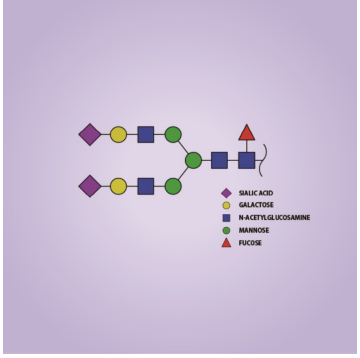
Application Note

Glycans & Glycoproteins



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Glycoproteins
N-glycans
Monosaccharides
Sialic acids

Sialic acids in glycoproteins

- **ALEXYS™ Carbohydrate Analyzer**
- **SweetSep™ AEX20 anion-exchange column**
- **Acid hydrolysis of sialic acids from glycoproteins**
- **Fast and sensitive analysis of Neu5Ac and Neu5Gc**

Summary

Sialic acids are a class of sugars that plays vital roles in biological functions, including brain development, cell—pathogen interactions, and as disease biomarkers such as cardiovascular diseases and cancer [1,2]. Although commonly found in animal tissues, sialic acids are rarely observed in plants. Over 50 types of sialic acids are known, with N-acetylneuraminic acid (Neu5Ac) and N-glycolyneuraminic acid (Neu5Gc) being the most commonly studied [3]. Given their importance in many biological processes, accurate monitoring Neu5Ac and Neu5Gc levels in glycoproteins is crucial.

In this application note, a HPAEC-PAD method is presented for the compositional analysis of Neu5Ac and Neu5Gc in hydrolyzed glycoprotein samples using the new SweetSep AEX20 anion-exchange column in combination with the ALEXYS carbohydrates analyzer. The method enables fast and sensitive analysis of Neu5Ac and Neu5Gc released from glycoproteins. To demonstrate the applicability of the method, three different glycoproteins were analyzed.

Introduction

Sialic acids are a class of sugars with a nine-carbon backbone. Two of the important sialic acids are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). These sialic acids can be found in many biomolecules such as glycoproteins, glycolipid, or glycosphingolipids [3]. They play crucial roles in stabilizing the protein structure and regulating the ion binding activity and the hydrophilicity [3]. In addition, sialic acids can protect molecules or cells from enzymatic degradation [4, 5].

Due to its important functions, sialic acids are often served as disease biomarkers. For example, elevated amount of sialic acids are associated with metastatic cancer cells [2]. Sialic acids are also part of therapeutic glycoprotein, where they contribute to a better solubility and improved biological activity [5, 6]. It is important to note that humans have a high level of Neu5Gc antibodies, as they typically do not produce Neu5Gc. This can cause immune response when a therapeutic glycoprotein with Neu5Gc is administered [1, 3, 4, 7]. Therefore, accurate analysis of sialic acids in glycoproteins are important, both for diagnostic tool in variety of diseases, and for quality control in therapeutic glycoproteins production.

A novel type of anion-exchange column with SweetSep™ AEX20 stationary phase based on 5 µm polymer particles is used for the compositional analysis of sialic acids released from glycoproteins. Typically, sialic acids are released from glycoproteins using either enzymatic method (neuraminidase) or acid hydrolysis method. In this application note, HPAEC-PAD analysis of sialic acids from glycoproteins with two different acid hydrolysis methods is presented. Several parameters including repeatability, linearity, limits of detection, as well as method's precision and accuracy are evaluated in this application note.

Method

The sialic acid analysis was performed using the ALEXYS™ Carbohydrate Analyzer (Figure 1), consisting of the ET210 eluent tray (for N2 blanketing), a P6.1L quaternary LPG pump, AS6.1L autosampler, CT2.1 column thermostat, and the DECADE Elite electrochemical detector. The SenCell™ with Au working electrode and HyREF (Pd/H_2) reference electrode was selected for sensitive detection of the sialic acids.

