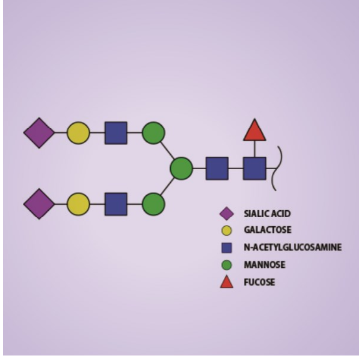
Application Note
Glycans & Glycoproteins



Monosaccharides in Glycoproteins

The finest HPAEC-PAD Applications for Carbohydrate analysis

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Artificial Sweeteners
Sugar alcohols

Glycans & Glycoproteins
Monosaccharides in glycoproteins
Analysis of *N*-glycans

- **SweetSep™ AEX20 high-resolution column**
- **Novel efficient amino acid trap, 5 µm**
- **Sensitive and selective analysis within 10 min**
- **Fetuin from fetal bovine serum and alpha-1-acid glycoprotein**

Summary

A large number of pharmaceutical products contain glycoproteins. Glycoproteins are proteins containing oligosaccharide chains (glycans) covalently attached to the polypeptide sidechain by the glycosylation process. The extent of glycosylation significantly influences the stability, activity, and pharmacodynamics of the glycoproteins. Therefore, monitoring the glycosylation by determining monosaccharide composition is one of the most important quality control methods for pharmaceutical industries. An attractive method for compositional analysis of released mono-saccharides from hydrolyzed glycoproteins is based on separation by High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). HPAEC-PAD combines efficient separation with direct sensitive detection without derivatization.

In this application note, the analysis of monosaccharides in glycoproteins is demonstrated using the ALEXYS carbohydrate analyzer in combination with the new AEX20 anion-exchange column based on highly monodisperse 5 µm particles, which enables fast, high-resolution separation of the monosaccharides of interest. The AEX20 is used in combination with the novel amino acid trap column to eliminate interference of amino acids, like lysine and glutamine, during analysis.

Introduction

Carbohydrates are the most abundant biomolecules in nature and play an important role in many physiological processes (metabolism, storage of energy, structure, etc.) and nutrition. The analysis of carbohydrates is of interest to the food industry and also many fields in life sciences such as glycomics [1]. Glycomics covers a range of scientific disciplines that are applied to study the composition, structure, and function of carbohydrates in biological systems. High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) can be used as a tool for the compositional analysis of monosaccharides in glycoproteins. It allows the quantification of the amount of individual monosaccharides and screening for compositional changes in glycosylation in proteins with pico- and femtomol sensitivity [2-4].

In this application note, the compositional analysis of monosaccharides from glycoproteins is based on an acid hydrolysis sample prep step prior to HPAEC-PAD analysis. Monosaccharides from glycoproteins can be released using prolonged heating in acidic conditions. The most commonly used acids are hydrochloric acid (HCl) and trifluoroacetic acid (TFA). It is recommended to use both acids for digestion due to their different hydrolysis activity. TFA is optimal for hydrolyzing most neutral sugars (fucose, galactose, glucose, and mannose), while HCl is more optimal for the hydrolysis of amino sugars (galactosamine and glucosamine) [5, 6].

In this application note, we demonstrate the performance of the new SweetSep™ AEX20 anion-exchange column for the fast high-resolution separation of the 6 important monosaccharides found in glycoprotein hydrolysates using HPAEC-PAD. The novel Antec amino acid trap column is used as a precolumn to retain interfering amino acids and peptides generated during the hydrolysis step. To demonstrate the applicability of the method, monosaccharides were released from two different proteins and analyzed using the SweetSep™ AEX20 as an example.

Method

The analysis was performed using the ALEXYS™ Carbohydrate Analyzer (Figure 1) equipped with 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 sugars.

Separation

Under alkaline conditions (pH > 12) carbohydrates can be separated using HPAEC. Carbohydrates are weak acids with pKa values ranging between 12 and 14. At high pH, they will be either completely or partially ionized depending on their pKa value. Due to the extreme alkaline conditions only polymeric anion-exchange columns are suitable for carbohydrate separation. The retention time of carbohydrates is inversely correlated with the pKa value and increases significantly with molecular weight.

