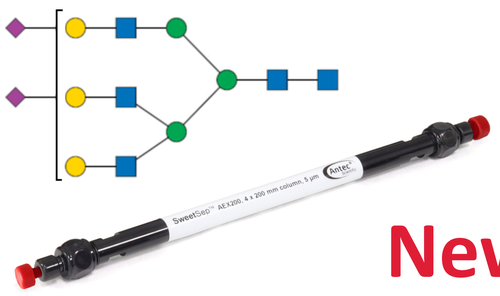

# New SweetSep™ Anion-Exchange Columns for the Analysis of *N*-Glycans Using HPAEC-PAD/MS

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## Introduction

One of the major challenges in the analysis of glycans in glycoproteins is the vast number of different glycan isomers present, each with varying abundancies. To improve the structure elucidation workflow in glycomics, the development of analytical methods to detect and quantify glycans is crucial. High-performance anion exchange chromatography combined with pulsed amperometric detection (HPAEC-PAD) and mass spectroscopy (MS) is an ideal analytical method that allows for rapid and sensitive detection of intact *N*-glycans and monosaccharides. A new line of polymeric anion-exchange stationary phases, SweetSep™, has been developed by Antec Scientific based on highly monodisperse latex-coated 5 µm particles functionalized with quaternary amine groups. In this study two 4 x 200 mm columns based on SweetSep™ AEX20 and SweetSep™ AEX200 were evaluated for their potential use in compositional analysis of glycoprotein monosaccharides using HPAEC-PAD and profiling of released N-glycans using HPLC-PAD/MS, respectively.

## Stationary phase

The SweetSep™ AEX20 & AEX200 stationary phases are specifically developed for the separation of mono-, oligo– and polysaccharides in HPAEC-PAD/MS applications.

- 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.*

## Method and instrumentation

**Table 1. PAD Method and Instrumentation**

| | |
|---|---|
| LC system | ALEXYS Carbohydrate Analyzer incl. DECADE Elite EC detector |
| Columns | SweetSep™ AEX20, 4.0 mm ID × 200 mm (monosaccharides analysis)<br>SweetSep™ AEX200, 4.0 mm ID × 200 mm (*N*-glycan analysis) |
| Temperature | 30°C for separation, 35°C for detection |
| PAD Potential waveform (4-step) | E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V<br>ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s |
| Data acquisition | DataApex Clarity CDS or Thermo Scientific™ Chromeleon™ CDS |

**Table 2. MS Method and Instrumentation**

| | |
|---|---|
| Detector | Bruker Daltonics HCT Plus (ESI-ion trap) |
| Data acquisition | Bruker Compass & EsquireControl software |
| Desalter | Thermo Scientific™ Dionex™ ERD™ 500 Electrolytically Regenerated Desalter |
| Desalter current | 500 mA |
| Desalter potential | 4.2 V |
| Capillary potential | 4000 V |
| End plate potential | 3500 V |

