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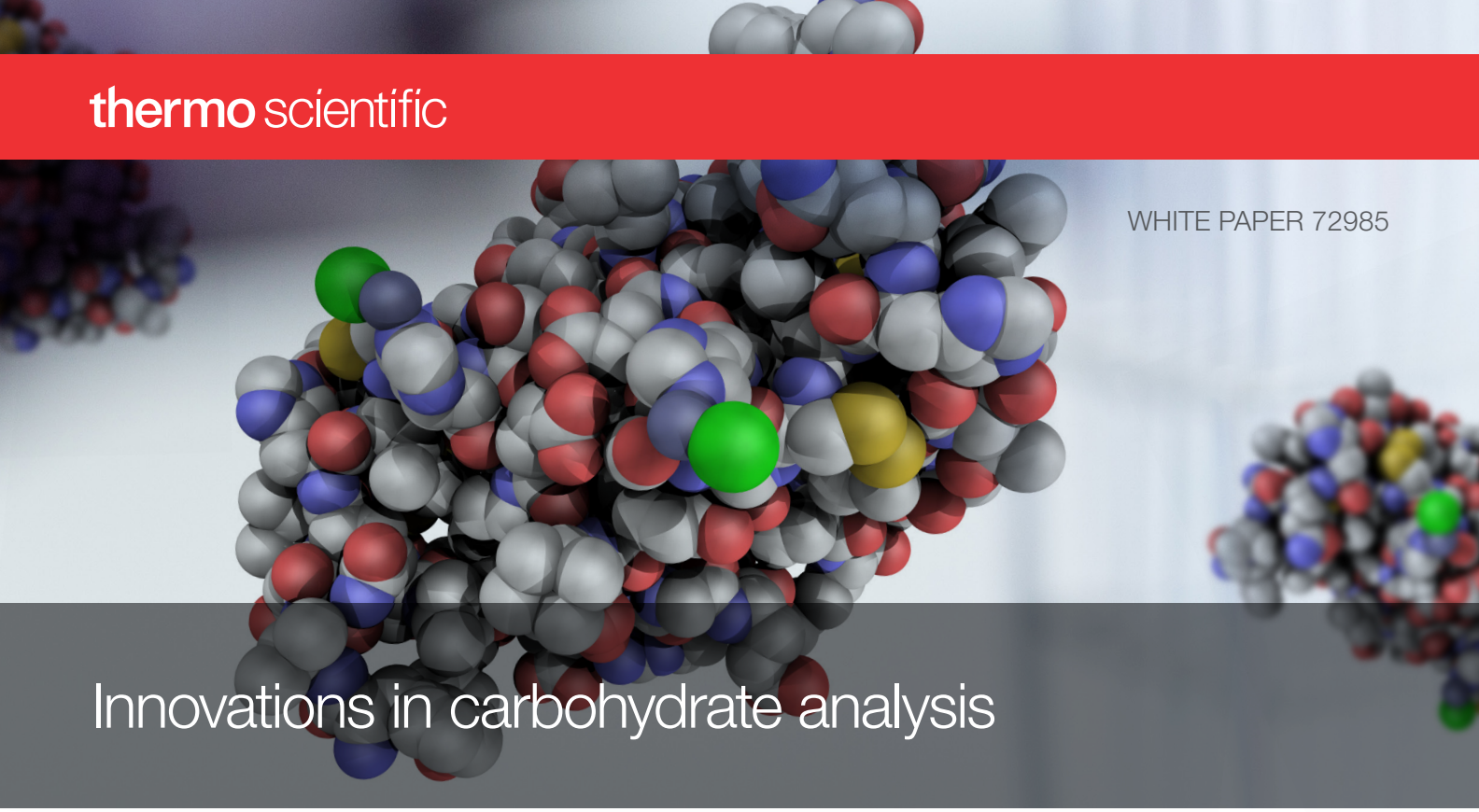

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Innovations in carbohydrate analysis

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Introduction

In liquid chromatography (LC) the manual preparation of eluents is one of the major contributors to the uncertainties of the analytical results obtained. This article describes electrolytic and highly automated inline Eluent Generation applied to carbohydrate separations using high-performance anion exchange chromatography (HPAE) in combination with pulsed amperometric detection (PAD).