Figure 1. ALEXYS Carbohydrate Analyzer consisting of the ET210 eluent tray (for N_2 blanketing), a P6.1L quaternary LPG pump, AS6.1L autosampler, CT2.1 column thermostat, and the DECADE Elite electrochemical detector.

Table 1

Conditions

LC system	ALEXYS Carbohydrate Analyzer - quaternary LPG (Antec Scientific)
Columns	SweetSep™ AEX20, 4 × 200 mm column, 5 µm Amino acid trap, 4 × 50 mm column, 5 µm Borate ion trap, 4 x 50 mm column, 10 µm (all columns Antec Scientific)
Mobile phase	MP A: H_2O MP B: 100 mM NaOH MP C: 200 mM NaOH All mobile phases were sparged and blanketed under Nitrogen 5.0
Flow rate	0.7 mL/min
System backpressure	About 265 bar, column backpressure about 200 bar
Injection	10 µL
Temperature	30 °C for separation, 35°C for detection
Flow cell	SenCell with 2 mm Au WE and HyREF, 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 0.3 µA
ADF	0.5 Hz
Range	5 µA/V for standard and sample measurements, 500 nA/V for LOD & LOQ determination.

Table 2

Step-gradient program

Time (min)	*Mobile phase*	*Description*
0 - 12	12 mM NaOH	Isocratic elution and detection
12 - 22	200 mM NaOH	Column cleanup
22 - 52	12 mM NaOH	Equilibration

AEX20 analytical column

Antec Scientific has introduced an innovative new stationary phase, AEX20. It consists of a highly monodisperse 5 µm ethylvinylbenzene-divinylbenzene copolymer (80% crosslinked) coated with functionalized nanoparticles with dual ion exchange sites (quaternary amine + tertiary amine). The resin is optimized for the separation of monosaccharides and due to the properties of the exchange groups the separation is more tolerant towards slight variations in mobile phase composition.

The 4 x 200 mm ID AEX20 analytical column without guard column was used for this evaluation. A guard column is not necessary, because of the use of an amino acid trap. The trap column will function as a guard to prevent the accumulation of contaminations and particulate matter on the analytical column.

Amino acid trap column

The novel amino acid trap column (4 x 50 mm) is also based on a monodisperse 5 µm polymeric resin which efficiently retain interfering amino acids and peptides that may be present in glycoprotein hydrolysates. The small particle size and distribution of the trap resin will also assure better peak efficiencies and resolution of the sugars of interest on the analytical column.

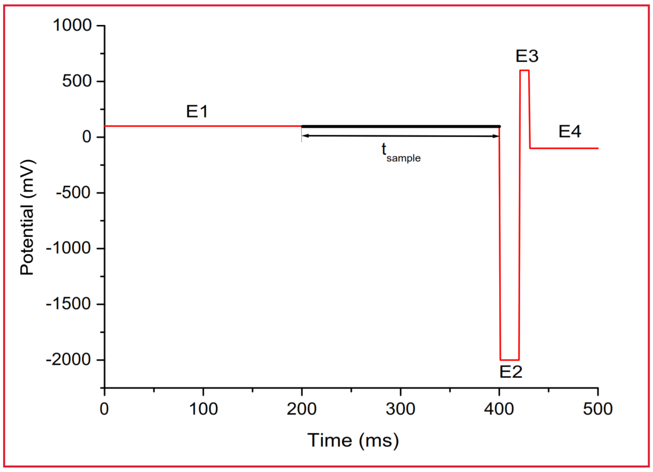

Figure 2. 4-step PAD potential waveform for the detection monosaccharides and other carbohydrates.

Borate ion trap column

In carbohydrate analysis, the peak shape of certain sugars, such as mannose, sugar alcohols and fructose, are deteriorated when traces of borate are present in the mobile phase. A borate ion trap column (4 x 50 mm) was installed in the solvent line between the pump and autosampler as a precaution to eliminate borate ions from the mobile phase.

The analysis of the monosaccharides is based on a step-gradient, see Table 2. At a concentration of 12 mM NaOH, carbonate ions (CO_3^{2-}) present in the mobile phase will bind strongly to the active sites of the stationary phase resulting in a loss of retention and column efficiency. A column clean-up / regeneration step after isocratic elution with 200 mM NaOH is therefore necessary to remove the bound carbonate ions and strongly retained components, like the amino acids and peptides trapped on the amino acid trap column. This regeneration step ensures reproducible retention behavior for each run.

