Comprehensive LC-PAD/MS Analysis of N-glycans using the SweetSep™ HPAEC Column

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Introduction

In the field of glycomics, understanding the functional aspects and biological implications of glycoproteins requires a comprehensive understanding of their structure, for example, the glycosylation sites or the type of glycan moieties. Despite the significance of these insights, the analysis of glycans in glycoproteins presents a major challenge due to the vast number of different glycan isomers present in glycoproteins, each with varying abundances. Therefore, to improve the structure elucidation workflow in glycomics, there is a need for the development of analytical methods that enable the separation, detection, and quantification of glycans from glycoproteins.

The typical approach for glycan analysis of glycoproteins involves enzymatic release of the glycans from the glycoproteins, followed by chemical derivatization of the glycans. Subsequently, the mixture of glycans can be separated and identified using liquid chromatography such as HILIC with fluorescence and MS detection. High-performance anion exchange chromatography combined with pulsed amperometric detection (HPAEC-PAD) is an alternative analytical technique that allows for the separation and detection of glycans without the need for derivatization. When coupled with MS, HPAEC-PAD becomes a powerful analytical workflow for the high-resolution separation, sensitive detection, quantification, and accurate identification of glycans from glycoproteins.

Stationary Phase

The SweetSep™ AEX200 stationary phase is specifically developed for the separation of all classes of carbohydrates ranging from mono– up to polysaccharides including N-glycans using HPAEC-PAD/MS.

- Strong polymeric anion-exchange resin
- Highly monodisperse latex-coated particles (5 µm)
- Fast, high-resolution separation
- Moderate column back pressure

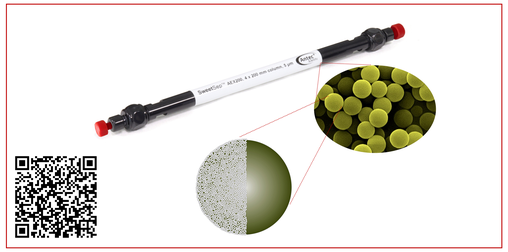

Figure 1. *SweetSep™ AEX 200 column based on a polymeric stationary phase consisting of monodisperse 5 µm particles coated with latex nano beads, functionalized with quaternary amine groups (for clarity, only half of the nano-beads are shown).*

Method and Instrumentation

Table 1. PAD Method and Instrumentation

LC system	ALEXYS™ Carbohydrate Analyzer with DECADE™ Elite electrochemical detector and SenCell™ (Antec Scientific)
Columns	2 × SweetSep™ AEX200 2.1 x 150 mm column in series (Antec Scientific)
Temperature	35°C for separation and detection
Flow rate	180 µL/min
Split ratio	1:1
PAD Potential waveform (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. MS Method and Instrumentation

Detector	Bruker Daltonics HCT Plus (ESI-ion trap)
Desalter	Thermo Scientific™ Dionex™ AERS™ 500e 2 mm
Desalter current	80 mA
Desalter potential	4.2 V
Capillary potential	4000 V
End plate potential	3500 V
Nebulizer gas	45 psi
Drying gas flow rate	5 L/min
Drying gas temperature	350°C

Instrumental setup HPAEC-PAD/MS for *N*-glycan analysis

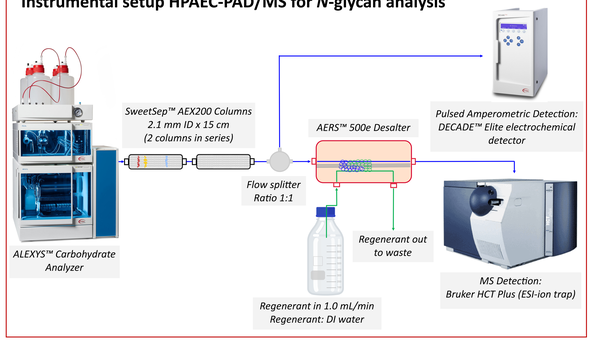

Figure 2. *Instrumental setup for the analysis of intact N-Glycans with HPAEC-PAD/MS. Desalter is installed upfront MS detection to suppress high concentration of sodium ions in the mobile phase. Regenerant: deionized water, 1.0 mL/min.*

Analysis of NIST N-Glycans Standard (SRM3655)

In biopharmaceutical development & QC, profiling and quantification of released intact N-linked glycans is an important step to assess glycosylation. In this study, a mix of the 13 most commonly observed N-glycans on therapeutic monoclonal antibodies (NIST SRM3655) was analyzed to illustrate the performance of the method. Identification of the N-glycan isomers was achieved by injecting the individual N-Glycan standards. The HPAEC-PAD/MS chromatograms are presented below.

