Application Note

Food & Beverage

The finest LC-EC applications for Food & Beverage analysis

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

Carbohydrates
Monosaccharides
Lactose
Other oligo- and polysaccharides

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

Carbohydrates in Food Products

- **ALEXYS® Carbohydrate Analyzer**
- **Pulsed amperometric detection**
- **Robust & reproducible analyses**
- **Examples from beverages and artificial sweetener**

Summary

The ALEXYS® Carbohydrate Analyzer is a dedicated analytical solution based on High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) for the analysis of sugars and oligosaccharides in a variety of samples.

In this application note typical results obtained with the ALEXYS® Carbohydrate Analyzer are reported, demonstrating its performance for the analysis of carbohydrates in food products.

Introduction

Carbohydrates not only provide the most easily accessible energy source for our body, they also play an important role in many physiological processes. They are involved in intercellular recognition, infection processes, and certain types of cancer. Carbohydrates analysis is of interest to the food industry but also many fields in life sciences.

Analytes of interest include simple mono- or disaccharides (such as glucose and sucrose), oligosaccharides (Maltodextrin), polysaccharides (starch, cellulose) and glycoproteins. High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) can be used for the compositional analysis and quantification of sugars in food samples.

The ALEXYS Carbohydrate Analyzer (Fig. 1) is a fully dedicated HPAEC-PAD system with integrated column oven, helium blanketing hardware (including pressure resistant bottles), and autosampler, based on the DECADE Elite electrochemical detector and can be completed with the gold electrode SenCell flow cell to work with the no-wear 4-step pulse option.

This application note shows some typical results obtained with the ALEXYS Carbohydrate Analyzer for the analysis of carbohydrates in various food products.

Figure 1: ALEXYS Carbohydrate Analyzer.

Method

Separation

Under alkaline conditions (pH > 12) carbohydrates can be separated by means of High Performance Anion-Exchange Chromatography (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 HPAEC 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.

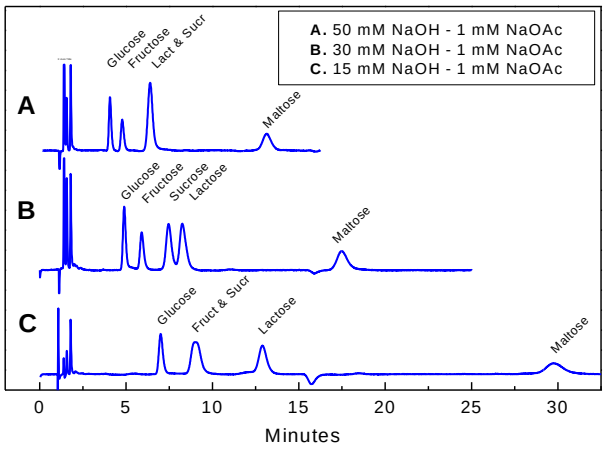

Figure 2: Retention times of common food carbohydrates as a function of sodium hydroxide concentration in the mobile phase

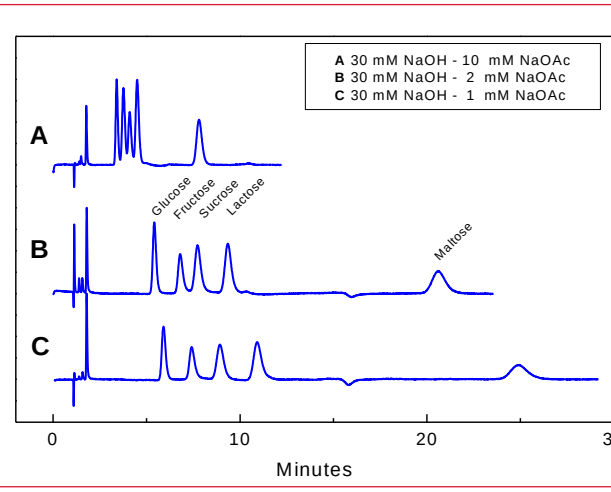

Figure 3: Retention times of common food carbohydrates as a function of the sodium acetate concentration in the mobile phase

The retention behavior of the carbohydrates can be controlled by the concentration of sodium hydroxide and sodium acetate in the mobile phase. An increase of the sodium hydroxide concentration [OH^-] has a dual effect on the retention of carbohydrates. The increase in ionic strength of the eluent causes a decrease in analyte retention, while the higher pH will increase the degree of dissociation resulting in an increase in analyte retention. If the pH > pKa (full dissociation), then the ionic strength will dominate the separation process and the retention decreases. This is illustrated in Fig. 2A. Sodium acetate is commonly used as a retention modifier to decrease the elution time of higher molecular weight carbohydrates, thus allowing faster analysis.