To minimize the introduction of carbonate ions in the mobile phase the eluents were prepared manually using a carbonate-free 50% w/w NaOH solution (commercially available). The diluent was deionized water (resistivity > 18 MΩ.cm) which was sonicated and sparged with nitrogen 5.0 prior to use. The mobile phase should be prepared in plastic bottles instead of glass. NaOH is a strong etching agent and will react with the inner glass wall resulting in the release of silicates and borates. The bottles with mobile phase and column clean-up solution were blanketed with nitrogen (0.2 bar overpressure) during the analysis to minimize the build-up of carbonate ions in the mobile phase and to ensure a reproducible analysis.

Detection

For the pulsed amperometric detection of the monosaccharides the SenCell is used [7]. This electrochemical flow cell has an Au working electrode (WE), HyREF (Pd/H_2) reference electrode (RE), and a stainless steel auxiliary electrode (AE). A 4-step potential waveform is applied as shown in Figure 2.

The temperature for separation was set to 30°C while detection is performed at 35°C The cell current was typically about 0.3 µA with these PAD settings under the specified conditions. This particular 4-step waveform with a pulse duration of 500 ms has several benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [8].

Preparation of standards and samples

Standards: 10 mM stock standards of the 6 individual sugars commonly found in glycoproteins (fucose, galactosamine, glucosamine, galactose, glucose, and mannose) and 2-deoxy-D-glucose (a glycosylation inhibitor) were prepared in a 95/5 (v/v%) water/acetonitrile. The 5% acetonitrile was added to suppress degradation and bacterial or fungal growth. Stock standards under these conditions are stable for more than a month in the fridge at 4°C. Working standards mix in the concentration range of 100 nM—100 µM were prepared by mixing and serial dilution of the stock standards with DI water.

Samples: monosaccharides from glycoproteins were released by acid hydrolysis using HCl and TFA. The detailed procedure for the acid hydrolysis has been described elsewhere. Two glycoproteins that were subjected to acid hydrolysis are fetuin from fetal bovine serum (Sigma Aldrich) and alpha-1-acid glycoprotein from human plasma (Sigma Aldrich).

Results

A chromatogram of a 10 µL injection of a 10 µM standard mix of 7 monosaccharides in water is shown in Figure 3.

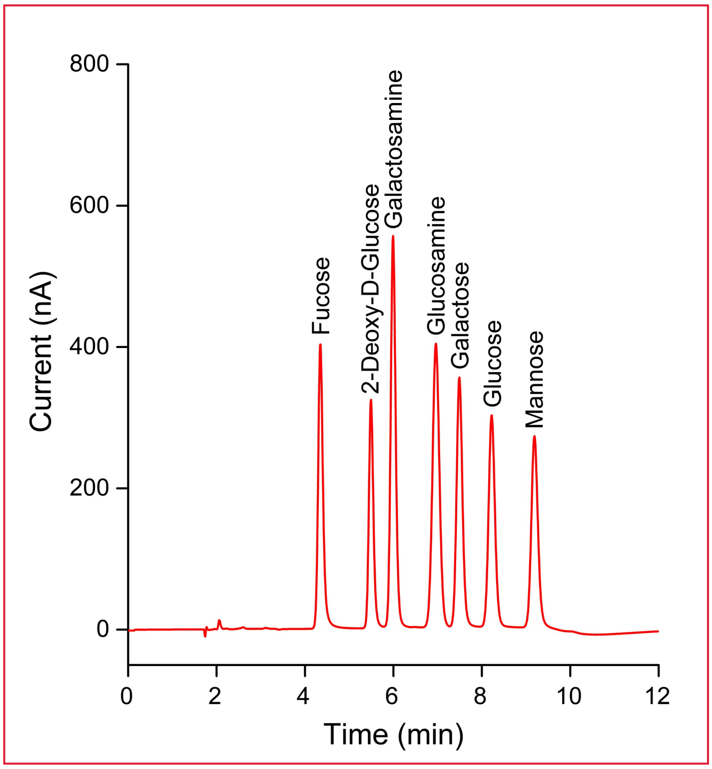

Figure 3. Chromatogram of a 10 µL injection of a 10 µM standard mix of 7 monosaccharides in water: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and (7) Mannose.