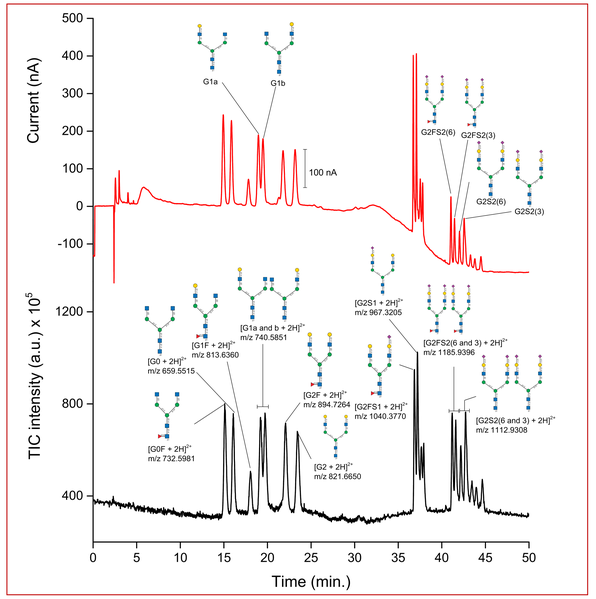

Figure 3. *Chromatograms obtained with an 10 µL injection of a 1 µM N-glycans standard containing desialylated, mono-, and disialylated oligosaccharides on SweetSep™ AEX200 column. Top: Pulsed Amperometric Detection with annotations of isomers. Bottom: TIC with full annotations. Gradient program: 0 min: 50 mM NaOH + 5 mM NaOAc, 25 min: 50 mM NaOH + 12 mM NaOAc, 35 min—50 min: 50 mM NaOH + 100 mM NaOAc. Desalter at 1.0 mL/min prior MS. 90 µL/min to ESI-MS.*

Compositional Analysis of Glycans

The U.S. FDA maintains strict regulation of glycoprotein for therapeutic use. Glycoprotein monosaccharides composition significantly influences its physical and chemical properties, its efficacy, and its safety to be used as medicine. Therefore, compositional analysis of glycans in glycoproteins becomes essential in maintaining the quality of therapeutic glycoproteins. Figure 4 shows the application of HPAEC-PAD for high-resolution separation and detection of commonly found monosaccharides in glycoproteins, facilitating comprehensive compositional analysis of glycans.

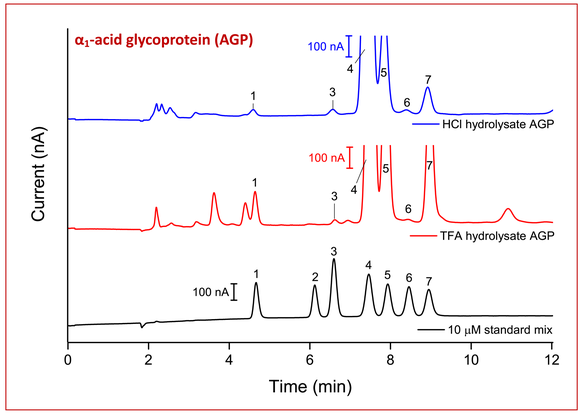

Figure 4. *Chromatograms obtained with an 10 µL injection of a 10 µM mix of monosaccharide standard commonly found in glycoproteins (black trace), a TFA hydrolysate of AGP (α_1-acid glycoprotein, 2 µg protein equiv., red trace), and a HCl hydrolysate of AGP (2 µg protein equiv., blue trace) on the SweetSep™ AEX20 column, 4.0 mm ID × 200 mm. Peak labels: (1) Fucose, (2) 2-deoxyglucose (internal standard), (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose, and (7) Mannose.*

Conclusion

- High-resolution separation, detection and identification of the 13 most commonly observed N-glycans in mAbs using HPAEC-PAD/MS in combination with the SweetSep™AEX200 column was successfully achieved.
- SweetSep™ AEX20 allows for the determination of monosaccharide composition of glycoproteins.

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