Application Note

Food, Beverage, Life Sciences

Mono- and Disaccharides

The finest LC-EC Applications for Food & Beverage analysis

Phenols
Bisphenol A
Catechins
Flavonoids
Phenols
Antioxidants
Resveratrol
Epicatechin
Quercetin
Other polyphenols

Carbohydrates
Monosaccharides
Lactose
Mono- and Disaccharides
Other oligo- and Polysaccharides

Vitamins, minerals etc.
A, C, D, E, and K
Iodide
Q10, ubiquinols

- **Mono- and disaccharides in food & life sciences**
- **ALEXYS Carbohydrate Analyzer based on HPAEC-PAD**
- **Flow cell with Au working electrode**
- **Sensitive and selective analysis**

Summary

In this publication the analysis of monosaccharides and other carbohydrates is demonstrated using an ALEXYS Carbohydrate Analyzer equipped with a DECADE Elite electrochemical detector. The method is based on separation by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a 4-step potential waveform.

Introduction

The 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.

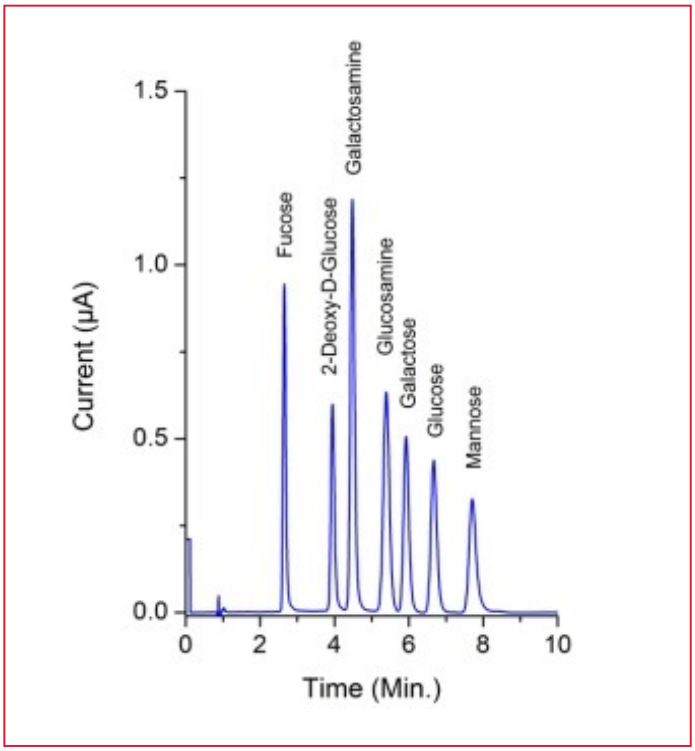

Figure 1: Chromatogram of a 10 µL injection of a 10 µM standard mix of 7 monosaccharides in water: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and (7) Mannose.

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.

An anion-exchange column and matching guard column was used for separation. In case of samples containing amino acids or small peptides, like in glycoproteins, an additional trap column (3 x 30mm) must be installed between the injector and the guard column. The use of such trap column will affect the peak performance (slight increase in retention time and peak width). An additional trap column (against borates) is recommended to install between the pump and injector to prevent borates to affect peak shapes. All chromatograms were recorded without trap columns unless stated otherwise.

Table 1

Conditions	
HPLC	ALEXYS Carbohydrate Analyzer
Columns	BorateTrap™ Inline Trap Column, 50 x 4.0 mm ID (placed between LC pump and injector) AminoTrap™, 30 x 3mm ID# CarboPac™ PA20, 150 x 3.0 mm ID + 30 x 3.0 mm ID All columns: Thermo Scientific™ Dionex™
Mobile phase	10 mM sodium hydroxide (analysis), 200 mM sodium hydroxide (column regeneration).
Flow rate	0.5 mL/min
Injection	10 µL (Full loop)
Temperature	30 °C for separation & 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 0.5 µA
ADF	0.5 Hz
Range	1 or 2 µA/V

*) Original work done with a flow cell containing a 3 mm Au WE, HyREF and 50 µm spacer
#) The trap column is only required when analyzing samples containing amino acids and small peptides.

