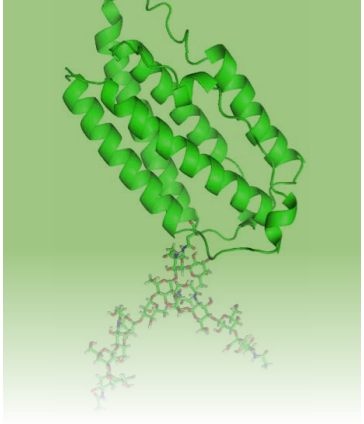
Application Note

Clinical/Pharma/ Food/Life science



Monosaccharides

The most reliable LC-EC applications for biotech analysis ever formulated

Aminoglycosides
- Amikacin
- Framycetin Sulphate
- Gentamicin Sulphate
- Kanamycin Sulphate
- Lincomycin
- Neomycin
- Spectinomycin
- Tobramycin

Bisphenol A

Catechins

Flavonoids and phenols

Phenols

Antioxidants

Polyphenols
- Resveratrol
- Epicatechin
- Quercetin
- other polyphenols

Carbohydrates

- **Mono- and disaccharides in food & life sciences**
- **ALEXYS Carbohydrate Analyzer with AEC**
- **Pulsed Amperometric Detection (PAD)**
- **Flow cell with Au working electrode**
- **Sensitive & Selective analysis**

Introduction

Carbohydrates (also called saccharides) are the most abundant biomolecules in nature and play an important role in many physiological processes (metabolism, storage of energy, structure etc.) and nutrition.Complex carbohydrates (oligo- and polysaccharides) are composed of monosaccharides that are covalently linked by glycosidic bonds, either in the α or β form. Due to the presence of hydroxyl groups which can be oxidized, carbohydrates can be detected using pulsed amperometric detection with pico- and femtomol sensitivity [2-4]. The analysis of carbohydrates is of interest to the food industry but also many fields in life sciences. One important field is glycomics [1]. Glycomics covers a range of scientific disciplines that are applied to study the composition, structure and function of carbohydrates in biologic 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.

Summary

In this publication the analysis of monosaccharides and other carbohydrates is demonstrated using an ALEXYS LC-EC analyzer based on the new DECADE Elite electrochemical detector. The method is based on separation by HPAEC in combination with PAD using a 4-step potential waveform.

Figure 1: ALEXYS Carbohydrate analyzer for isocratic HPAEC (mono- and disaccharides).

Method

Separation

Under alkaline conditions (pH > 12) carbohydrates can be separated by means of 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 pKa value and increases significantly with molecular weight. The elution order of carbohydrates on such anion-exchange columns is usually as follows: sugar alcohols elute first, followed by mono-, di-, tri-, and higher oligosaccharides.

The CarboPac PA20 (3 x 150 mm) Anion-Exchange column with a guard column was chosen for the method evaluation. In case of samples containing amino acids or small peptides, like in glycoproteins, an additional AminoTrap column (3 x 30 mm) must be installed between the injector and the guard column. The use of an Aminotrap column will affect the peak performance (slight increase in retention time and peak width). All chromatograms are recorded without AminoTrap column unless otherwise stated.

Table 1

LC-EC Conditions	
HPLC	ALEXYS LC-EC Analyzer with low-pressure mobile phase selector
Column	CarboPac PA20 150 x 3 mm ID analytical column + CarboPac PA20 30 x 3 mm ID guard column.
Mobile phase	10 mM sodium hydroxide (analysis), 200 mM sodium hydroxide (column regeneration).
Flow rate	0.5 mL/min
Vinjection	10 µL (Full loop)
Temperature	30°C for separation & detection
Flow cell	VT-03™ with Au WE, stainless steel AE and HyREF™ (Pd/H_2) RE, spacer 50 µm
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 0.5 µA
ADF	0.5 Hz
Range	1 or 2 µA/V

The analysis is based on a step-gradient, see table 2. At a concentration of 10 mM NaOH, carbonate ions (C032-) 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 other contaminants like amino acids/peptides. This regeneration step assures reproducible retention behavior for each run. The LC-EC system was equipped with only one pump and a Vici electrically-actuated low pressure (LP) valve in the pump LP suction line to switch between mobile phase and the solution for column clean-up.