Pulsed amperometric detectors are relatively insensitive to ionic strength changes of a sodium acetate gradient, as long as the sodium hydroxide concentration remains constant during the gradient run. High purity grade sodium acetate should be used for the preparation of the mobile phase, as impurities can cause large baseline shifts during a gradient run.

Mobile phase preparation

Carbon dioxide gas present in air will dissolved as CO_3^{2-} ions in the strong alkaline eluent. The dissolved carbonate ions will increase the ionic strength of the mobile phase, resulting in a shortening of the retention times of the carbohydrate analytes. Therefore, keeping the mobile phase free of carbonate is one of the key factors towards reproducible carbohydrate analyses in an HPAEC-PAD set-up.

Take the following precautions to prepare a carbonate-free mobile phase:

- Use only deionized water (> 18 MOhm.cm, TOC<10ppb) freshly supplied from a water filtering apparatus. Most of the carbon dioxide dissolved in the water can be removed by first degassing it in an ultrasonic bath for 10 – 15 minutes, and subsequent sparging with Helium 5.0 gas.

- Prepare the mobile phase using a commercially available 50% w/w carbonate-free NaOH stock solution. Commercially available NaOH pellets are not acceptable for mobile phase preparation, because they are always covered with a thin layer of sodium carbonate (adsorbed from the air).

- The mobile phase should be prepared in plastic bottles; NaOH is a strong etching agent and will react with the inner glass wall resulting in the release of silicates and borates when using glass bottles.

- Add the appropriate amount of 50% w/w NaOH solution to obtain the final eluent. Always pipette the necessary amount of NaOH from the middle of the 50% NaOH solution and do not leave the bottle open for unnecessary long times.

- Only high-purity grade sodium acetate should be used for preparation of a mobile phase, as impurities can cause large unnecessary baseline shifts when running a gradient.

Whence the mobile phase is ready it should be sparged continuously or blanketed with a small overpressure of helium during the analysis to prevent carbonates dissolving back into the mobile phase (which would destabilize the retention times).

Detection

Pulsed Amperometric Detection (PAD) with a gold (Au) working electrode is applied for carbohydrate analysis. A DECADE Elite electrochemical detector is equipped with a SenCell™ with Au working electrode (WE) and maintenance-free HyREF (Pd/H_2) reference electrode. The fully optimized 4-step potential waveform as shown in Figure 4 is advised. This particular waveform results in an excellent reproducibility and minimal electrode wear [1]; i.e. resulting in less flow cell maintenance and system down time. The cell current is typical about 1 – 2 µA for this set-up.

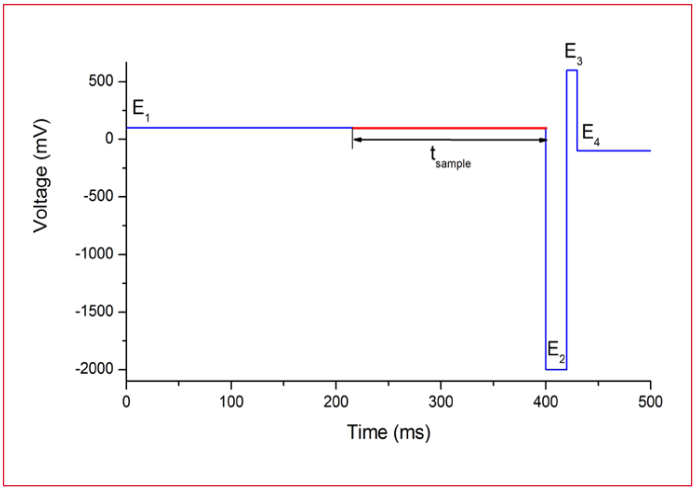

Figure 4: 4-step PAD potential waveform for the detection of carbohydrates

Column regeneration

Especially, during the isocratic analysis of carbohydrates with weak eluents ([NaOH] < 50 mM) a gradual loss of retention is observed due to the slow build up of interfering anions on the column. If during the isocratic analysis of carbohydrates a loss of retention is observed, regeneration of the column is necessary. Regeneration of the column can be achieved by flushing the column with a volume of 30 – 60 mL of carbonate-free 0.2 M NaOH. After regeneration, the column should be allowed to re-equilibrate again with mobile phase. Stabile retention times (RSD < 0.4%) can be achieved again after flushing the column for 5 hour with eluent at a flow rate of 2 mL/min.