Separation

Neu5Ac and Neu5Gc are relatively stronger acids compared to carbohydrates, with a pKa of 2.6 and 2.92, respectively [8]. In

Figure 1. ALEXYS Carbohydrate Analyzer.

an alkaline conditions, Neu5Ac and Neu5Gc are negatively charged, and therefore can be separated using HPAEC. Due to the extreme alkaline conditions, only polymeric anion-exchange columns are suitable for separation of sialic acids. The separation of sialic acids was carried out using SweetSep™ AEX20 columns (4 × 200 mm analytical column and 4 × 50 mm precolumn), which are anion-exchange columns containing highly monodisperse 5 µm ethylvinylbenzene-divinylbenzene copolymer (80% crosslinked) coated with functionalized nanoparticles with dual ion exchange sites (quaternary amine + tertiary amine). In addition, a borate ion trap was installed between the pump and the injector.

The gradient profile described in Table 2 was employed for the separation of the sialic acids. Sialic acids are retained stronger compared to neutral carbohydrates, and therefore required a strong modifier for fast elution [9]. The method started with linear gradient elution at 100 mM NaOH + 70 mM NaOAc from t=0 min to 100 mM NaOH + 300 mM NaOAc at t = 7.5 minute. This mobile phase composition was kept until t = 9 minute to elute any late eluting component and to remove carbonate ions (CO_3^{2-}) build up on the column. After the clean-up step the column is equilibrated for 21 minutes to the starting conditions, resulting in a total run time of 30 minutes. The temperature for separation was set at 30°C.

To minimize the introduction of carbonate ions in the mobile phase the eluents were carefully prepared manually using a carbonate-free 50% w/w NaOH solution and electrochemical

Table 1

HPAEC-ECD Conditions

HPLC system	ALEXYS™ Carbohydrate Analyzer
Detector	DECADE™ Elite electrochemical detector
Columns	SweetSep™AEX20, 4 x 200 mm analytical column, 5 µm SweetSep™AEX20, 4 x 50 mm precolumn, 5 µm Borate ion trap, 4 x 50 mm column, 10 µm (Antec Scientific)
Mobile phase (MP)	A: 100 mM NaOH B: 100 mM NaOH + 500 mM NaOAc Eluents blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
Back pressure	about 200 bar
Injection	10 µL
Temperature	30 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF Palladium RE, AST 2
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
I-cell	about 0.2— 0.4 µA
ADF	0.1 Hz
Range	2 µA/V

Table 2

Gradient program

Time (min)	*Mobile phase*	*%A*	*%B*	*Description*
0	100 mM NaOH, 70 mM NaOAc	86	14	Elution & detection
7.5	100 mM NaOH, 300 mM NaOAc	40	60	
7.5—9	100 mM NaOH, 300 mM NaOAc	40	60	Column clean-up and regeneration
9—30	100 mM NaOH, 70 mM NaOAc	86	14	Equilibration, starting conditions

grade sodium acetate salt (all commercially available). The diluent was DI water (resistivity >18 MΩ.cm, TOC <5 ppb), which was sparged with Nitrogen 5.0 (purity 99.999%). During analysis the mobile phase headspace is also blanketed with Nitrogen gas (0.2—0.4 bar N_2 overpressure) using the ET210. The inert gas atmosphere will minimizes the introduction of CO_2 in the mobile phase and the subsequent formation of CO_3^{2-} ions, ensuring reproducible analysis.

Detection

For the pulsed amperometric detection of the analytes, the Antec SenCell™ electrochemical flow cell is used. This flow cell [10] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/ H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform was applied as described in Table 1. The temperature for detection was set to 35°C. The cell current was typically about 0.2—0.4 µA using these PAD settings under the specified conditions. This particular 4-step waveform with a pulse duration of 500 ms has been claimed to have benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [11], resulting in less flow cell maintenance and system down time.

Preparation of standards, reagents and samples

Standards: stock standards of Neu5Ac and Neu5Gc were prepared by dissolving 5 mg of the individual compounds in 10 mL H_2O. This corresponds to the concentration of 1.62 mM of Neu5Ac and 1.54 mM of Neu5Gc. Stock standards under these conditions are approximately stable for more than a month in the freezer at –30°C. A combined stock standards in the concentration of 100 µM of Neu5Ac and 10 µM of Neu5Gc were made by mixing 620 µL of Neu5Ac and 65 µM of Neu5Gc and diluting the mix up to 10 mL with H_2O. Working standards were prepared by serial dilution of the combined stock standards with DI water. The working standards were prepared ranging from 0.1 µM—25 µM for Neu5Ac, and 0.01 µM—2.5 µM for Neu5Gc.