This standard mix represents a group of monosaccharides (hexoses and hexosamines) commonly found in glycoproteins. It is evident from Figure 3 that all compounds elute within 10 minutes. The monosaccharides are baseline separated (R ≥ 1.5) with peak efficiencies in the range of 7000 – 17000 theoretical plates. Several column parameters of the chromatogram in Figure 2 are shown in Table 3.

Table 3

Peak table, 10 µL injection of a 10 µM standard mix of 7 monosaccharides in water

Compound	*t_R (min)*	*k′ (-)*	*N (-)*	*R (-)*	*Tailing (-)*
Fucose	4.35	1.5	7702	-	1.21
2-Deoxy-D-Glucose	5.49	2.2	12275	5.8	1.17
Galactosamine	5.99	2.5	11187	2.4	1.11
Glucosamine	6.96	3.1	8759	3.7	1.06
Galactose	7.49	3.4	13819	1.9	1.11
Glucose	8.23	3.8	13492	2.7	1.06
Mannose	9.19	4.4	16850	3.4	1.13

Linearity, repeatability, and LOD

The linearity was investigated in the concentration range of 0.1 - 100 µmol/L. In this concentration range the linearity is excellent and correlation coefficients for peak area were better than 0.999 for all 6 monosaccharides commonly found in the glycoproteins. For 2-deoxy-D-glucose, the correlation coefficient is 0.997.

Table 4

Limit of Detection (LOD)

Compound	*LOD (nmol/L)*
Fucose	13.9
2-Deoxy-D-Glucose	16.6
Galactosamine	10.7
Glucosamine	15.0
Galactose	16.4
Glucose	19.3
Mannose	19.7

The Limit of Detection (LOD) for all monosaccharides is shown in Table 4. The LODs were calculated as the analyte response corresponding to 3x the ASTM noise (average peak-to-peak baseline noise of 10 segments of 0.5 min). The responses of a chromatogram obtained with a 100 nM standard mix with a 500 nA/V range were used to calculate the LOD. Concentration detection limits of the monosaccharides were in the range of

10 – 20 nmol/L, which corresponds to 0.15 – 0.25 pmol on-column.

The relative standard deviation (RSD) of the retention time, peak area, and peak height were determined for 10 replicate injections of two different concentrations of monosaccharides standard mix in water. The results are shown in Table 5. RSDs for retention time were < 0.3%. For the peak areas, the RSDs were < 0.5% for all monosaccharides in both the 10 µM and 1 µM standard. These data demonstrate that with this method reproducible analysis of monosaccharides can be achieved.

Table 5

Repeatability of 10 µL injections of a 10 and 1 µM standard mix in water (n=10)

Compound	*RSDs (%) 10 µmol/L*			*RSD(%) 1 µmol/L*		
	t_R	*Area*	*Height*	*t_R*	*Area*	*Height*
Fucose	0.25	0.19	0.26	0.30	0.70	0.28
2-deoxy-D-glucose	0.18	0.35	0.37	0.26	0.35	0.48
Galactosamine	0.19	0.16	0.12	0.27	0.40	0.23
Glucosamine	0.16	0.10	0.29	0.25	0.52	0.33
Galactose	0.16	0.23	0.24	0.22	0.62	0.41
Glucose	0.17	0.23	0.29	0.21	0.60	0.32
Mannose	0.15	0.28	0.39	0.22	0.78	0.47

Retention of Amino Acids

Amino acids present in glycoproteins can be detected using HPAEC-PAD. If the concentration of amino acids, in particular lysine, is high enough compared to that of the released monosaccharides, they will interfere with the monosaccharide quantification because of coelution. Moreover, amino acids are less efficiently removed from the Au electrode surface due to the suboptimal potential waveform applied for monosaccharide detection, which might lead to fouling and loss of response. To eliminate the interference of amino acids and to assure optimal performance of the monosaccharide analysis using HPAEC-PAD the amino acid trap column was used as a precolumn. The performance of the amino acid trap is demonstrated in figure 4. A mix of 6 monosaccharides and 4 amino acid standards was injected onto the SweetSep™ AEX20 column with and without trap column. Peak responses of