Table 1

LC-ECD conditions - isocratic analysis of sugars

HPLC	ALEXYS Carbohydrate Analyzer with SSV
Column	RCX-10 250 x 4.6 mm ID, 7 µm (Hamilton)
Mobile phase	30 mM NaOH and 1 mM NaOAc, continuously sparged or blanketed with Helium 5.0
Column cleaning	100 mM potassium hydroxide
Flow rate	2 mL/mL
Temperature	30 °C for separation and detection
Back pressure	about 125 bar
$V_{injection}$	20 µL
Flow cell*	SenCell™ with 2 mm Au and HyREF (Pd/H_2), AST pos. 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
Range	5 µA/V
ADF	0.5 Hz
I-cell	About 1.5 µA

Table 2

LC-ECD conditions - gradient analysis of oligosaccharides

HPLC	ALEXYS Carbohydrate Analyzer with LPG gradient mixer
Column	RCX-10 250 x 4.6 mm ID, 7 µm (Hamilton)
Mobile phase	A) 60 mM NaOH B) 60 mM NaOH – 500 mM NaOAc Mobile phases are continuous sparged with Helium 5.0
Flow rate	2 mL/mL
Gradient	t = 0 min: 90% A, 10% B t = 15 min: 10% A, 90% B
Temperature	30 °C for separation and detection
Back pressure	about 145 bar
$V_{injection}$	20 µL
Flow cell*	SenCell™ with 2 mm Au and HyREF (Pd/H_2), AST pos. 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
Range	20 µA/V
ADF	0.5 Hz
I-cell	-0.5 - 1 µA

* Original data recorded with a flow cell with 3 mm Au, spacer 50 µm, 3-step pulse (E1, E2, E3: 0.05, 0.75, -0.80 V; ts, t1, t2, t3: 0.06, 0.5, 0.13, 0.12 s)

Results - Isocratic analysis of sugars

Mixtures of simple sugars, such as mono and disaccharides can be determined using HPAEC-PAD under isocratic conditions (Table 1) with high sensitivity and good reproducibility. This method is particularly attractive for the analysis of sugars in a wide range of food products such as beverages, fruit juices, milk products and beer.

Linearity

An excellent linear detector response in the concentration range between 20 nM and 10 µM was observed for a standard mixture of sugars (Figure 5 and Table 3). The analysis of these carbohydrates at the low level of 0.1 µM range can be achieved routinely.

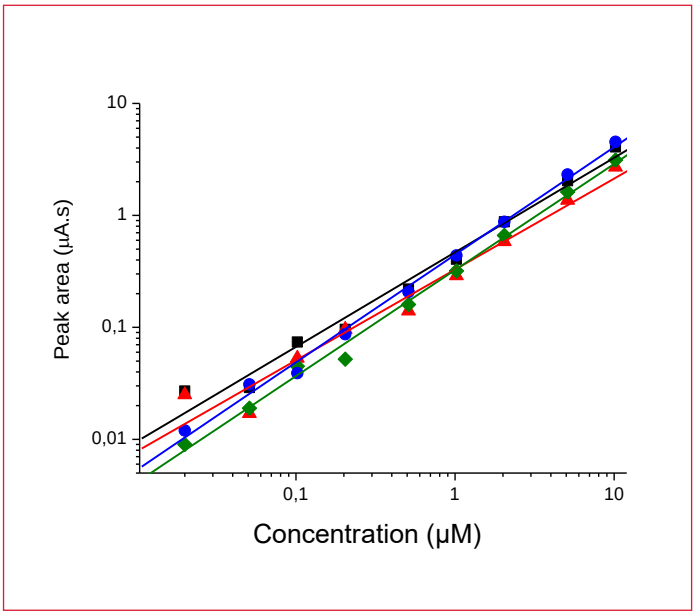

Figure 5: Calibration plots of glucose, fructose, sucrose and lactose in the concentration range 20 nM - 10 µM (n=10 per concentration). Conditions as given in Table 1.