Results and discussion

Neutral carbohydrates are polyalcohols with pK_a above 12 that can be separated at high pH using HPAE. Under these conditions, their detection predominantly is by pulsed amperometry (PAD).[1,2,3] Modern column chemistry and detection capabilities allow for a comprehensive analysis starting with alditols and monosaccharides to oligo- and polysaccharides.[4,5] Driven by the application of continuously and dynamically regenerated desalting devices, known as electrolytically regenerated suppressors in ion chromatography (IC), the hyphenation of HPAE with mass spectrometry became a tool in modern glycan analysis.[6] High pH eluents in HPAE are based on aqueous solutions of sodium or potassium hydroxide. The manual preparation of such solutions requires the impeccable execution of precautions to avoid contamination by the absorption of carbon dioxide from the surrounding atmosphere.[7] Most importantly these steps need to be performed by every of the tasked laboratory members in the same way. Slight deviations of the manual eluent preparation influence the chromatographic measurements and contribute to the uncertainties of the analytical results;[8] ideally, they should be avoided.

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A technique originally developed for its application in IC is capable of generating high purity hydroxide eluents based on an electrolytic process. It is known as Dionex Reagent-Free Ion Chromatography (RFIC) and allows the in situ eluent preparation, avoiding the manual handling of external chemicals.[9,10] Figure 1 shows the general setup of an RFIC instrument applied to HPAE-PAD. The electrolytic process, combining hydroxide ions from the feed water with the counter ions from the Thermo Scientific™ Dionex™ Eluent Generator Cartridge (EGC), happens in the high-pressure part of the IC instrument. This approach prevents carbon dioxide-contamination resulting in automatically, and user-independently generated high-purity eluents. The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software, version 7.2, controls the defined hydroxide concentration and chosen elution program (isocratic, gradient) by automatically adjusting the electrolytic process. In essence, the user only has to deploy deionized water of ASTM Type I quality or better.

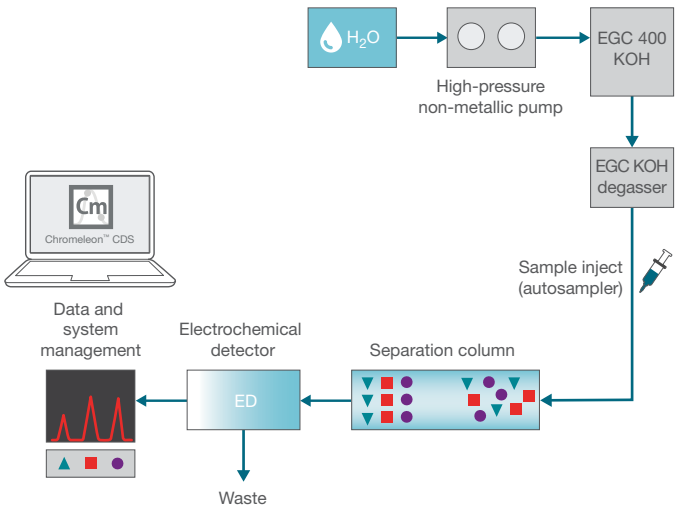

Figure 1. Schematic Setup of electrolytic Eluent Generation (RFIC); the KOH eluent is produced electrolytically in the Eluent Generator Cartridge (EGC).

This instrument setup can be used for the determination of mono-, di- to oligosaccharides and aminoglycosides. Figure 2 shows the fast separation of biofuel sugars from lignocellulosic-biomass-derived samples. Patil et al. used a Thermo Scientific™ Dionex™ CarboPac™ SA10-4µm column reducing the analytical cycle time (<10 min) while improving the chromatographic resolution.[11] To handle the high sugar concentrations present in the hydrolysates, to avoid a massive dilution of the sample and shifting the linearity of the calibration functions towards higher concentrations a small injection volume was applied, combined with a thicker gasket.[12] Please note the low eluent concentration and the high carbohydrate concentrations used in Figure 2.