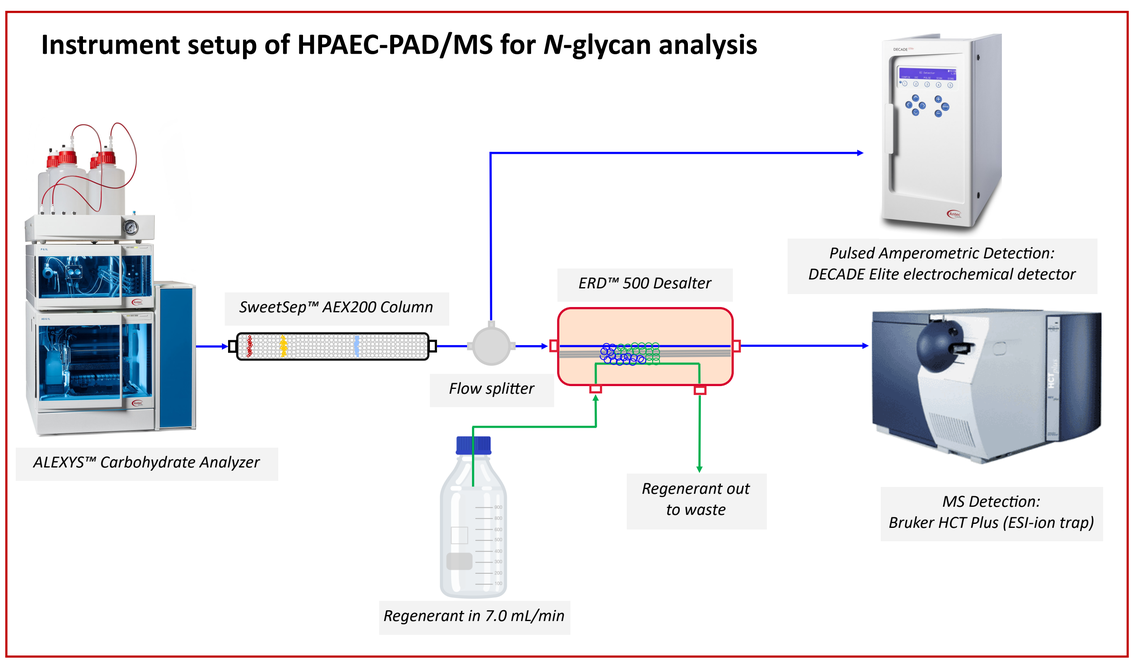


*Figure 2. Instrument setup for the analysis of intact N-Glycans with HPAEC-PAD/MS. Desalter is installed upfront MS detection to suppress high concentration of sodium ions in the mobile phase. Regenerant: deionized water.*

## Results

### 1. Compositional analysis of glycoprotein monosaccharides

N-glycans and O-glycans consist of various monosaccharides including fucose (Fuc), mannose (Man) , N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), galactose (Gal), glucose (Glc) and sialic acids. Determination of the glycan monosaccharides composition is an important orthogonal method to confirm the consistency of glycosylation in quality control testing of glycoprotein therapeutics. Acid hydrolysis of glycoproteins followed by HPAEC-PAD analysis is an simple and effective method to assay the monosaccharides contents without the need for sample derivatization. Separation of all 6 glycan monosaccharides of interest and 2-Deoxy-D-Glucose (glycosylation inhibitor) was achieved within 7 minutes using the new SweetSep™ AEX20 column under isocratic elution conditions (Figure 3).

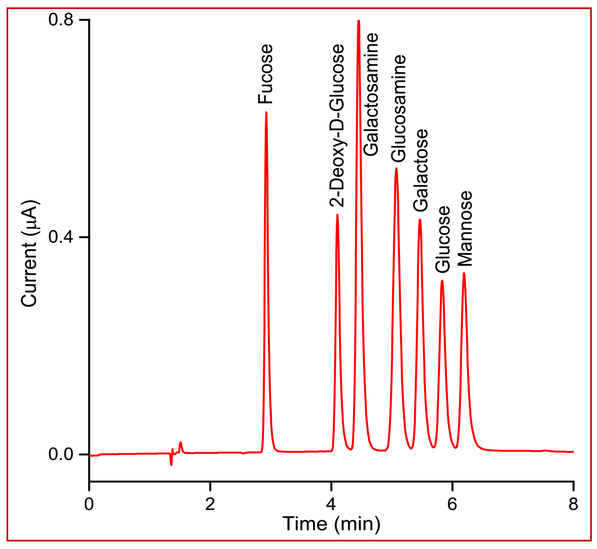


**Table 3. Column Efficiency and Peak Resolution**

| *Compound* | *N* | *Rs* |
|---|---|---|
| Fucose | 10604 | |
| 2-Deoxy-D-Glucose | 16489 | 9.787 |
| Galactosamine | 13007 | 2.478 |
| Glucosamine | 8000 | 3.278 |
| Galactose | 12090 | 1.809 |
| Glucose | 13810 | 1.896 |
| Mannose | 13556 | 1.750 |