Table 3

Correlation coefficient r, determined via weighted linear regression method in the range of 20 nM - 10 µM (Fig. 8)

Component	*R*
Glucose	0.9993
Fructose	0.9979
Sucrose	0.9992
Lactose	0.9995

Reproducibility

The performance of the ALEXYS Carbohydrate Analyzer is demonstrated using a standard mixture of glucose, fructose, lactose and sucrose in water. Figure 6 shows an overlay of 23 consecutively recorded chromatograms.

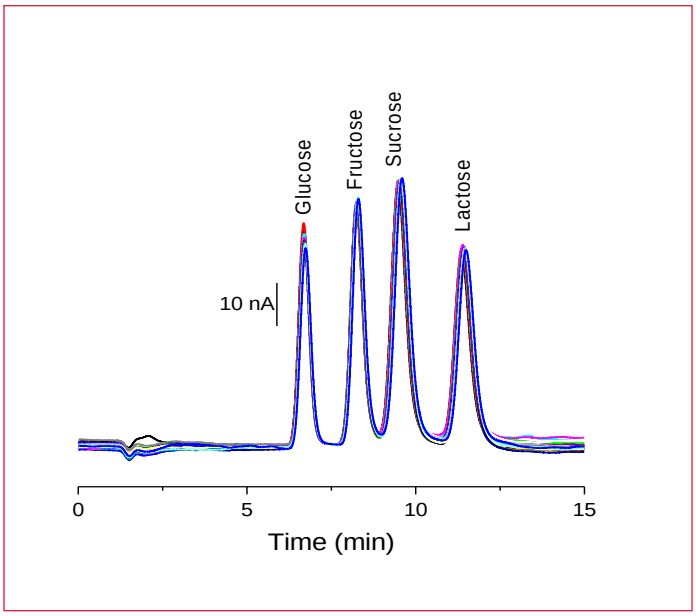

Figure 6: Overlay of 23 chromatograms of a standard mixture of 2 µM glucose, 2 µM lactose, 4 µM fructose and 4 µM sucrose in water (20 µl injected). The theoretical plate numbers for the components are 14.900, 10.900, 12.000 and 17.300 plates/meter, respectively. Conditions as given in Table 1, except ADF set to 0.01 Hz.

The data of the retention times and peak areas is plotted in Figures 7 and 8 respectively. The relative standard deviations (RSD) of the retention times and peak areas were determined for the 23 consecutive injections of the sugar mixture, and they are summarized in Table 4. The excellent reproducibility of the method is evident from the obtained RSD values of <0.4% for retention time and <3% for peak area.

Table 4

Reproducibility (n = 23)		
Component	*Retention time %RSD*	*Peak area %RSD*
Glucose	0.24	2.9
Fructose	0.23	2.4
Sucrose	0.36	2.5
Lactose	0.33	1.4

Detection limits

The chromatogram of the standard mixture was used to calculate the concentration Limit of Detection (cLOD) of the HPAEC-PAD method. The cLOD is defined as the concentration that gives a signal that is three times the peak-to-peak noise. The on-column LOD takes into account the injection volume and is the minimum amount of molecules that can be detected. The LOD of the applied HPAEC-PAD method is summarized in Table 5.

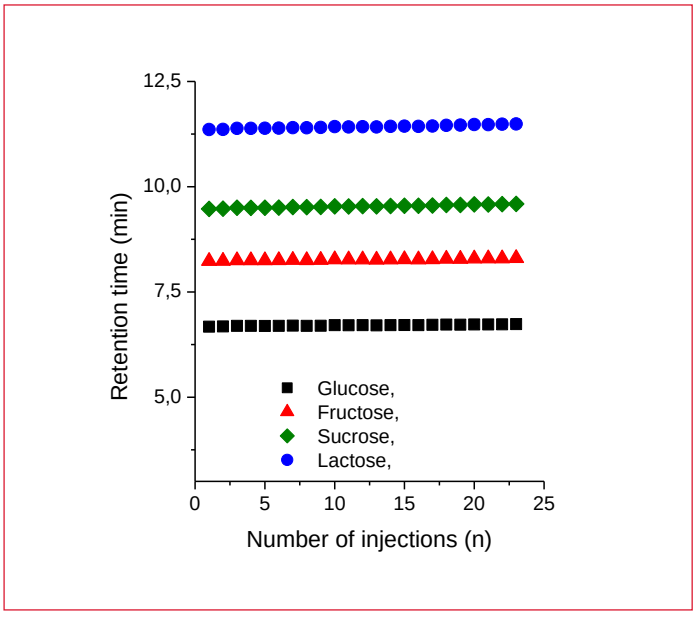

Figure 7: Retention times from 23 consecutively analyzed injection of a standard mixture of 2 µM glucose, 2 µM lactose, 4 µM fructose and 4 µM sucrose in water, 20 µL injections. Conditions as given in Table 1.