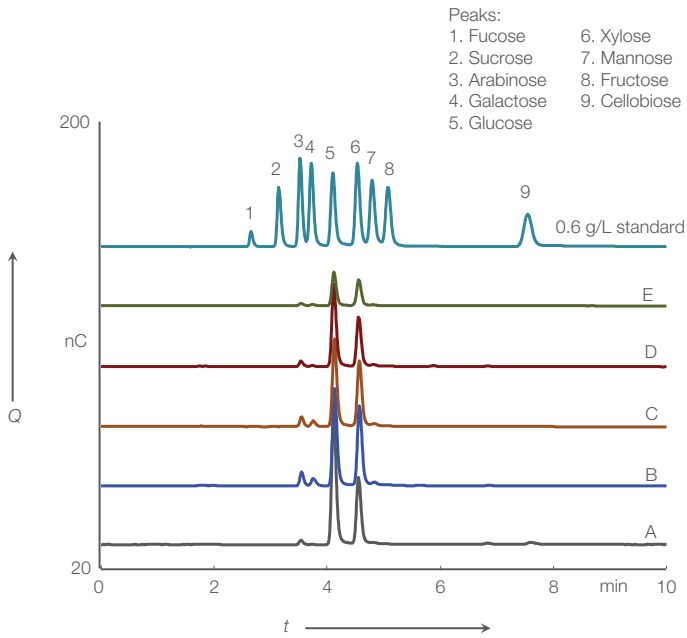

Figure 2. Representative chromatograms of five hydrolyzed lignocellulosic biomass-based biofuel samples. Column: Thermo Scientific Dionex CarboPac SA10-4µm, Eluent: 1 mmol/L KOH (Eluent source: RFIC-EG), Flow: 1.5 mL/min, Injection vol.: 0.4 µL, Temp.: 45 °C, Detection: PAD (Au-electrode, gasket: 1.57 mm [62 mil]).

The same Eluent Generation approach can be used to assay the tobramycin content and quantify the related impurities of tobramycin-based antibiotics. The aminoglycosides were separated at 0.5 mL/min on the Thermo Scientific™ Dionex™ CarboPac™ PA1 Analytical & Guard Column using a 2 mM KOH eluent and detected by PAD. Figure 3 shows the separation of tobramycin with kanamycin impurities.[13] The cycle-time was adjusted to 16 min to ensure that the oxygen dip (a dip in the baseline that is the result of having less dissolved oxygen in the sample than in the eluent) from the previous injection did not appear near a peak of interest. The analytical conditions support the trace determination of impurities in tobramycin and other aminoglycosides like neomycin, paromomycin.[14,15] The elution of streptomycin, kanamycin, and amikacin requires elevated hydroxide concentrations.[16,17] In addition to the HPAE approaches, a new column, the Thermo Scientific™ Dionex™ IonPac™ AmG-3µm C18, facilitates conventional ion-pair separations of aminoglycosides, followed by PAD or Corona CAD detection.[18]

