Application Note

Glycans & Glycoprotein

Analysis of *N*-Glycans using HPAEC-PAD/MS

Introduction

Glycosylation is a fundamental biological process in which polysaccharides, referred to as glycans, become covalently bound to proteins. In the field of glycomics, one of the major challenges is the multitude of distinct glycan isomers found within a glycoprotein, each varying in their abundances [1]. To improve the structure elucidation workflow in glycomics, the development of analytical methods to detect and quantify glycans is crucial. Many widely used analytical techniques for glycans detection and quantification involve the derivatization of glycan samples after their release from glycoprotein. However, the derivatization may cause some issues such as incomplete labelling or loss of sialic acids, ultimately leading to incomplete structure elucidation [2]. Therefore, a glycan analysis method that eliminates the need for derivatization is preferrable.

High-performance anion exchange chromatography combined with pulsed amperometric detection (HPAEC-PAD) coupled with mass spectroscopy (MS) detection offers an optimal analytical approach for sensitive detection of *N*-glycans without sample derivatization. Previous studies have highlighted the use of HPAEC-PAD [3-4] or the use of a mass spectrometer [5-6] for the detection of *N*-glycans. Notably, several studies have combined mass spectrometry for glycan identification with PAD for glycan quantification [7-9]. In this application note, a glycan analysis method is presented based on the HPAEC-PAD/MS technique. A mix of the 13 most commonly observed *N*-glycans on therapeutic monoclonal antibodies (NIST SRM3655) was analyzed as a proof of principle to demonstrate the performance of the method.

Method

Separation of the *N*-glycans is based on HPAEC using the new SweetSep™ AEX200 anion-exchange column. Carbohydrates can be separated using HPAEC under alkaline conditions (pH > 12). *N*-glycans are polysaccharides, which under alkaline conditions will be retained stronger than simple monosaccharides or disaccharides. The retention behavior of *N*-glycans is also dependent on the sialic acid units in a *N*-glycan structure. Therefore, gradient elution with an increasing amount of sodium acetate modifier is used to speed up the elution of the *N*-glycans (see gradient program in Table 1). It is essential to perform a desalting step after the separation on the column. Sodium hydroxide and acetate eluents are non-volatile and high conductance solvents, thus, they are not compatible with electrospray ionization MS and may