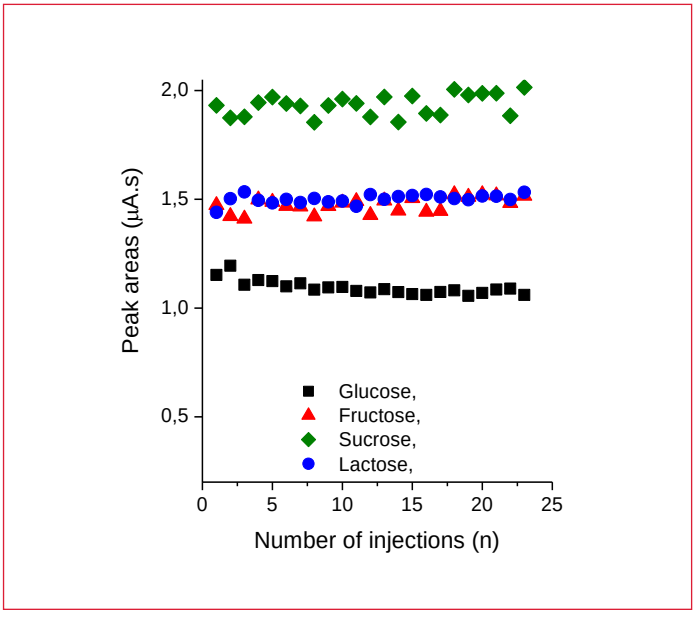

Figure 8: Peak area from 23 consecutively analyzed injection of a standard mixture of 2 µM glucose, 2 µM lactose, 4 µM fructose and 4 µM sucrose in water, 20 µL injections. Conditions as given in Table 1.

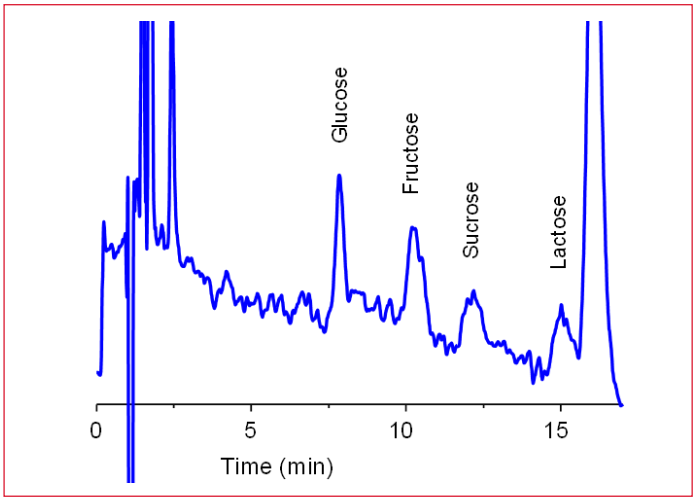

Figure 9: Chromatogram of a 20 nM glucose, lactose, fructose and sucrose in water, 20 µL injections. Conditions as given in Table 1.

Table 5

Limit of Detection (LOD)

Component	*cLOD (nM)*	*on-column LOD (pmol)*
Glucose	10	0.2
Fructose	15	0.3
Sucrose	15	0.3
Lactose	10	0.2

With the ALEXYS Carbohydrate Analyzer, an on-column detection limit of 0.2 pmol could be reached for glucose and lactose under the specified conditions of Table 1. To demonstrate the sensitivity of the method, a near-LOD concentration of 20 nM sugars was analyzed (Figure 9).

Beverage samples

In the following section several example chromatograms are shown of the analysis of mono- and disaccharides, namely apple juice, buttermilk and malt beer (Figures 8 - 10). Such aqueous samples, only require little sample preparation. These samples only need to be sonicated, diluted and filtered prior to injection. Other food products contain carbohydrates that are physically associated or chemically bound to other components, e.g., nuts, cereals, fruit, breads and vegetables would need more intensive sample preparation to isolate the carbohydrate from the rest of the food before it can be analyzed.