Table 2

Step-gradient program		
Time (min)	*Mobile phase*	*Description*
0 – 10	10 mM NaOH	Isocratic elution & detection
10 – 20	200 mM NaOH	Column clean-up/regeneration
20 – 50	10 mM NaOH	Re-equilibration to starting conditions

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 (commercially available). The diluent was deionized water (resistivity >18 MΩ-cm) which was sonificated and sparged with Helium 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 appropriate amount of 50% w/w NaOH solution was carefully pipetting into the diluent under gently stirring and Helium sparging to prepare the required the mobile phase solutions. The bottles with mobile phase and column clean-up solution were blanketed with Helium (0.2 bar overpressure) during the analysis to minimize the build-up of carbonate ions in the mobile phase and to assure a reproducible analysis.

Detection

For the pulsed amperometric detection of monosaccharides and other carbohydrates an Antec VT-03 electrochemical flow cell is used for this evaluation. This flow cell has an Au working electrode (WE), HyREF (Pd/H2) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform is applied as shown in figure 2.

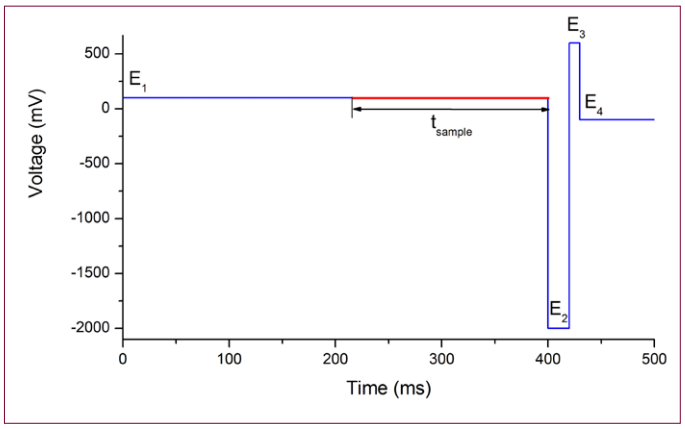


Figure 2: 4-step PAD potential waveform for the detection monosaccharides and other carbohydrates.

The temperature for separation and detection was set to 30°C. The cell current was typical about 0.5 µA with these PAD settings under the specified conditions. This particular 4-step waveform with a pulse duration of 500 ms has been claimed to have as benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [5]. Note that an ALEXYS LC-EC system with the new DECADE Elite electrochemical detector is required for PAD detection using a 4-step potential waveform.

Results

In figure 3 a chromatogram is shown of a 10µL injection of a 10µM standard mix of 7 mono-saccharides in water obtained with the HPAEC-PAD system using the specified conditions in table 1 and 2. All compounds elute within 10 minutes, the total run time is 50 minutes due to the step-gradient program to regenerate and re-equilibrate the column, which is repeated every run.

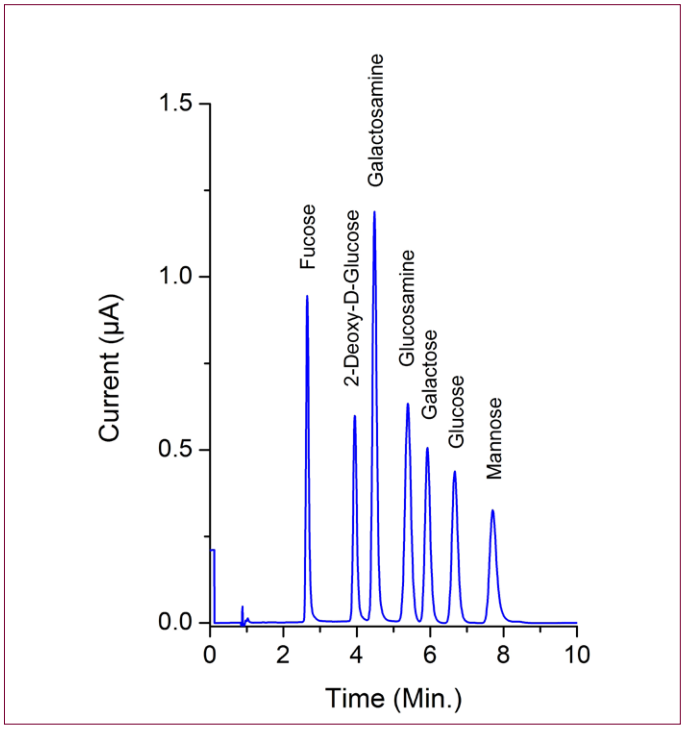


Figure 3: Chromatogram of a 10µL injection of a 10µM standard mix of 7 monosaccharides in water: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and (7) Mannose.

