Application Note

Food, Beverage, Life Sciences

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

Carbohydrates in food according to AOAC

- **Mono– and disaccharides in food**
- **Gradient separation (quaternary LPG)**
- **Pulsed Amperometric Detection (PAD)**
- **Flow cell with Au working electrode**
- **First Action official AOAC method**

Summary

In this application note the analysis of common monosaccharides, disaccharides and more complex oligosaccharides is demonstrated using the ALEXYS Carbohydrates analyzer based on the DECADE Elite electrochemical detector. The sugar profile method published by the AOAC for the analysis of carbohydrates in food, dietary supplements, pet food and animal feeds was used in this study [1]. This method was granted the AOAC first action official method status in 2019.

The analysis is based on gradient separation on a High-Performance Anion-Exchange column followed by Pulsed Amperometric detection using a 4-step potential waveform.

Introduction

Carbohydrates are the most abundant biomolecules found in living organisms. These carbohydrates, which are also called saccharides, originate as products of photosynthesis and play an important role in metabolism, storage of energy end nutrition. [2] Carbohydrates can be separated in different groups based on their structures of which monosaccharides are the simplest molecules. Common examples of monosaccharides are glucose and fructose. Monosaccharides can be described as aldehyde– or ketone-alcohols containing three to six carbon atoms and are the building blocks for more complex carbohydrates such as disaccharides and polysaccharides. Maltose is an example of a disaccharide which is mainly found in grains and cereals. Due to the presence of oxidizable hydroxyl groups in all carbohydrates, they can be detected with electrochemical detection. [3-5].

Because carbohydrates are one of the key components in many foods, the analysis of these molecules becomes very important. For multiple reasons carbohydrate quantification in foods is performed: Foods should have compositions according to regulations and customers must be informed about the nutritional contents of food product. High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) can be used as a tool for the compositional analysis/profiling of carbohydrates in a wide range of foods.

Figure 1: ALEXYS Carbohydrates analyzer consisting of the ET 210 eluent tray, P6.1L analytical pump, AS6.1L autosampler, CT2.1 column thermostat and DECADE Elite electrochemical detector (the additional post-column pump is not displayed in the photograph).

Table 1

Conditions	
HPLC	ALEXYS Carbohydrates analyzer
Columns	CarboPac™ PA20, 150 x 3.0 mm ID + 30 x 3.0 mm ID BorateTrap™ Inline Trap Column, 50 x 4.0 mm ID, 20 µm All columns: Thermo Scientific™ Dionex™,
Mobile phases	MP A: deionized water (>18 MOhm.cm)* MP B: 100 mM NaOH* MP C: 200 mM NaOH* MP D: 600 mM NaOH* Post-column: 200 mM NaOH *) Eluents bottles kept under inert helium 5.0 atmosphere using the ET 210 eluent tray to avoid the introduction of carbonate ions.
Flow rate	Analytical pump: 0.5 mL/min Post column pump: 0.2 mL/min
Injection	25 µL (full loop)
Temperature	28 °C for separation & 35 °C for detection
Flow cell	SenCell with 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 0.6 µA
ADF	0.1 Hz
Range	2 µA/V

Method

Separation

The separation of carbohydrates can be performed under alkaline conditions using anion-exchange columns. As carbohydrates are weak acids, with pKa values between 12 and 14, they will be either partially or completely ionized at high pH. The retention time of most carbohydrates are inversely correlated with pKa value and increases significantly with molecular weight. The elution order of carbohydrates on such anion-exchange columns is typically as follows: sugar alcohols elute first, followed by mono-, di-, tri-, and higher oligosaccharides. The AOAC method describes the use of a 15 cm analytical anion-exchange column together with a 3 cm guard column (Table 1). An inline trap column was installed in the solvent line between pump and autosampler (Table 1). Borate contamination in eluents can cause a significant loss of peak efficiency by tailing , especially for sugar alcohols (reduced monosaccharides) and carbohydrates with vicinal hydroxyl groups like for example Fructose.