Acids for hydrolysis: for releasing sialic acids from the glycoproteins, two different acids were prepared.
Acetic acid, 4M: in a 100 mL volumetric flask, 22.5 mL of glacial acetic acid was diluted with H_2O up to the total volume of 100 mL.
Trifluoroacetic acid (TFA), 0.2%: in a 100 mL volumetric flask, 200 µL of trifluoroacetic acid (99.5%) was diluted with H_2O up to the total volume of 100 mL.

Samples: Three different glycoproteins were subjected to acid hydrolysis to release its sialic acids content:

- Apo-transferrin from bovine
- Fetuin from fetal bovine serum
- α_1-acid glycoprotein from human plasma

The samples were prepared as follows: stock solutions of the individual glycoprotein were prepared by dissolving 3 mg of

glycoprotein in 1 mL water. The glycoprotein stock solutions were frozen in several aliquots to avoid degradation due to freeze-thaw cycle. For hydrolysis, 35 µL of glycoprotein stock solution was mixed with 65 µL H_2O and 100 µL of acid (4M of acetic acid or 0.2% of TFA), resulting in a total volume of 200 µL. The mixture was shortly vortexed and heated in a water bath at 80°C for 2 hours. The samples were dried in a rotary vacuum concentrator at 30°C for 2.5 hours. Note that this hydrolysis method may not be optimal for all samples. The hydrolysis duration as well as delay time between the hydrolysis and evaporation of the acidic medium may affect the sialic acids amount found due to incomplete release or acidic degradation of the free sialic acids [7, 12-14]. The dried samples were reconstituted with 2 mL of H_2O. The samples were then split into two vials, in which one vial was spiked with a known amounts of Neu5Ac and Neu5Gc as described in the Table 3. The samples were ready for injection and analyzed within 24 hours after reconstitution.

Table 3

Added amounts of Neu5Ac and Neu5Gc standards for spike recovery experiment

Protein	Neu5Ac *Added (µM)*	*Neu5Gc Added (µM)*
Apo-transferrin	1	1
Fetuin	10	0.25
α1-acid glycoprotein	10	1

Results

A chromatogram obtained with the 10 µL injection of 10 µM of Neu5Ac and 1 µM of Neu5Gc is depicted in Figure 2. The chromatogram shows baseline separation of Neu5Ac and Neu5Gc. Under this condition Neu5Ac eluted at 4 minute, and Neu5Gc eluted at 6.9 minute. Note, that the use of acetate gradient results in a slight baseline drift.

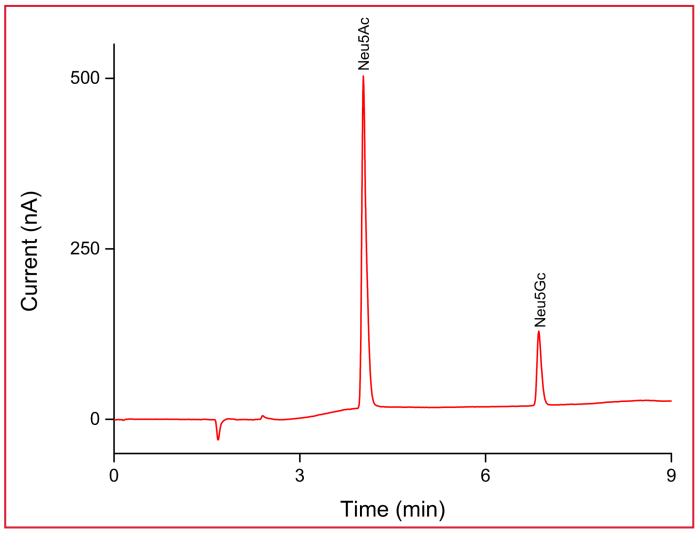

Figure 2. The chromatogram obtained from a 10 µL injection of 10 µM Neu5Ac and 1 µM of Neu5Gc in DI water.