Table 2

Step-gradient program

Time (min)	*Mob phase*	*Description*
0 - 10	10 mM NaOH	Isocratic elution and detection
10 - 20	200 mM NaOH	Column clean-up and regeneration
20 - 50	10 mM NaOH	Equilibration, starting conditions

The analysis is based on a step-gradient, see Table 2. At a concentration of 10 mM NaOH, carbonate ions (CO3 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 other contaminants like amino acids/peptides. This regeneration step assures reproducible retention behavior for each run. The LC-EC system was equipped with a P6.1L pump with integrated solvent selection valve and degasser.

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 sonicated 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 a Antec electrochemical flow cell is used for this evaluation . This flow cell has an Au working electrode (WE), HyREF (Pd/H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform is applied as shown in Figure 2.

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

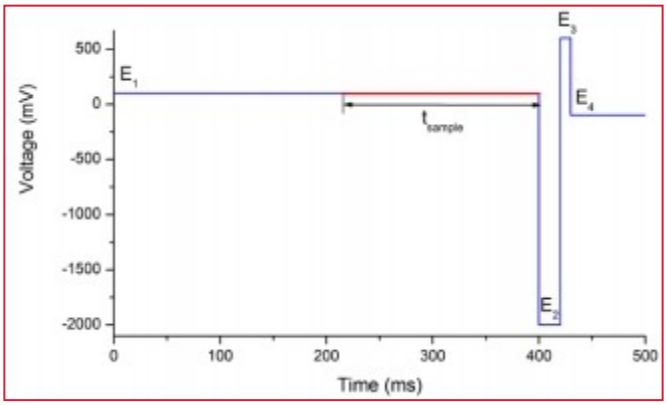

Figure 2: 4-step PAD potential waveform for the detection mono-saccharides and other carbohydrates.

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]. The DECADE Elite electrochemical detector is required for PAD detection using a 4-step potential waveform.

Results

In Figure 1 a chromatogram is shown of a 10 µL injection of a 10 µM 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.

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 sidechain 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 1 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 1 is 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)*	*Height (nA)*	*K' (-)*	*Eff. (-)*	*Res. (-)*	*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 and 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.

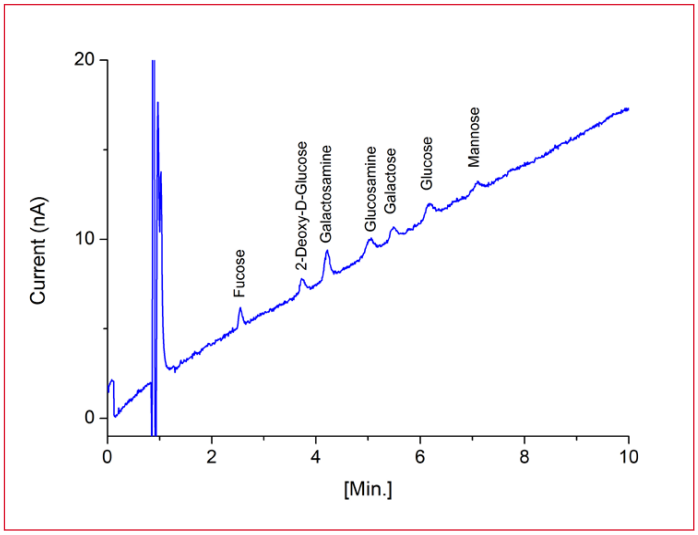

Figure 3: Chromatogram of a 10 µL injection of a 10 nM standard mix of 7 monosaccharides in water: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and (7) Mannose.

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 3.