All solvents were kept under an inert atmosphere and free of any dissolved CO_2 (from the air). Above pH 12 carbon dioxide will be converted into carbonate ions. Carbonate is a divalent anion and is a stronger eluent than hydroxide. The presence of carbonate ions in the mobile phase will cause a loss of retention and resolution and should be avoided at all time. The ET 210 eluent tray in the carbohydrate analyzer is equipped with a helium delivery system for that purpose. It facilitates sparging and blanket of all LC mobile phases with an inert helium gas atmosphere in an user-friendly way. The system is delivered with rugged plastic bottles made of polypropylene copolymer (PPCO), because the mobile phases cannot be prepared and kept in glass bottles. NaOH is a strong etching agent and will react with the inner glass wall resulting in the release of silicates and borates.

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 (15 minutes) and subsequently sparged with helium 5.0 (15 minutes) prior to use. The appropriate amount of 50 % w/w NaOH solution was carefully pipetted into the diluent under gentle stirring and helium sparging to prepare the required mobile phase solutions. The bottles with mobile phase and column clean-up solution were blanketed with helium (0.2—0.4 bar Helium overpressure) during the analysis.

Table 2

Gradient program

Time (min)	*A %*	*B %*	*C %*	*D %*	*Description*
0 .00	90	10	0	0	Isocratic elution
13.00	90	10	0	0	Gradient elution
25.00	0	0	100	0	
25.01	0	0	0	100	Column clean-up and regeneration
28.00	0	0	0	100	
28.01	90	10	0	0	Equilibration to starting conditions
50.00	90	10	0	0	

The separation was performed at 28 °C with the analytical anion-exchange column inside the CT 2.1 column thermostat. In table 2 the gradient profile is listed which was used for the separation of the mixture of carbohydrates. The equilibration step after the column clean-up with 600 mM NaOH was slightly extended compared to the profile reported in the Journal of the AOAC [1].

Detection

For detection 200 mM NaOH was added post-column using a second P6.1L pump. The AOAC method is based on post-column addition of NaOH for detection and claims that this will improve baseline stability during the gradient elution [1]

Pulsed amperometric detection of the carbohydrates is performed using a SenCell with gold 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.

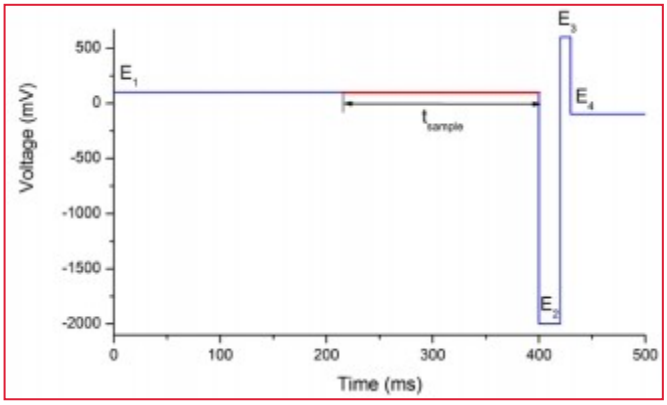

Figure 2: 4-step PAD potential waveform for the detection of carbohydrates.

The temperature for detection was set to 35°C. The cell current typically is about 0.6 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 [6].

Results

In Figure 3 a chromatogram of a 25 µL injection of a 10 µg/mL standard mix of 8 carbohydrates in water is shown, obtained with the HPAEC-PAD system using the specified conditions in Table 1 and 2. All relevant compounds elute within 30 minutes, the total run time is 50 minutes due to the gradient program to re-equilibrate the column and to remove late eluting compounds which might be present in real food samples. This mix of standards represents a group of mono– and disaccharides commonly found in foods. Maltotriose and

maltotetraose illustrate the capability of the method to separate even larger carbohydrates.

It is evident from Figure 3 that all carbohydrates are well separated (R ≥ 1.6) with peak efficiencies in the range of 4800 – 45500 theoretical plates, which demonstrates the suitability of the system for compositional analysis . The peak table of the chromatogram in Figure 3 is shown in Table 3.