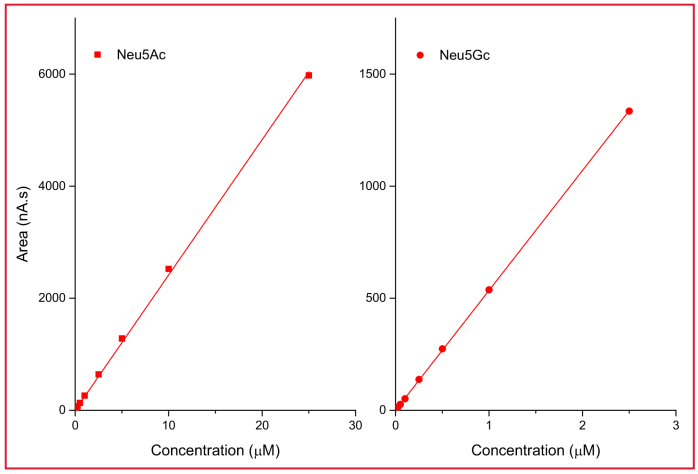

Figure 3. Calibration curves obtained with 10 µL injections of Neu5Ac (0.1—25 µM) and Neu5Gc (0.01—2.5 µM) standards in DI water.

Table 4

Repeatability of 10 µL injections of Neu5Ac and Neu5Gc standards in DI water (n = 8)

	RSDs (%) 10 µM Neu5Ac and 1 µM		*RSDs (%) 1 µM Neu5Ac and 0.1 µM*	
Compound	t_R	*Area*	t_R	*Area*
Neu5Ac	0.10	0.09	0.09	0.49
Neu5Gc	0.06	0.21	0.06	0.78

Linearity and repeatability

The linearity was evaluated for the two sialic acid standards in the concentration range of 0.1—25 µM for Neu5Ac and 0.01—2.5 µM for Neu5Gc. Excellent linearity was achieved with correlation coefficients based on the peak area better than 0.999 for both analytes. Calibration curves for Neu5Ac and Neu5Gc are depicted in Figure 3.

The relative standard deviations (RSDs) of the retention time and peak area were determined by repetitive injections of the 10 µM Neu5Ac and 1 µM Neu5Gc standard mix, as well as 1 µM Neu5Ac and 0.1 µM Neu5Gc standard mix in DI water. The retention times were stable as shown in Table 4, with RSD values ≤ 0.11% for both Neu5Ac and Neu5Gc in two different concentrations. The RSDs for peak areas were < 0.3% for the 10 µM Neu5Ac and 1 µM Neu5Gc standard mix and < 0.8% for the 1 µM Neu5Ac and 0.1 µM Neu5Gc standard mix. These data demonstrate good reproducibility for the analysis of sialic acids using the presented method.

Limit of detection (LOD) and limit of quantification (LOQ)

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for both sialic acids are shown in Table 5. The LODs were

calculated as the analyte response corresponding to 3× the ASTM noise (average peak-to-peak baseline noise of 10 segments of 0.5 min). The noise was calculated based on a 5-minute section of the baseline from t = 25 minutes to t = 30 minutes. The average response based on 8 replicate injections obtained with the 1 µM Neu5Ac and 0.1 µM Neu5Gc standard mix was used to calculate the LOD and LOQ. The LOQs were calculated in a similar way to LODs, with 10× S/N ratio instead of 3×. The calculated LODs of Neu5Ac (17.0 nM) and Neu5Gc (7.8 nM) demonstrate the excellent sensitivity of the method.

Table 5

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

	Limit of detection		*Limit of quantification*	
Compound	*nM*	*ppb*	*nM*	*ppb*
Neu5Ac	17.0	5.3	56.7	17.5
Neu5Gc	7.8	2.5	25.8	8.4

Sample analysis

Three glycoprotein samples were hydrolyzed using two different acids. The 2 M acetic acid hydrolysis is a commonly used method to release sialic acids from glycoprotein. It is important to note that in an earlier attempt (data not shown), a small amount of acetic acid left after hydrolysis procedure is detrimental to the released sialic acid, causing appearance of another unknown peak next to the corresponding sialic acid peaks. Hydrolysis using trifluoroacetic acid is a common practice to hydrolyze glycan into its monosaccharides building blocks. However, it is a lesser-known method to release sialic acids from glycoprotein due to its stronger acidic properties [15]. In this application note we described the use of a very low concentration of trifluoroacetic acid as the alternative of using acetic acid, which is relatively more difficult to remove due to its higher boiling point.