*Figure 3. Chromatogram obtained with an 10 µL injection of a 10 µM mix of glycoprotein monosaccharides in DI water on a SweetSep™ AEX20, 4.0 mm ID × 200 mm column. Isocratic elution: 17.5 mM NaOH, 0.7 mL/min.*

### 2. Profiling of released N-glycans by HPAEC-PAD/MS

In biopharmaceutical development & QC, profiling of released, intact N-linked glycans is one of the important analysis tools to assess glycosylation in glycoprotein therapeutics. Release of the *N*-glycans from the protein backbone using *N*-glycosidase F enzyme (PNGase F), followed by HPAEC-PAD/MS analysis is a powerful method to characterize the glycan profile of glycoproteins, without the need for sample derivatization. The new AEX200 column is particularly suitable for high-resolution separation of glycan oligosaccharides. This is demonstrated by the example HPAEC-PAD/MS chromatograms shown in Figure 4, obtained from an sialylated N-glycan standard from fetuin.

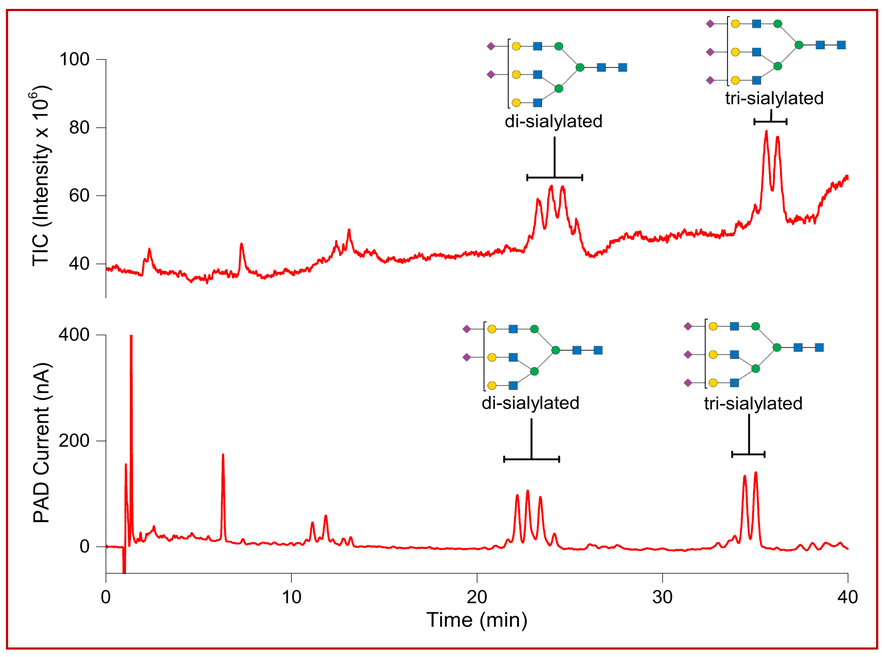


*Figure 4. Chromatograms obtained with an 10 µL injection of a 25 µM N-glycans standard containing di– and tri–sialylated oligosaccharides on a SweetSep™ AEX 200 column, 4.0 mm ID × 200 mm. Top: Total Ion Current (TIC) Chromatogram. MS conditions: nebulizer gas pressure 70 psi, drying gas flow rate 12 L/min, drying gas temperature 365°C. Bottom: Pulsed Amperometric Detection Chromatogram. Gradient elution: 0 min: 100 mM NaOH + 6 mM NaOAc, 70 min: 100 mM NaOH + 190 mM NaOAc, 1.0 mL/min.*

## Conclusion

Two novel 4 x 200 mm anion-exchange columns, based on highly monodisperse 5 µm particles, **SweetSep™ AEX20 and SweetSep™ AEX200** were evaluated for their potential use in compositional analysis of glycoprotein monosaccharides and profiling of released N-glycans using HPLC-PAD/MS, respectively. The presented data demonstrate:

- **Fast, high-resolution separation** of all 6 glycoprotein monosaccharides using the AEX20
- High-resolution separation of sialylated N-glycan oligosaccharides using the AEX200

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