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 detection method is presented based on the HPAEC-PAD/MS technique. A sialylated *N*-glycans standard from fetuin 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. 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). Subsequently, a column clean-up step (100 mM NaOH + 200 mM NaOAc) is executed for 5 minutes, followed by 15 minutes of equilibration to the starting conditions. 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 and are not compatible with

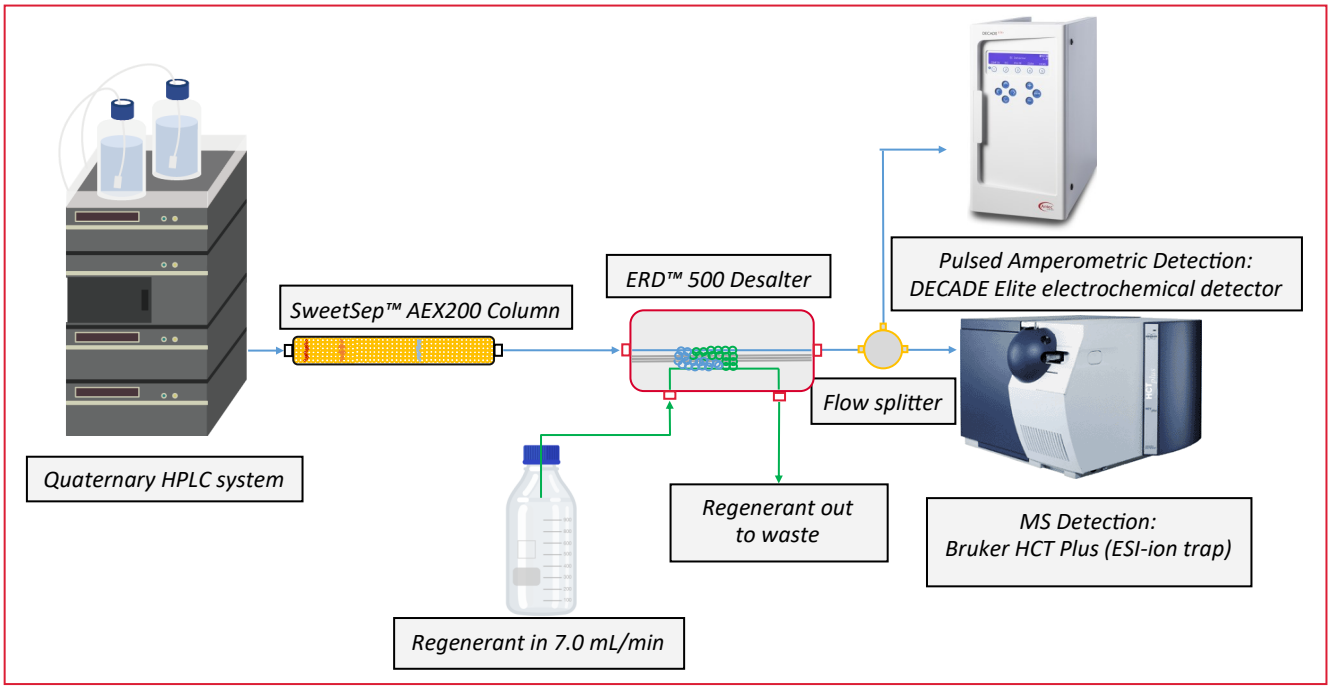


Figure 1. Instrument setup for N-glycan analysis using HPAEC-PAD/MS.

Table 1. HPAEC-PAD conditions

LC system	Quaternary HPLC system
Detector	DECADE Elite electrochemical detector
Columns	SweetSep™ AEX200, 4.0 mm ID × 200 mm
Flow rate	0.7 mL/min and 1 mL/min
Gradient program	0 min: 100 mM NaOH + 6 mM NaOAc 70 min: 100 mM NaOH + 190 mM NaOAc 70—75 min: 100 mM NaOH + 200 mM NaOAc 75—90 min: 100 mM NaOH + 6 mM NaOAc
System backpressure	About 310 bar
Temperature	30°C for separation, 35°C for detection
Flow cell	FlexCell Au WE, HyREF, carbon filled PTFE AUX
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. Desalting & MS conditions

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	4.0 V
Capillary potential	4000 V
End plate potential	3500 V
Nebulizer pressure	60 psi
Drying gas flow rate	10 L/min
Drying gas temperature	365°C

electrospray ionization MS and may 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 detection, the effluent from the desalter 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 25 µM fetuin oligosaccharide standard 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). The assignment of peaks was done by comparative analysis of the acquired chromatograms and existing literature references [13, 14], as