The chromatograms obtained from 10 µL injections of the glycoprotein hydrolysates are shown in Figure 4—9 on the next page. Neu5Ac and Neu5Ac are sufficiently separated from the small, early-eluting peaks in all samples. Both Neu5Ac and Neu5Gc were found in the apo-transferrin and fetuin hydrolysates. As expected, Neu5Gc was not detected in the α_1-acid glycoprotein sample, because the sample is originated from the human plasma. The amount found for Neu5Ac and Neu5Gc from the acetic acid hydrolysates are similar to the corresponding TFA hydrolysates, indicating that a small amount of TFA can be used to release sialic acids from glycoprotein without significant degradation.

The average amount of Neu5Ac and Neu5Gc for all samples are shown in the Table 6. The samples were measured in triplicate and the peak area RSDs for Neu5Ac in the samples range from 0.08% to 1.18%, while for Neu5Gc the peak area RSDs range from 0.34% to 1.36%. It shows that precise measurements of the sialic acids can be achieved with this method. The accuracy of the sialic acid released from glycoprotein cannot be easily evaluated due to the possibility of less optimal hydrolysis. However, comparison with the published value shows that the amount of sialic acids are in the similar range [7]. The method accuracy was assessed based on the sample recovery. The sample recovery can be calculated based on the average amount of the analytes in the sample and spiked sample and compared to the added amount to the spiked sample.

$$\text{Recovery (\%)} = 100\% * \frac{\text{Amount}_{\text{spiked sample}} - \text{Amount}_{\text{sample}}}{\text{Amount}_{\text{standard}}}$$

The sample recoveries are also listed in the Table 6. The sample recovery found for all samples ranged between 90% - 110%.

Table 6

Average sialic acid content in three glycoprotein acid hydrolysates (n = 3)

Protein	*Neu5Ac*				*Neu5Gc*			
	Acetic acid hydrolysates		*TFA hydrolysates*		*Acetic acid hydrolysates*		*TFA hydrolysates*	
	Amount (µM)	*Recovery (%)*	*Amount (µM)*	*Recovery (%)*	*Amount (µM)*	*Recovery (%)*	*Amount (µM)*	*Recovery (%)*
Apo-transferrin	0.62	110.3	0.62	108.5	0.72	102.7	0.74	101.2
Fetuin	9.04	101.9	10.29	99.9	0.13	102.9	0.15	101.3
α1-acid glycoprotein	20.53	96.2	20.58	94.3	n.d.	103.5	n.d.	100.8

*n.d. = not detected

Acetic acid hydrolysates

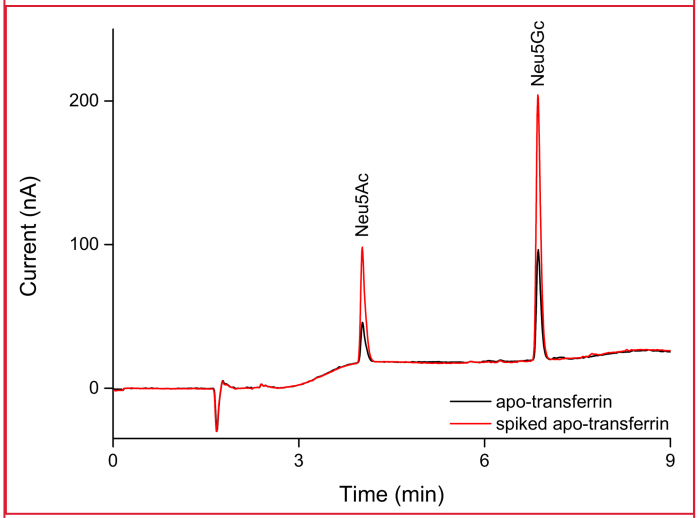

Figure 4. The chromatogram of a 10 µL injection of acetic acid hydrolysates of apo-transferrin sample (black line) and spiked sample (red line).