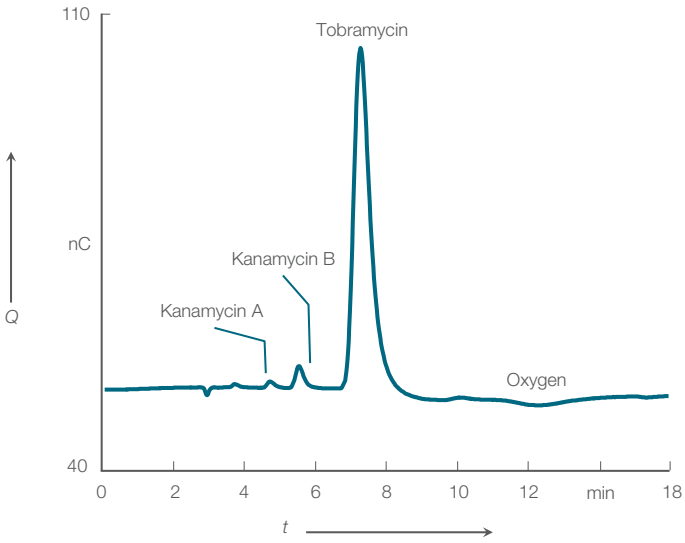

Figure 3. Tobramycin with trace amounts of Kanamycin A and B. Column: Thermo Scientific Dionex CarboPac PA1, Eluent: 2 mmol/L KOH (Eluent source: RFIC-EG), Flow: 0.5 mL/min, Injection vol.: 20 µL, Temp.: 30 °C, Detection: PAD (Au-electrode, gasket: 51 µm [2 mil]; AAA pulse sequence).

Figure 4 shows a third example of the use of KOH-Eluent Generation in HPAE-PAD. Patil et al. used Thermo Scientific™ Dionex™ CarboPac™ PA20-Fast-4µm columns to optimize the separation of glycoprotein monosaccharides and amino sugars in HCl and TFA hydrolysates. The smaller particle size of this column offers higher peak efficiencies, leading to high-resolution separations and allowing a shorter column format and, ultimately, significantly shorter run times. It results in baseline-resolved peaks that elute within 6 min off the column. The total run time is 20 min to allow for washing and re-equilibration after the column regeneration step. The chromatogram shows not only the region where the monosaccharides and amino sugars elute but also the column wash and re-equilibration.[19]

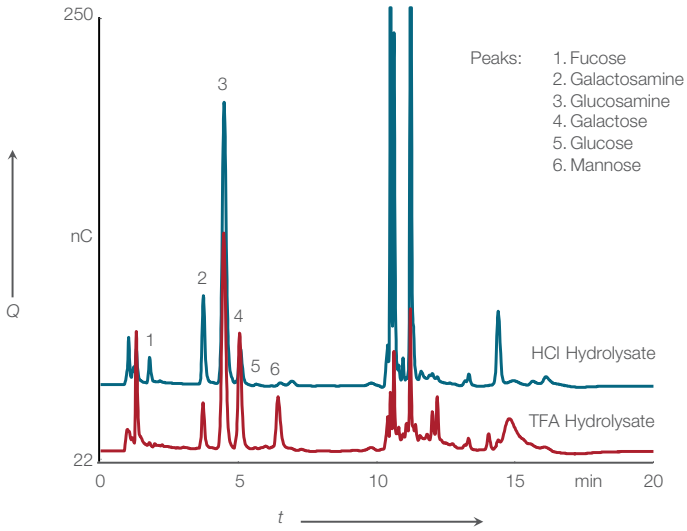

Figure 4. Analysis of TFA and HCl hydrolysates of bovine Fetuin. Column: Thermo Scientific Dionex CarboPac PA20-Fast-4µm (2 × 100) mm, Eluent: KOH-Gradient (Eluent source: RFIC-EG), Flow: 0.22 mL/min, Injection vol.: 2.5 µL, Temp.: 30 °C, Detection: PAD (Au-electrode, gasket: 51 µm [2 mil]).

Aggrawal et al. demonstrated the use of RFIC for HPAE-PAD for the analysis of mono- to oligosaccharides in honey. They used a Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast-4µm Column to separate low concentrations of carbohydrates in the presence of high concentrations of glucose and fructose. This method enabled the detection of honey-adulteration by the addition of industrial sugar syrups.[20]

The above examples present the user-independent inline, and high-pressure electrolytic eluent generation of KOH-eluents, both isocratic and gradient. This provides accurate eluent concentrations and precise retention times resulting in reproducible and reliable analytical data.

The elution of oligo- and polysaccharides requires the use of a "pushing ion," like acetate or nitrate.[5,21] The "pusher" accelerates the elution of strongly bound species without compromising selectivity and without interfering with the pulsed amperometric detection. Until recently applications requiring such eluent compositions had to be performed using manually prepared eluents. With the Dual Eluent Generation Cartridge (Dual EGC) mode of operation (Figure 5) electrolytically generated eluents become available for the analysis of complex carbohydrates. In this new 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 is applicable with the 1 mm column format. Huang et al. demonstrated its use for profiling galactosyl-oligosaccharides (GOS)[22] and the analysis of N-linked oligosaccharides from glycoproteins.[23] They used a Thermo Scientific™ Dionex™ CarboPac™ PA200 Column set in the 1 mm format. As a representative example for the elution for even stronger retained polysaccharides, Figure 6 shows an inulin-separation in the Dual EGC operation mode.