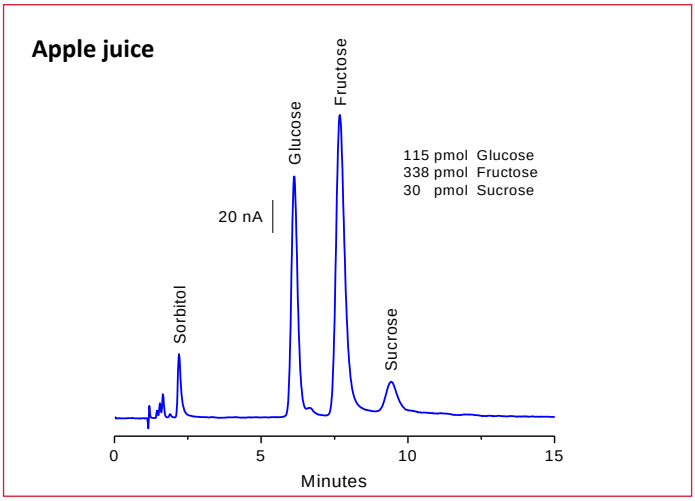

Figure 8: Chromatogram of apple juice. Sample diluted 10.000 x with water. Conditions as in Table 1, except 10 µL injection, and mobile phase: 30 mM NaOH.

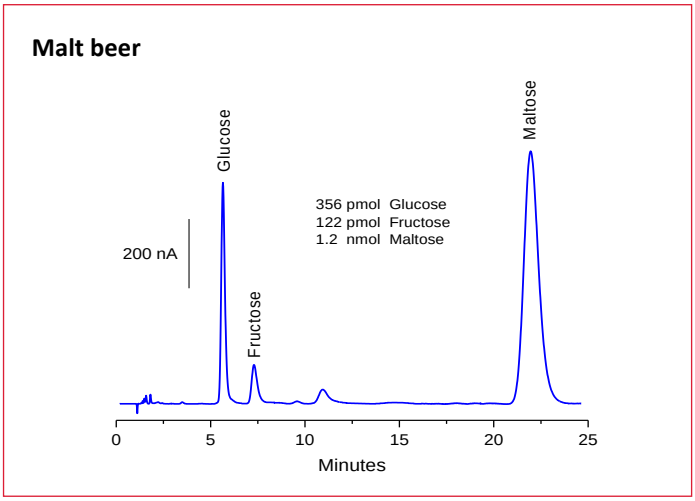

Figure 9: Chromatogram of malt beer. Sample was degassed for 10 minutes (ultrasonic bath) to remove dissolved CO_2 and diluted 1.000 x with water. Conditions as in Table 1, except 10 µL injection, and mobile phase: 30 mM NaOH, 2 mM NaOAc.

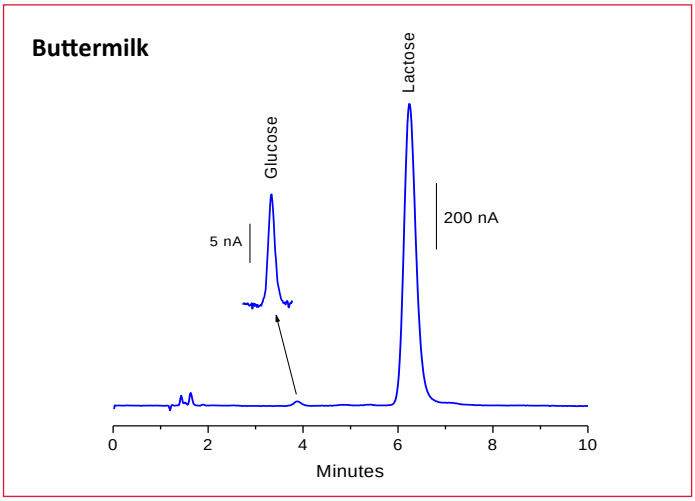

Figure 10: Chromatogram of buttermilk. Sample was diluted 1.000 x with water and filtered over a 0.2 µm membrane. Conditions as in Table 1, except 10 µL injection, and mobile phase: 30 mM NaOH.