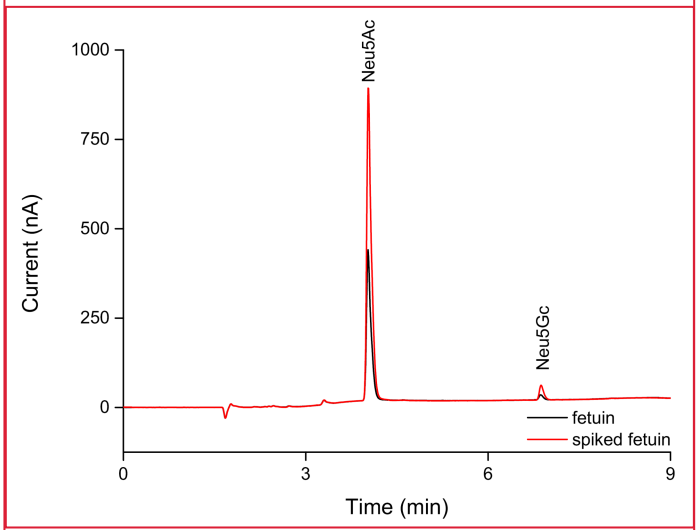

Figure 5. The chromatogram of a 10 µL injection of acetic acid hydrolysates of fetuin sample (black line) and spiked sample (red line).

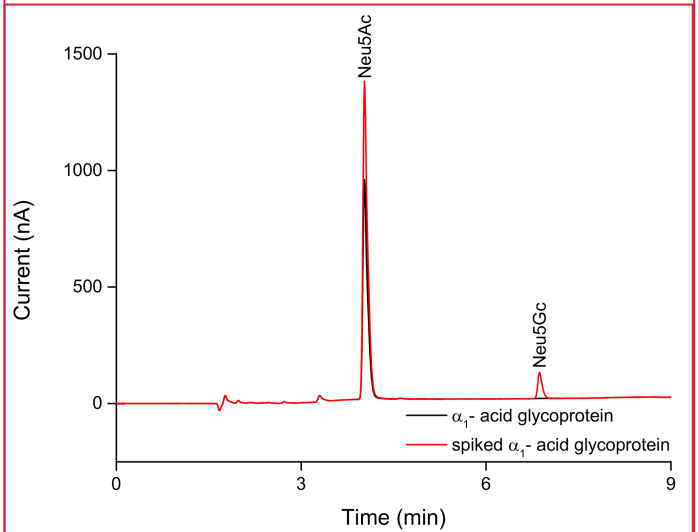

Figure 6. The chromatogram of a 10 µL injection of acetic acid hydrolysates of α_1-acid glycoprotein sample (black line) and spiked sample (red line).

Trifluoroacetic acid (TFA) hydrolysates

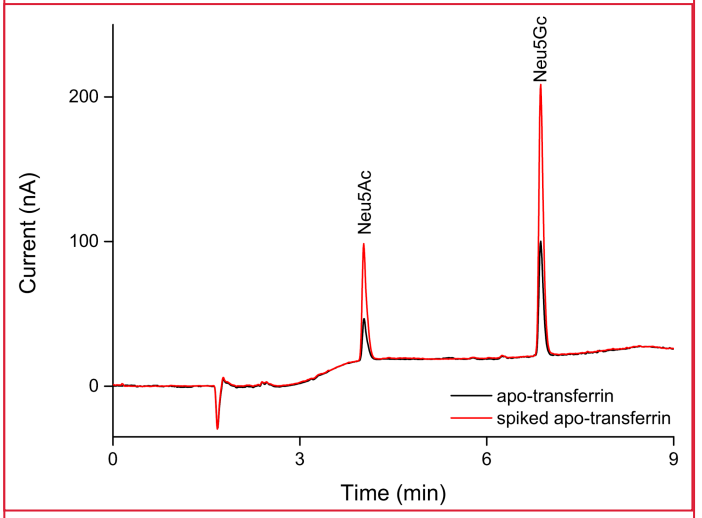

Figure 7. The chromatogram of a 10 µL injection of TFA hydrolysates of apo-transferrin sample (black line) and spiked sample (red line).