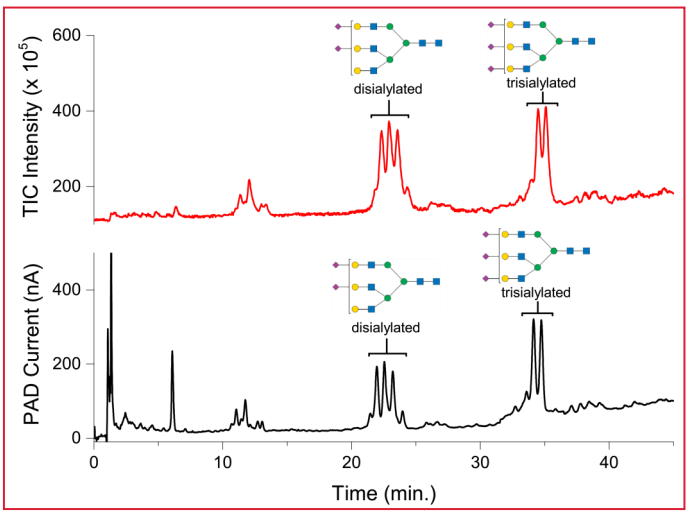


Figure 2. Chromatograms obtained with an 10 µL injection of a 25 µM N-glycans standard containing di– and tri–sialylated oligosaccharides on a SweetSep™ AEX 200 column, 4.0 mm ID × 200 mm. Top: Total Ion Current (TIC) Chromatogram. Bottom: Pulsed Amperometric Detection Chromatogram.

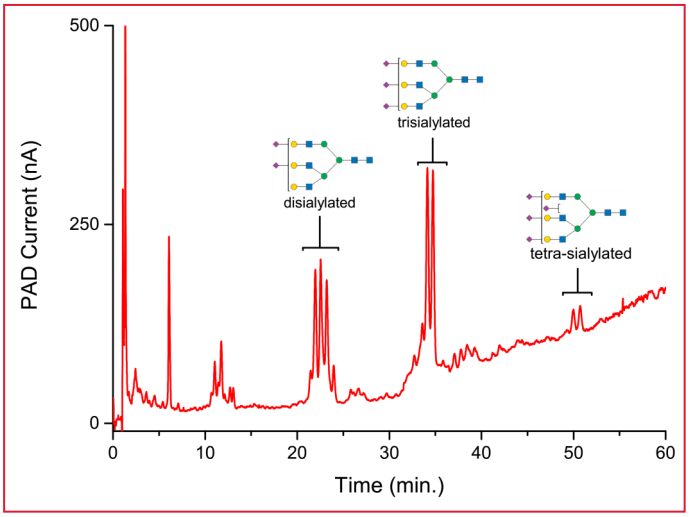


Figure 3. PAD Chromatograms obtained with an 10 µL injection of a 25 µM N-glycans standard showing the separation between the di-, tri-, and tetrasialylated N-glycans.

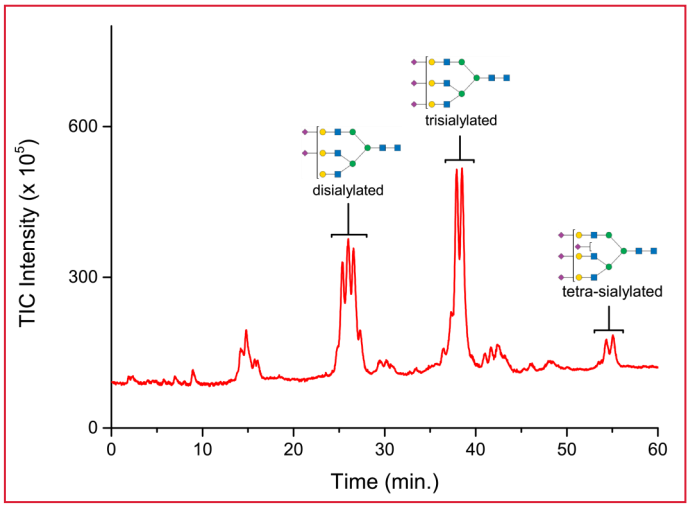
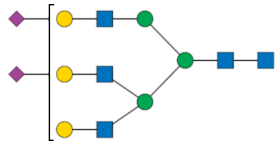


Figure 4. TIC Chromatograms obtained with an 10 µL injection of a 25 µM N-glycans standard on 0.7 mL/min flow rate.



the individual standards were not available. The oligosaccharide standard consists of a mixture of di-, tri-, and tetrasialylated *N*-glycans. Both PAD and TIC signals show consistent data corresponding to the disialylated and trisialylated *N*-glycans. However, tetrasialylated *N*-glycans are only detected in the PAD mode under these conditions (Figure 3). This issue arises from the limited capacity of the desalter, resulting in suppressed ion signals caused by the presence of salts [10].

The presence of salts can be avoided using the pre-desalter flow splitting. The pre-desalter flow splitting is a popular option in several references because desalting is only needed for MS detection [7-9]. However, when implementing pre-desalting flow splitting, it is important to note that the flow rate of the effluent after the desalter is significantly decreased. Consequently, the reduced amount of *N*-glycans going into the MS could lead to a decrease in the signal-to-noise ratio and a potential loss of resolution. Alternatively, salt contamination on MS can be avoided by decreasing the concentration of the salt or the flow rate. Although effective in minimizing salt contamination, this option requires a longer run time to separate *N*-glycans. A TIC chromatogram in Figure 4 demonstrates that *N*-glycans were retained longer when the flow rate is decreased to 0.7 mL/min. However, the detection of tetrasialylated N-glycans is now enabled and in addition, the peak response is higher compared to the higher flow rate configuration.

In conclusion, the presented method shows the potential of parallel PAD and MS detection for intact *N*-glycan analysis. Further, this method can be extended into tandem MS/MS for the identification of *N*-glycans coupled with PAD for the quantification of *N*-glycans.

References

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