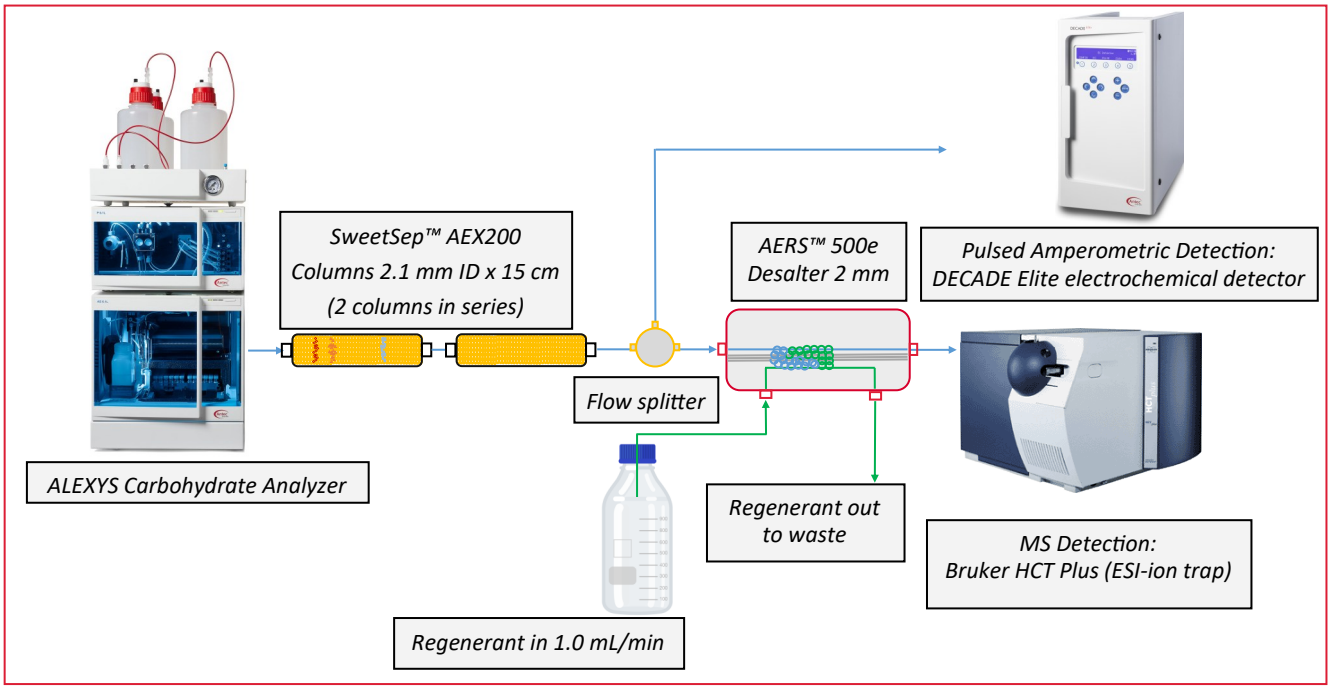

Figure 1. Instrument setup for N-glycan analysis using HPAEC-PAD/MS.

Table 1. HPAEC-PAD conditions

LC system	Quaternary HPLC system (ALEXYS)
Detector	DECADE Elite electrochemical detector
Columns	2 × SweetSep™ AEX200, 2 × 150 mm column, 5 µm
Initial flow rate	180 µL/min
Split ratio	1:1
Gradient program	0 min: 50 mM NaOH + 5 mM NaOAc 25 min: 50 mM NaOH + 12 mM NaOAc 35—50 min: 50 mM NaOH + 100 mM NaOAc 50—130 min: 50 mM NaOH + 5 mM NaOAc
System backpressure	About 220 bar
Temperature	30°C for separation, 35°C for detection
Flow cell	FlexCell Au WE, HyREF Pd RE, carbon-filled PTFE AUX
PAD Potential wave-form (4-step)	E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s

Table 2. Desalting & MS conditions

Detector	Bruker Daltonics HCT Plus (ESI-ion trap)
Data acquisition	Bruker Compass & EsquireControl software
Desalter	Thermo Scientific™ Dionex™ AERS™ 500e Electrolytically Regenerated Desalter 2 mm
Desalter regenerant	DI water
Desalter current	80 mA
Desalter potential	4.2 V
Capillary potential	4000 V
End plate potential	3500 V
Nebulizer pressure	45 psi
Drying gas flow rate	5 L/min
Drying gas temperature	350°C

lead to damage of the ion source. Moreover, the presence of non-volatile salts can lead to ion suppression and thus a loss in sensitivity of MS detection [10]. The use of the desalter can also be circumvented using volatile buffer system such as ammonium formate [10, 11].

Prior to the desalter, the effluent from the column is split with a 1:1 split ratio to allow simultaneous PAD & MS detection (Figure 1). The HPAEC-PAD conditions are listed in Table 1. For PAD detection, a 4-step potential waveform was applied. The choice of the 4-step potential waveform resulted in excellent reproducibility and minimal electrode wear [12]. The detection temperature was set at 35°C. MS and desalting conditions are listed in Table 2.

Results

An overlay of chromatograms of 10 µL injections of 1 µM mix of 13 *N*-glycan standards is shown in Figure 2. The overlay consists of chromatograms obtained from the pulsed amperometric detection and the MS detection, represented by the Total Ion Current (TIC) chromatogram. Peak identification was based on the *m/z* value as well as injection of individual *N*-glycan standard. The mix of 13 *N*-glycan standards consists of non-sialylated, monosialylated, and disialylated *N*-glycans. All 13 *N*-glycans are biantennary and belongs to complex *N*-glycan structures.

Both PAD and TIC signals show a similar chromatographic separation profile of the *N*-glycans. There are several observed trends in the separation of *N*-glycans using HPAEC-PAD in this condition. First of all, the separation of *N*-glycans is based on the number of sialic acid units in a glycan structure, i.e. the more sialic acid , the later the retention time of the glycan is. In this case, the non-sialylated *N*-glycans eluted under 25 minutes, while mono– and disialylated *N*-glycans eluted in about 37 and 45 minutes, respectively. If the number of sialic acid is the same for two or more glycans, then the number of galactose governs the retention time behaviour of the glycan. Glycan with more galactose units will be retained longer, for example G2 eluted later than G1a, and G1a eluted later than G0. It seems like the observation leads to the trend of the larger the glycan structure, the more retained it will be. However, the fucosylated *N*-glycans elute earlier compared to non-fucosylated *N*-glycans (of the same structure), for example G0F eluted earlier than G0. The most interesting observation is the separation of linkage isomers, differing in only the linkage position of sialic acids, for instance, G2FS2(6) eluted earlier than G2FS2(3). This may be related to the acidity of the hydroxyl group, which in a reducing sugar follows a hierarchy of 2-OH > 6-OH > 3-OH > 4-OH. These observations are in line with the reported trend in several literatures [13,14]. Although most of the *N*-glycans are identified, there are several *N*-glycans peaks that are unidentified. These unidentified peaks are described in the literature as impurities, and these impurities are related to a singly and asialylated glycoforms [15], which may corresponds to trace amounts of different sialic acids such as *N*-glycolyl neuraminic acid (NGNA) or 2-Keto-3-deoxynonic acid (Kdn).