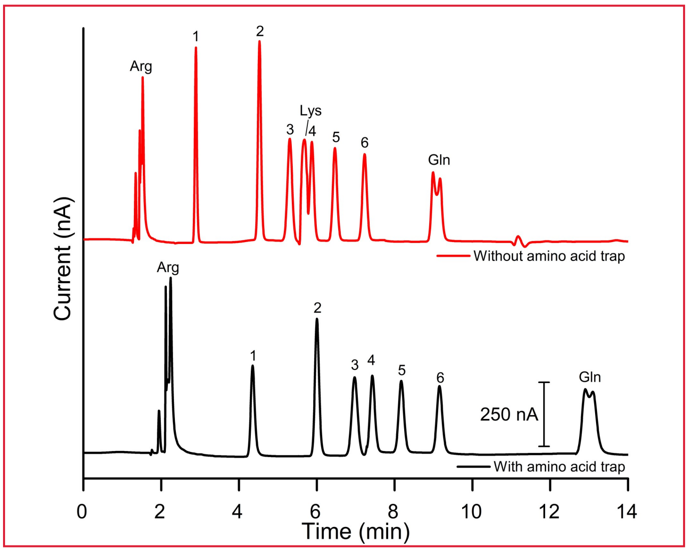

Figure 4. Overlay chromatograms of a 10 µL injection of 10 µM monosaccharide standard mix + 4 amino acids with amino acid trap (black trace) and without amino acid trap (red trace). Peak labels: (1) L-Fucose, (2) Galactosamine, (3) Glucosamine, (4) Galactose, (5) Glucose, (6) Mannose, Arginine (Arg), Lysine (Lys), and Glutamine (Gln). Valine is not shown and elutes during the column-cleaning step.

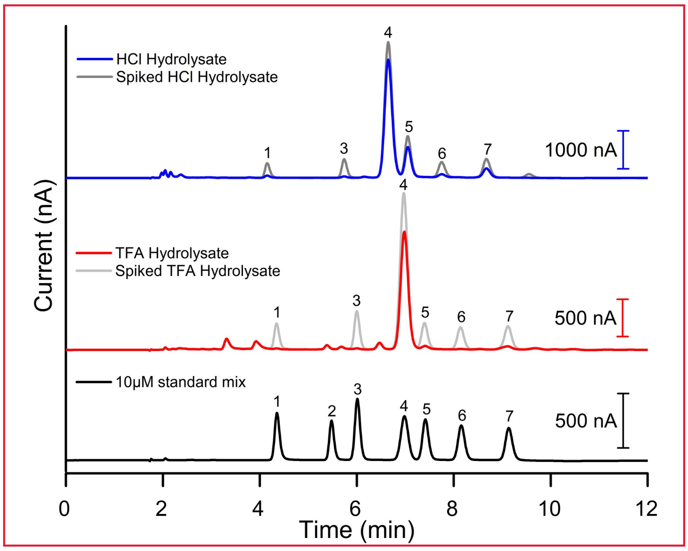

Figure 5. Overlay chromatograms of a 10 µL injection of: a 10 µM standard mix (black trace), TFA hydrolysate of fetuin equivalent to 2 µg protein (red trace), and HCl hydrolysate of fetuin equivalent to 2 µg protein (blue trace). The spiked samples were depicted as grey traces in the background. Peak labels: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and (7) Mannose.

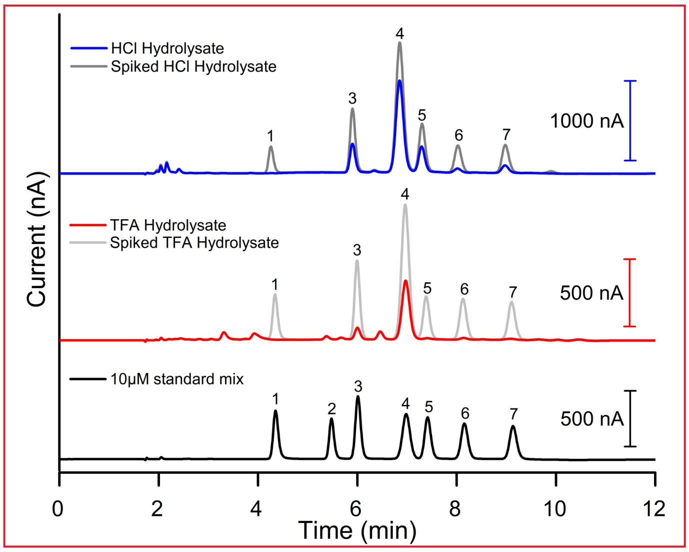

Figure 6. Overlay chromatograms of a 10 µL injection of: a 10 µM standard mix (black trace), TFA hydrolysate of AGP equivalent to 2 µg protein (red trace), and HCl hydrolysate of AGP equivalent to 2 µg protein (blue trace). Peak labels: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and (7) Mannose.

amino acids using the 4-step pulse, are significantly reduced compared to those of the monosaccharides. Therefore, to achieve comparable signals, the concentration of amino acids in the standard mix was intentionally made 100 times higher (1 mM) than that of the monosaccharides (10 µM). Without an amino acid trap in the system lysine is coeluting with galactose under these elution conditions.