Table 4

Limit of Detection (LOD), based on a 100 nM standard

Compound	*LOD (nmol/L)*
Fucose	5
2-Deoxy-D-Glucose	8
Galactosamine	4
Glucosamine	7
Galactose	9
Glucose	10
Mannose	12

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

Peak table, 10 µL injections of 10 and 0.1 µM carbohydrate standard mix in water (n=10)

	10 µmol/L			*100 nmol/L*		
	%RSD			*%RSD*		
Compound	t_R	*Area*	*Height*	t_R	*Area*	*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

Table 6

Peak table, 10 µL injection of a 10 µM standard mix of 10 mono– and disaccharides in water

Compound	t_R *(min)*	*Height (nA)*	*K' (-)*	*Eff. (-)*	*Res. (-)*	*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

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 4 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]. The peak table of the chromatogram in Figure 4 is shown in Table 6.

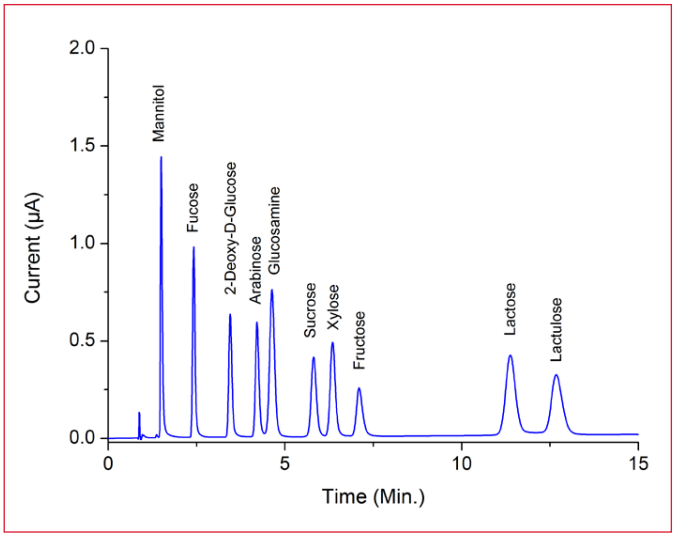

Figure 4: Chromatogram of a 10 µL injection of a 10 nM standard mix of 10 mono- and disaccharides in water: (1) Mannitol, (2) Fucose, (3) 2-Deoxy-DGlucose, (4) Arabinose, (5) Glucosamine, (6) Sucrose, (7) Xylose, (8) Fructose, (9) Lactose and (10) Lactulose.

References

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Conclusion

The ALEXYS Carbohydrate Analyzer based on the DECADE Elite detector provides a selective and sensitive 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.

5. R.D. Rocklin, A.P. Clarke, M. Weitzhandler, Improved longterm reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform, Anal. Chem, 70 (1998), 1496 – 1501
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7. S.H. Sørensen, F.J. Proud, A. Adam, H.C. Rutgers, R.M. Batt, A novel HPLC method for the simultaneous quantification of monosaccharides and disaccharides used in tests of intestinal function and permeability, Clin. Chim. Acta, 221 (1993), 115 - 124

Figure 5: Recommended instrument configuration for this application: the ALEXYS Carbohydrate Analyzer with Solvent Switch Valve.
The system consists of a P6.1L pump with integrated degasser and Solvent Switch Valve (SSV) for the option to run step gradients or automated column clean-up, an AS6.1L autosampler, an ET 210 Eluent tray for helium blanketing, and the DECADE Elite electrochemical detector. A CT 2.1 column oven with broad temperature range can be added optionally for separations under near-ambient temperatures. The ALEXYS Carbohydrate Analyzer can be operated under different Chromatography Data System (CDS) software: DataApex™ Clarity™ CDS (version 8.3 and up) or Thermo Scientific™ Chromeleon™ CDS (version 7.2 SR 5 and up).

Ordering information

Detector only	
176.0035A	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
ALEXYS analyzers	
180.0055W	ALEXYS Carbohydrate Analyzer with Solvent Switch Valve
186.A05852	CT 2.1 column thermostat
116.4321	SenCell 2 mm Au HyREF
Software	
195.0035[#]	Clarity CDS single instr. incl LC, AS module

#) alternative option: Antec ECD drivers are available for use with Chromeleon CDS , OpenLAB CDS or OpenLAB Chemstation CDS .

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