In conclusion, HPAEC shows good separation of all 13 *N*-glycans, and is capable to distinguish the isomeric structure of the glycans. The presented method shows the potential of parallel PAD and MS detection for *N*-glycan analysis. Further, this method can be extended into tandem MS/MS for the identification of *N*-glycans for linkage position determination, and the pulse amperometric detection can be used for the quantification of *N*-glycans.

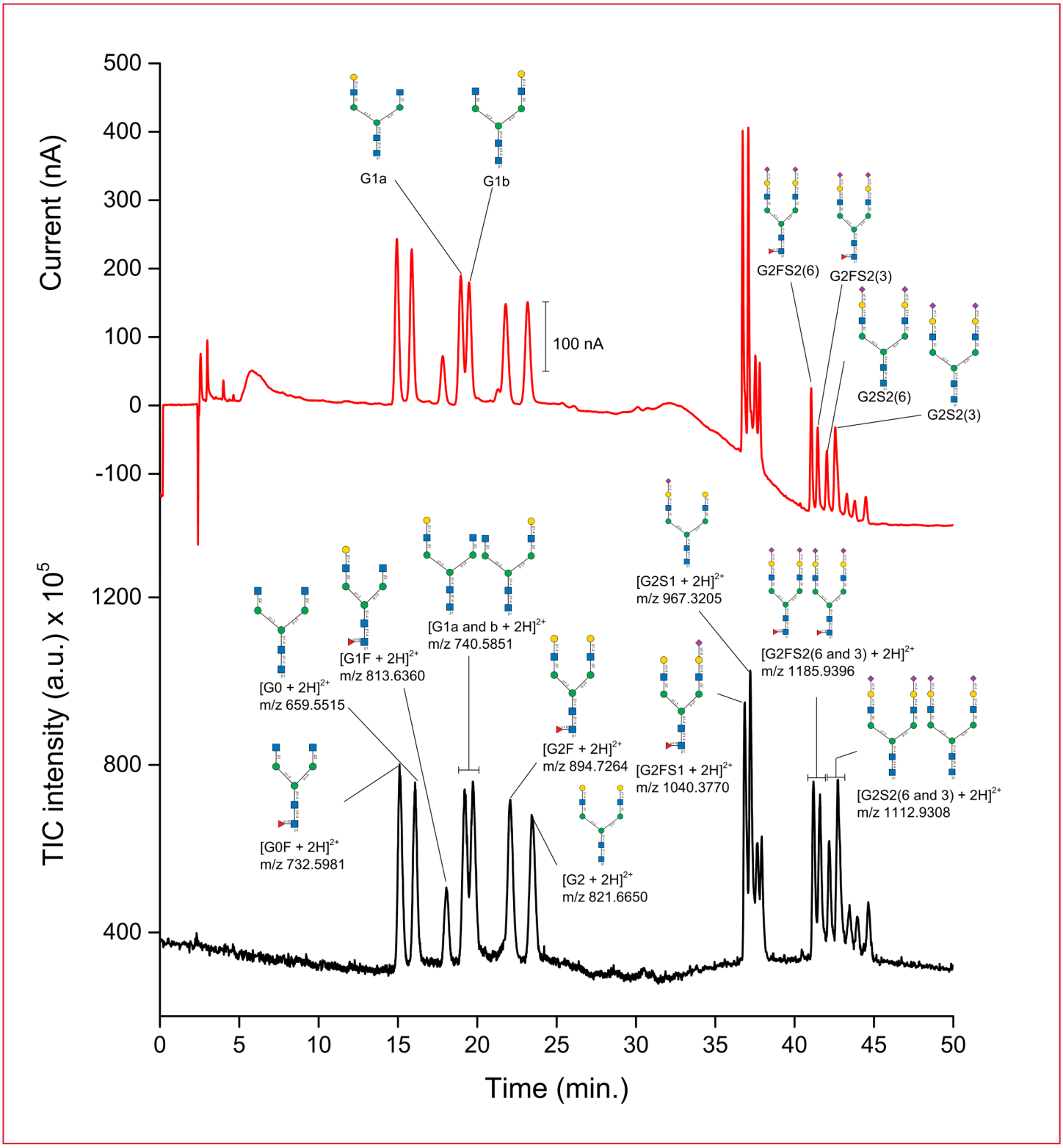

Figure 2. Chromatograms obtained with an 10 µL injection of a 1 µM mix of N-glycans standard containing 13 biantennary N-glycans (non-sialylated, mono– and di-sialylated) on 2 × SweetSep™ AEX 200 column, 2.1 mm ID × 150 mm (2 columns in series). Top: Pulsed Amperometric Detection Chromatogram. Bottom: Total Ion Current (TIC) Chromatogram. Peak annotations are based on the m/z values and injections of individual N-glycan standard.

References

1. Spiro, R.G., Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds, *Glycobiology*, 2002, 12(4), 43R-56R
2. Szabo, Z., Thayer, J.R., Rohrer, J., Instrument configuration for native *N*-linked oligosaccharide characterization by HPAE-PAD/MS, ThermoFisher Scientific Technical Note 72478
3. Townsend, R.R., et al., Separation of branched sialylated oligosaccharides using high-pH HPAEC-PAD, *Anal. Biochem.*, 1989, 182, 1-8
4. Rohrer, J.S., Separation of asparagine-linked oligosaccharides by high-pH anion-exchange chromatography with pulsed amperometric detection: empirical relationships between oligosaccharide structure and chromatographic retention, *Glycobiology*, 1995, 5(4), 359-363
5. Han, L., Costello, C.E., Mass spectrometry of glycans, *Biochemistry*, 2013, 78(7), 710-720
6. Liew, C.Y., et al., Structural identification of N-glycan isomers using logically derived sequence tandem mass spectrometry, Commun. Chem., 2021, 4:92
7. Maier, M., et al., Applying mini-bore HPAEC-MS/MS for the characterization and quantification of Fc N-glycans from heterogeneously glycosylated IgGs, *J. Chrom. B.*, 2016, 1033-1034, 342-352