This standard mix represents a group of monosaccharides (hexoses and aminohexoses) commonly found in glycoproteins. Glycoproteins are proteins containing oligosaccharide chains (glycans) covalently attached to the polypeptide side-chain by glycosylation. 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. To release the monosaccharides from the glycan chains acid hydrolysis with TFA and/or HCl is performed prior to HPAEC-PAD analysis. It is evident from figure 3 that all relevant monosaccharides are well separated ($R \geq 1.8$) with peak efficiencies in the range of 4500 – 7000 theoretical plates, which demonstrates the suitability of the system for the compositional analysis of glycoproteins. The peak table of the chromatogram in figure 3 is shown in table 3.

Table 3

Peak table (10µL injection of a 10µM standard mix of 7 monosaccharides in water)						
Compound	*tr (min)*	*Height (nA)*	*Capacity (-)*	*Eff (th.pl)*	*Resolution (-)*	*Tailing (-)*
Fucose	2.65	941.5	2.2	4630	-	1.28
2-Deoxy-D-Glucose	3.94	594.2	3.8	6324	7.3	1.29
Galactosamine	4.48	1182.8	4.4	6264	2.6	1.17
Glucosamine	5.39	630.6	5.5	4792	3.4	1.07
Galactose	5.93	505.1	6.1	7001	1.8	1.13
Glucose	6.67	437.7	7.0	6702	2.4	1.14
Mannose	7.70	325.1	8.3	6997	3.0	1.35

Linearity, Repeatability & LOD

The linearity was investigated in the concentration range of 1 - 10 µmol/L. In this concentration range the linearity is excellent and correlation coefficients for peak area were better than 0.999 for all monosaccharides. In the low concentration range between 10 – 100 nmol/L the correlation coefficients were 0.99 for all compounds.

The Limit of Detection (LOD) for all monosaccharides are shown in table 4. The LOD's were calculated as the analyte response corresponding to 3x the ASTM noise (average peak-to-peak baseline noise of 30 segments of 0.5 min). The responses of a chromatogram obtained with a 100 nM standard mix were used to calculate the LOD. Concentration detection limits of the monosaccharides were in the range of 4 – 12 nmol/L, which corresponds to 40 – 120 fmol on-column. To demonstrate the good detection sensitivity of the ALEXYS HPAEC-PAD system a chromatogram of a 10 µL injection of a 10 nM standard mix is shown in figure 4.

Table 4

Limit of Detection (LOD), based on a 100 nM standard	
Compound	*CLOD (nmol/L)*
Fucose	5
2-Deoxy-D-Glucose	8
Galactosamine	4
Glucosamine	7
Galactose	9
Glucose	10
Mannose	12

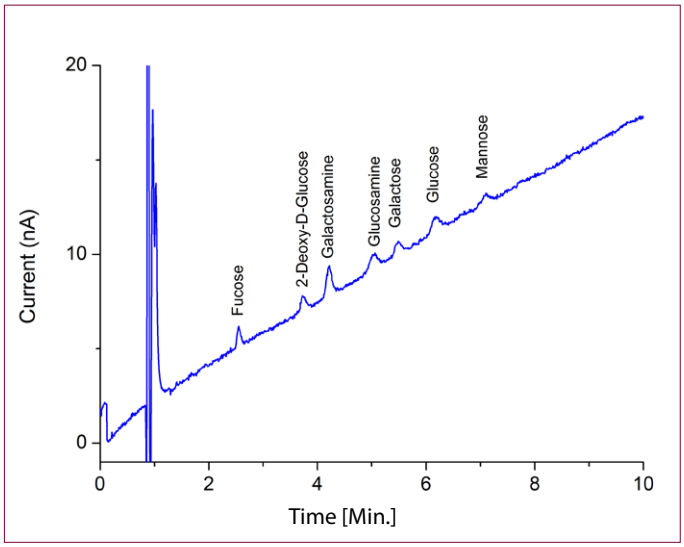


Figure 4: Chromatogram of a 10µL injection of a 10 nM standard mix of 7 monosaccharides in water: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and (7) Mannose.

The relative standard deviation (RSD) of the retention time and peak area were determined for 10 replicate injections of a low and high concentration monosaccharides standard in water. The results are shown in table 5. RSD's for retention time were ≤ 0.3%. For the peak areas the RSD's were < 1% for all monosaccharides in the 10 µM standard and < 2% for the 100 nM standard. These data demonstrate that with this method reproducible analysis of monosaccharides can be achieved.