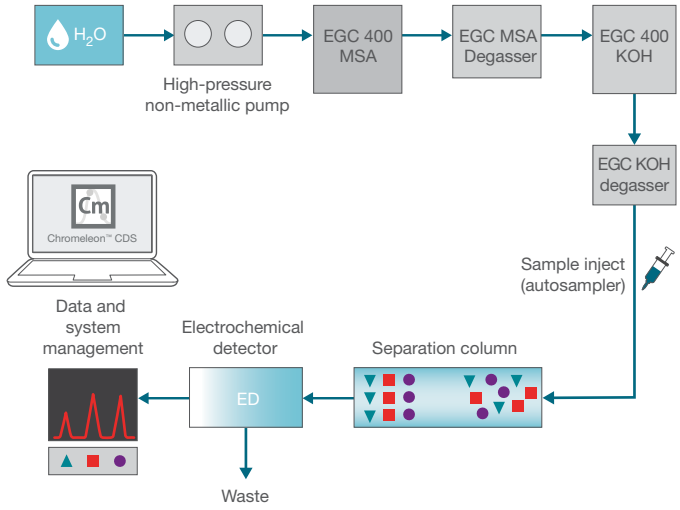

Figure 5. Ion chromatography system Dual EGC mode workflow.

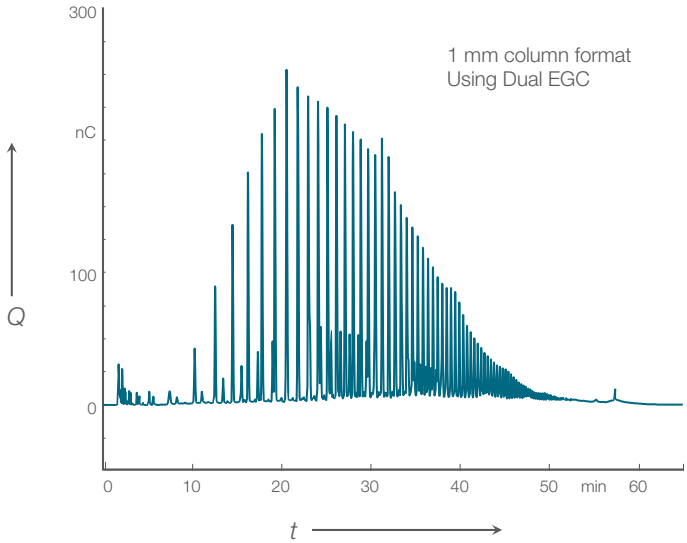

Figure 6. Inulin Analysis using Dual EGC. Column: Thermo Scientific Dionex CarboPac PA200 (1 × 250) mm, Eluent: KOH/KMSA-Gradient (Eluent source: RFIC-EG), Flow: 0.063 mL/min, Injection vol.: 0.4 µL, Temp.: 30 °C, Detection: PAD (Au-electrode, gasket: 25.4 µm [1 mil]) [24].

Conclusion

The use of electrolytic eluent generation applied to HPAE-PAD improves the consistency in producing low KOH-concentration eluents and KOH-gradients for the elution of smaller saccharides, making the method user-independent, reproducible, and rugged concerning retention time and peak area precision.

The Dual EGC mode enables the analyst to run gradient methods using electrolytically generated KOH/KMSA to elute stronger retained oligo- and polysaccharides offering improved reproducibility, eliminating manual preparation of eluents, thus maximizing instrument uptime. The Dual EGC mode shows excellent performance for HPAE-PAD of complex carbohydrates.

In its different modes of operation, RFIC improves the reproducibility of the analytical HPAE-PAD measurement and assures greater accuracy and comparability between instruments as well as between laboratories.

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