8. Kandzia, S., Costa, J., N-glycosylation analysis by HPAEC-PAD and mass spectrometry, *Methods Mol. Biol.*, 2013, 1049, 301-312
9. Bruggink, C., et al., Analysis of carbohydrates by anion exchange chromatography and mass spectrometry, *J. Chrom. A.*, 2005, 1085, 104-109
10. Flick, T.G., et al., Solution additives that desalt protein ions in native mass spectrometry, *Anal. Chem.*, 2012, 84, 7511-7517
11. van Schaick, G., et al., Anion exchange chromatography—mass spectrometry for monitoring multiple quality attributes of erythropoietin biopharmaceuticals, *Anal. Chim. Acta*, 2021, 1143, 166-172
12. Rocklin, R.D., Clarke, A.P., Weitzhandler, M., Improved long-term reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform, *Anal. Chem*, 1998, 70, 1496-1501
13. Lee, Y.C., Carbohydrate analyses with high-performance anion -exchange chromatography, *J. Chrom. A.*, 1996, 720, 137-149
14. Hard, M.R., Townsend, R.R., Separation of positional isomers of oligosaccharides and glycopeptides by high-performance anion-exchange chromatography with pulsed amperometric detection, *PNAS*, 1988, 85, 3289—3293
15. Certificate of Analysis Standard Reference Material® 3655, National Institute of Standards and Technology, issued 21 April 2022, expiry date 01 September 2026.

Figure 3. Recommended HPAEC-PAD instrument configuration for this application: the ALEXYS Carbohydrate Analyzer. The system consists of an ET210 Eluent tray for nitrogen blanketing, a P6.1L quaternary LPG pump capable of running gradient program, an AS6.1L autosampler, a CT2.1 column thermostat, and a DECADE Elite electrochemical detector. The ALEXYS Carbohydrate Analyzer can be fully controlled by different Chromatography Data System (CDS) software, namely DataApex™ Clarity™ CDS (version 8.3 and up) or Thermo Scientific™ Chromeleon™ CDS (version 7.2 SR5 and up).

Antec Scientific (USA)
info@AntecScientific.com
www.AntecScientific.com
T 888 572 0012

Antec Scientific (worldwide)
info@AntecScientific.com
www.AntecScientific.com
T +31 172 268888