Table 3

Peak table, 25 µL injection of a 10 µg/mL standard mix of 8 saccharides in water

Compound	*Tr (min)*	*Height (nA)*	*Capacity*	*Efficiency*	*Resolution*	*Tailing*
Galactose	6.1	2203	5.1	5182	-	1.1
Glucose	6.9	1805	5.9	4833	2.1	0.9
Sucrose	7.8	689	6.8	4997	2.1	1.0
Fructose	8.5	772	7.5	4986	1.6	1.1
Lactose	14.8	643	13.8	4865	9.5	1.0
Maltose	25.8	813	24.8	59079	17.3	0.9
Maltotriose	31.1	1015	30.1	455102	17.2	0.9
Maltotetraose	32.7	201	31.7	36911	3.7	1.4

Linearity, repeatability and LOD

The linearity was investigated in the concentration range of 0.05 – 30 µg/mL. In this concentration range the linearity is excellent and correlation coefficients for peak area are at least 0.999 for all saccharides.

Table 4

Limit of Detection (LOD), based on a 0.5 µg/mL standard

Compound	*LOD (ng/mL)*	*LOD (nM)*
Galactose	2.6	14
Glucose	2.7	15
Sucrose	8.8	26
Fructose	7.4	41
Lactose	8.8	26
Maltose	5.9	17

The Limit of Detection (LOD) for all carbohydrates is 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 15 segments of 0.3 min). The responses of a chromatogram obtained with a 0.5 µg/mL standard mix were used to calculate the LOD. Concentration detection limits of the carbohydrates were in the range of 3 – 9 ng/mL, which corresponds to 14 - 41 nM (350 – 1022 fmol on-column).

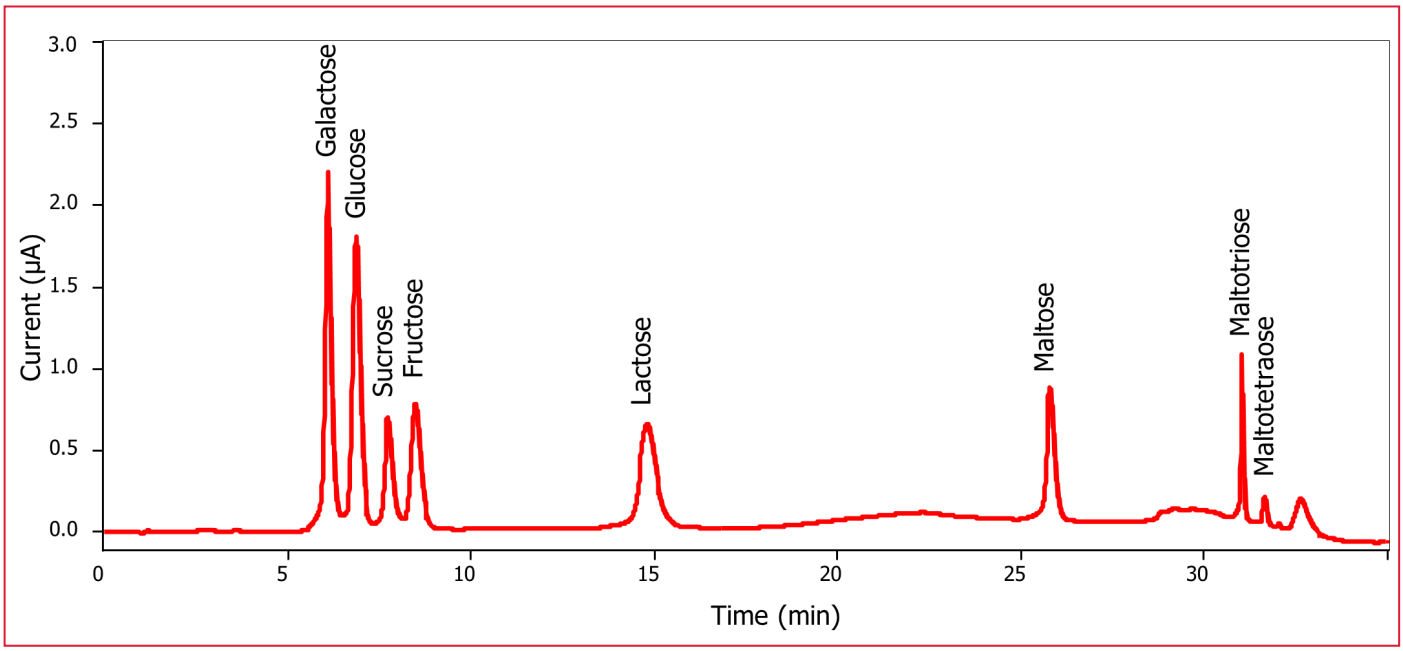

Figure 3: Chromatogram of a 25 µL injection of a 10 µg/mL standard mix of 8 saccharides in water: (1) Galactose, (2) Glucose, (3) Sucrose, (4) Fructose, (5) Lactose, (6) Maltose, (7) Maltotriose and (8) Maltotetraose.