It is evident that with amino trap column, the problem of coelution of amino acids is effectively eliminated. Note that with amino acid trap installed the retention times of the monosaccharides are shifted about 1.6 min.

Glycoprotein sample analysis

To demonstrate the applicability of the method, two glycoproteins were digested using TFA to release neutral sugars and using HCl to release amino sugars.

Table 6

Estimated monosaccharides contents in fetuin HCl hydrolysate (2 µg fetuin) and sample recovery

Monosaccharides	*Concentration (µM)*		*Recovery (%)*
	Calibration curves	*Standard addition*	
Fucose	0.04	0.04	96.7
Galactosamine	6.78	6.94	90.0
Glucosamine	30.38	32.06	86.7
Galactose	9.29	8.90	98.0
Glucose	1.65	1.57	100.0
Mannose	3.37	3.22	97.1

Table 7

Estimated monosaccharides contents in fetuin TFA hydrolysate (2 µg fetuin) and sample recovery

Monosaccharides	*Concentration (µM)*		*Recovery (%)*
	Calibration curves	*Standard addition*	
Fucose	0.00	0.00	98.6
Galactosamine	1.61	1.45	102.9
Glucosamine	11.61	10.59	102.4
Galactose	0.32	0.29	103.9
Glucose	0.41	0.39	102.0
Mannose	0.39	0.35	102.8

The samples were spiked with the 6 mono-saccharides commonly found in the glycoprotein to ensure peak identification. The chromatograms of the fetuin and AGP samples are shown in Figure 5 and Figure 6.

Monosaccharide contents in the fetuin sample were estimated using two different methods:

- Calibration curve based on standards (0.1 µM—100 µM)
- Standard addition

The estimation of monosaccharide contents using the standard addition method was based on a single point calibration by spiking the sample with a known amount of standards containing fucose, galactosamine, glucosamine, galactose, glucose, and mannose. The spike concentration was 10 µM for glucosamine and 5 µM for fucose, galactosamine, galactose, glucose, and mannose in the final sample. The estimated monosaccharide contents in the fetuin sample are shown in Table 6 and Table 7.

The method accuracy can be assessed using the standard addition method by calculating the sample recovery based on the responses of the analytes in the sample, the spiked sample, and the standards corresponding to the final spike concentration.

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

As shown in Table 6 and Table 7, amino sugars (glucosamine and galactosamine) are the most dominant monosaccharides present in both HCl and TFA hydrolysate of fetuin. The other 4 monosaccharides are also present, although the estimated quantities are significantly smaller than the amino sugars. Excellent recoveries were obtained for all sugars ranging between 90—104 % [10].

References

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Conclusion

The presented method using an ALEXYS carbohydrates analyzer in combination with the SweetSep™ AEX20 anion exchange column provides a reliable solution for the compositional analysis of glycoprotein hydrolysates using HPAEC-PAD . The use of an amino acid trap precolumn effectively eliminates the interference of amino acids which might be generated during the hydrolysis of the glycoprotein. The monosaccharide peaks were well resolved and baseline separation was achieved for all sugars in both TFA as well as HCl hydrolyzed samples. The method demonstrates excellent linearity, reproducibility and sensitivity.

Ordering information

Detector only	
176.0035B	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
ALEXYS carbohydrate analyzer	
180.0057W	ALEXYS Carbohydrate Analyzer - quaternary LPG
186.ATC00	CT 2.1 column thermostat
116.4321	SenCell 2 mm Au HyREF
Columns	
260.0020	SweetSep™ AEX20, 4 × 200 mm column, 5 µm
260.0040	Amino acid trap, 4 × 50 mm column, 5 µm
260.0030	Borate ion trap, 4 × 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.

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