, tri- and tetra-antennary carbohydrate chains. Human plasma AGPs were purchased from two vendors and treated with PNGase F, followed by α2-3, 6, 8 neuraminidase. The *N*-linked glycan profiles before and after neuraminidase treatments are shown in Figure 8. These data indicate that the complex chromatogram of human AGP oligosaccharides is due to sialylated oligosaccharides.

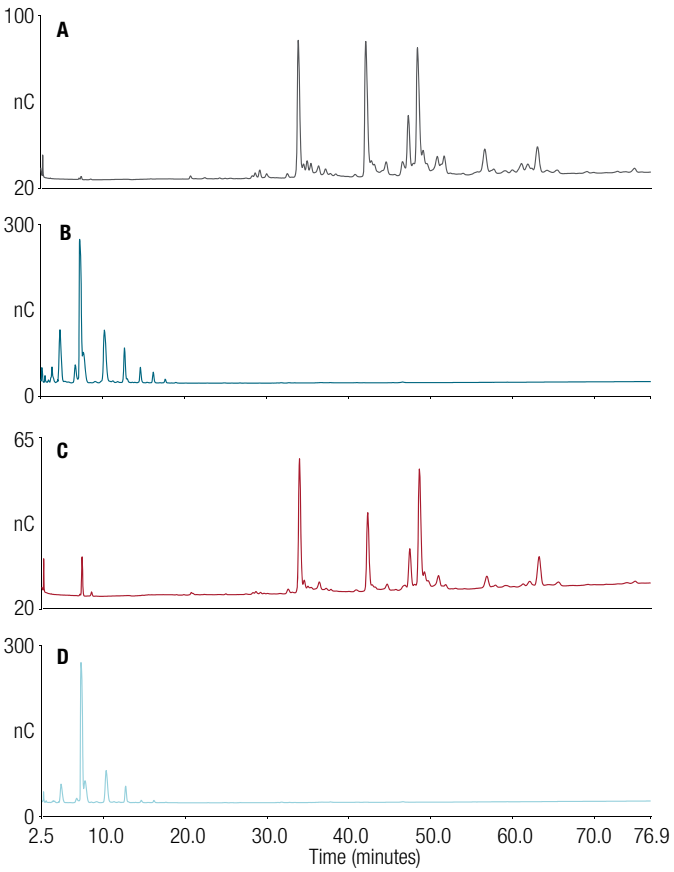

Figure 8. Separation of human plasma AGP *N*-linked oligosaccharides with neuraminidase treatment using Dual EGC. Chromatographic conditions are shown in the Conditions section. Chromatograms: (A) PNGase F digest of human plasma AGP from EMD Millipore; (B) AGP oligosaccharides from EMD Millipore with neuraminidase treatment; (C) PNGase F digest of human plasma AGP from Sigma; (D) AGP oligosaccharides from Sigma with neuraminidase treatment

Reproducibility of *N*-linked oligosaccharides separation using Dual EGC

Retention time and peak area stability for *N*-linked oligosaccharide separation using Dual EGC mode was assessed by the overlay of multiple consecutive injections of the same samples.

Human serum IgG can be treated with Rapid PNGase F with and without denaturation. Figure 3 showed the glycan profile without denaturation using Dual EGC. In Figure 9 we demonstrate that Dual EGC also achieves good separation and reproducibility of the denatured IgG glycan samples, which were prepared as described in the subsection Glycan purification of the Sample preparation section. Figure 9 shows the overlay of three consecutive injections of the same PNGase F digest from human serum IgG. We also demonstrated Dual EGC reproducibility in Figure 10 that is an overlay of five consecutive injections of AGP *N*-linked oligosaccharides. Both figures show good separation reproducibility is achieved by Dual EGC mode on a 1 mm Dionex CarboPac PA200 column.

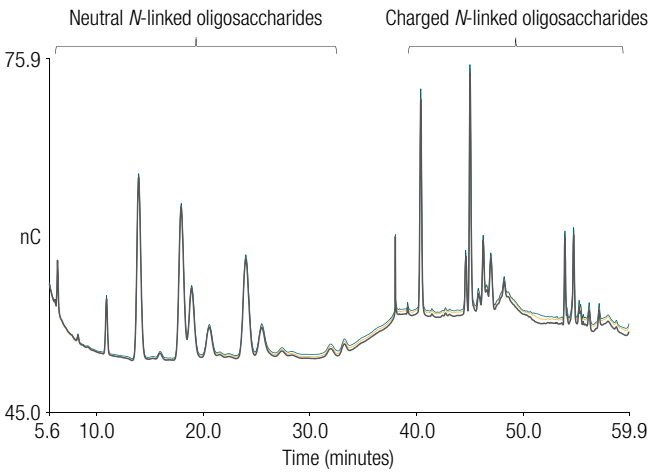

Figure 9. Overlaid chromatograms of three consecutive injections of *N*-linked oligosaccharides released from human serum IgG after the purification

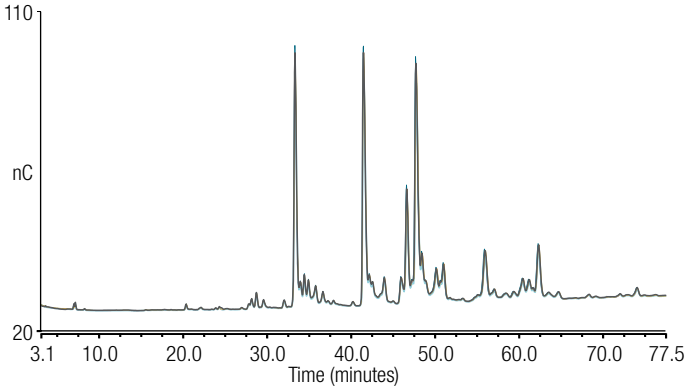

Figure 10. Overlaid chromatograms of five consecutive injections of *N*-linked oligosaccharides released from human AGP

Conclusion

In summary, the dual electrolytic eluent generation platform provides a convenient way to generate high-purity KMSA/KOH eluent for the separation of *N*-linked oligosaccharides released from glycoproteins. Separation was demonstrated to be comparable to that using the conventional manually prepared NaOAc/NaOH eluent. The Thermo Scientific Dionex ICS-6000 system with Dual EG mode provided consistent performance that enhances lab-to-lab reproducibility. Where the eluent concentration and gradient conditions need to be optimized frequently for each different type of sample, this automated eluent generation significantly reduces the time required for method development.

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