Table 5

Repeatability 10 µL injections of a 10µM and 100 nM monosaccharides standard in water (n=10)						
Compound	*10 µmol/L standard*			*100 nmol/L standard*		
	RSD% Retention time	*RSD% Area*	*RSD% Height*	*RSD% Retention time*	*RSD% Area*	*RSD% Height*
Fucose	0.16	0.34	0.68	0.20	1.44	1.31
2-Deoxy-D-Glucose	0.26	0.33	0.65	0.19	1.90	0.82
Galactosamine	0.23	0.43	0.59	0.16	1.28	1.30
Glucosamine	0.28	0.50	0.59	0.18	1.64	0.86
Galactose	0.30	0.63	0.76	0.20	1.97	1.34
Glucose	0.29	0.65	0.70	0.23	1.37	1.17
Mannose	0.30	0.35	0.73	0.28	1.95	1.22

Analysis of other mono- and disaccharides

To demonstrate the versatility of the HPAEC-PAD method for the analysis of carbohydrates, also another mix of mono- and disaccharide standards was analyzed, see figure 5 below. This standard contains a mix of carbohydrates among which relevant sugars used as probes in intestinal permeability studies (mannitol, Lactulose and Xylose). In such diagnostic studies these non-metabolized (inert) sugars are orally administered and the urinary recovery determined. With this non-invasive approach intestinal damage can be assessed in both humans and animals. HPAEC-PAD offers a selective and sensitive method for the quantification of these sugars (and other carbohydrates commonly found) in urine, without requiring sample pre-treatment or (post-column) derivatization [7].

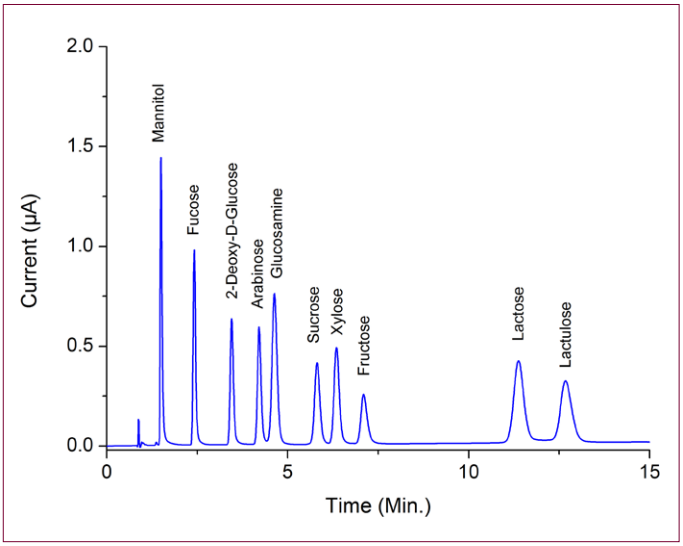


Figure 5: Chromatogram of a 10 µL injection of a 10nM standard mix of 10 mono- and disaccharides in water: (1) Mannitol, (2) Fucose, (3) 2-Deoxy-D-Glucose, (4) Arabinose, (5) Glucosamine, (6) Sucrose, (7) Xylose, (8) Fructose, (9) Lactose and (10) Lactulose.

Table 6

Peak table (10µL injection of a 10µM standard mix of 10 mono- and disaccharides in water)						
Compound	*tr (min)*	*Height (nA)*	*Capacity (-)*	*Eff (th.pl)*	*Resolution (-)*	*Tailing (-)*
Mannitol	1.50	1449	0.8	2805	-	1.42
Fucose	2.42	981	1.9	4659	7.2	1.22
2-Deoxy-D-Glucose	3.45	631	3.2	6562	6.6	1.35
Arabinose	4.21	590	4.1	7208	4.2	1.23
Glucosamine	4.63	757	4.6	4282	1.8	1.08
Sucrose	5.80	411	6.0	6085	4.0	1.13
Xylose	6.35	485	6.7	8042	1.9	1.13
Fructose	7.10	250	7.6	7602	2.5	1.33
Lactose	11.38	410	12.7	7540	10.1	1.08
Lactulose	12.67	308	14.3	7614	2.3	1.19

The peak table of the chromatogram in figure 5 is shown in table 6.

Conclusion

The ALEXYS Carbohydrates Analyzer based on the new DECADE Elite detector provides a selective and sensitive analysis solution for the analysis of mono- and disaccharides. At 10 µL injection volume LOD's in the range of 5 – 10 nmol/L has been obtained, which demonstrates the excellent detection sensitivity of the system.

References

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Ordering number

180.0054E	ALEXYS Carbohydrate analyzer (1 Channel) including column and flow cell
250.0033E	LP solvent selector option, 4-port,1/4-28
250.1078	CarboPac PA20 analytical column, 150 x 3.0 mm ID
250.1079	CarboPac PA20 guard column, 50 x 3.0 mm ID
250.1085	AminoTrap column, 50 x 3.0 mm ID

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