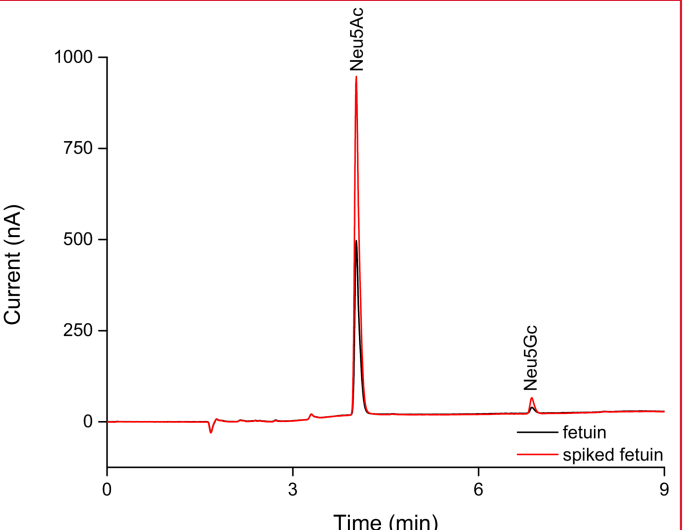

Figure 8. The chromatogram of a 10 µL injection of TFA hydrolysates of fetuin sample (black line) and spiked sample (red line).

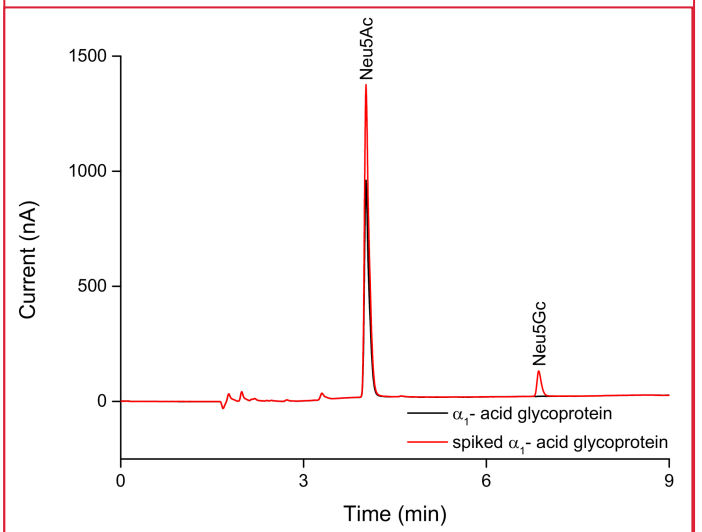

Figure 9. The chromatogram of a 10 µL injection of TFA hydrolysates of α_1-acid glycoprotein sample (black line) and spiked sample (red line).

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Conclusion

The ALEXYS™ Carbohydrate Analyzer in combination with the new SweetSep™ AEX20 allows high-resolution separation and sensitive detection of sialic acids released from glycoproteins. Neu5Ac and Neu5Gc are well separated using the presented method and eluted within 7 minutes. The method demonstrates excellent linearity, good repeatability and high detection sensitivity, as evidenced by the low Limit of Detection (LOD) for both sialic acids. Good method accuracy and precision was demonstrated by successful analysis of three different glycoproteins using two different hydrolysis methods. Overall, the presented method enables reliable sialic acid analysis from glycoproteins.

Ordering information

ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Columns	
260.0020	SweetSep™ AEX20, 4 x 200 mm column, 5 µm
260.0025	SweetSep™ AEX20, 4 x 50 mm precolumn, 5 µm
260.0030	Borate ion trap, 4 x 50 mm column, 10 µm
260.0100*	Pre-column filter PEEK, 0.5 µm
Software#	
195.0035	Clarity CDS single instr. incl LC, AS module

*) In case samples might contain particulate matter it is advised to use a pre-column filter.

#) Antec ECD drivers are available for Chromeleon CDS, OpenLAB CDS and Empower CDS. The ALEXYS Carbohydrates Analyzer (full system) can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system and DECADE Elite detector. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this application note are subject to change without further notice.

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