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 ≤ 0.35%. For the peak areas the RSD's were < 1% for all monosaccharides in the 10 µg/mL standard and < 1.5% for the 1 µg/mL standard. These data demonstrate that with this method reproducible analysis of carbohydrates can be achieved.

Table 5

Repeatability of 25 µL injections of a 10 and 1 µg/mL carbohydrate standard mix in water (n=10)

	10 µg/mL			*1 µg/mL*		
	%RSD			*%RSD*		
Compound	t_R	*Area*	*Height*	t_R	*Area*	*Height*
Galactose	0.25	0.27	0.14	0.28	0.41	0.31
Glucose	0.24	0.31	0.15	0.28	0.54	0.36
Sucrose	0.34	0.47	0.21	0.34	1.16	0.62
Fructose	0.26	0.51	0.08	0.32	0.94	0.35
Lactose	0.28	0.89	0.61	0.29	0.73	1.07
Maltose	0.10	0.56	0.31	0.09	1.40	0.53

Sample analysis

To illustrate the performance of the method with a real food sample an commercial infant cereal product was analyzed as an example. The sample for analysis was prepared in the following way:

- 0.5 g infant cereal sample was transferred to 50 mL centrifuge tubes and diluted in 30 mL hot deionized water.
- After mixing (vortex), the tube is placed in a 70°C water bath for 25 minutes.
- Subsequently, the sample solution is allowed to cool down, vortexed and transferred to a 50 mL volumetric flask and brought to volume using deionized water.
- After mixing, aliquots of the sample solution were transferred to 2 mL Eppendorf tubes and centrifuged for 20 minutes.
- The supernatant is diluted (1:20) with water and 25 µL injected into the LC system.

An example chromatogram of the cereal sample is shown in figure 4. The quantified amounts of sugars are listed in table 6. The obtained amounts are in correspondence with the reference values on the product label.

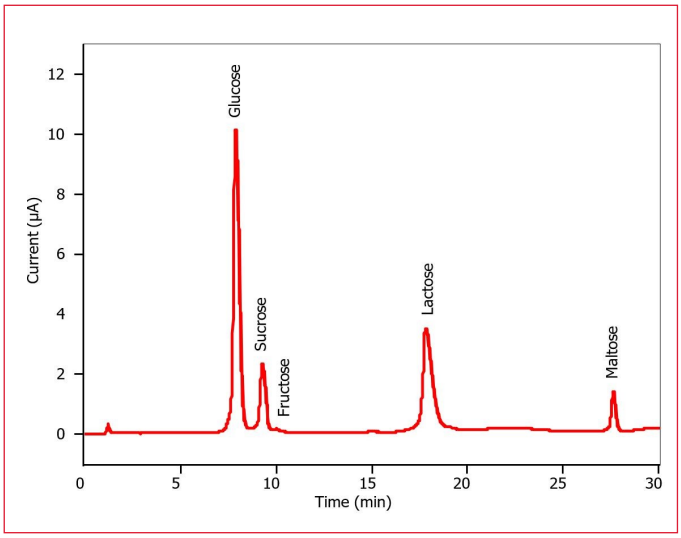

Figure 4: Chromatogram of a 25 µL injection of a 0.5 g/L infant cereal sample (red).

Table 6

Carbohydrate content of infant cereal sample

Compound	*Quantified amount (g/100g)*
Glucose	12.0
Sucrose	6.6
Fructose	0.3
Lactose	9.6
Maltose	2.6

References

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Conclusion

The ALEXYS Carbohydrates Analyzer provides a solution for the routine analysis of carbohydrates in food using HPAEC-PAD. As an example, it is demonstrated in this application note that the system is suitable for the analysis of carbohydrates as described in the AOAC first action method 2018.16 [1].

Ordering information

ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
180.0605	Post Column Kit Carbohydrates
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

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

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.

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