Results - Gradient analysis of oligosaccharides

The HPLC analysis of oligo- and polysaccharides is increasingly important in the study of human nutrition. Starch, a polymer (polysaccharide) based on glucose subunits is one of the important base materials used in the food industry nowadays. The food and beverage industry uses the hydrolysis products of corn or potato starch in a wide variety of food products. Starch can be de-polymerized into smaller chains (oligosaccharides) resulting in syrups or maltodextrin. Corn syrup is widely used as a food and beverage sweetener. Maltodextrins, which are non-sweet nutritive oligomers, are often used as a filler or binder in food products. In maltodextrins, the glucose subunits are joined by α1,4 linkages (y) with occasional branches of α1,6 linked glucose (x), see structural formula in Figure 11.

The ALEXYS Carbohydrate Analyzer (binary gradient version) is particularly suitable for the more demanding analysis of complex carbohydrate mixtures such as oligosaccharides. With this HPAEC-PAD gradient system, “fingerprints” of oligosaccharides and other complex carbohydrate mixtures can be recorded. It can serve as a tool for estimating the chain length (DP) distribution.

Figure 11 shows the chromatograms of a filtered solution of 100 mg/L artificial sweetener in water. The different chain lengths were identified, ranging from maltose (DP 2), and maltotriose (DP=3) up to DP = 14.

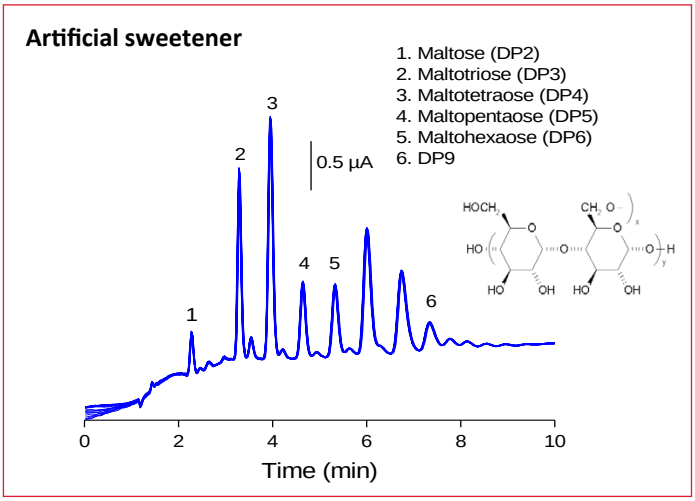

Figure 11: Overlay of 25 chromatograms of a 100 mg/L solution of artificial sweetener containing maltodextrin. Conditions as in Table 2.

Reproducibility

The relative standard deviations (RSD) of the retention times and peak areas were determined for DP2 up to DP6 based on the 25 consecutive injections of the artificial sweetener (Figure 11).

The good reproducibility of the method is evident from the obtained RSD values (n=25) of < 0.3% for the retention times and < 1.5% for the peak areas of the different oligomers (Figure 12 and Table 6).

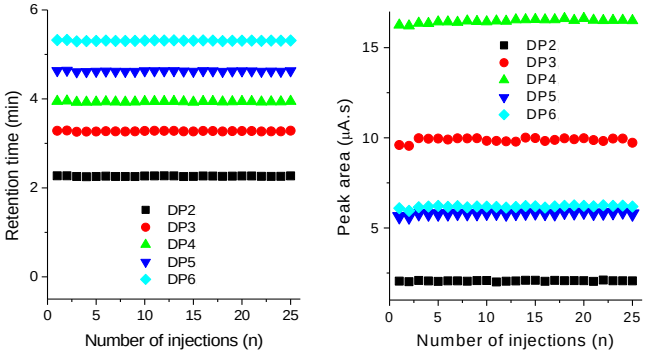

Figure 12: Retention times (left) and peak areas (right) of 25 subsequent analyses of a 100 mg/L solution of artificial sweetener containing maltodextrin. Top: retention time, Bottom: peak area. Conditions as in Table 2.

Table 6

Reproducibility (n = 25)

Component	*Retention time %RSD*	*Peak area %RSD*
DP2	0.27	1.2
DP3	0.23	1.2
DP4	0.19	0.6
DP5	0.17	1.1
DP6	0.14	1.0

Linearity and sensitivity

For DP3, DP5 and DP9 the linearity in detector response (peak area) was checked by diluting the 100 mg/L solution of artificial sweetener in the concentration range 2.5 mg/L - 100 mg/L.

The oligomers with DP3, DP5 and DP9 showed an excellent linear response in the specified concentration range (Figure 13 and Table 7). Amounts of 1 ng (on-column) of artificial sweetener gave well detectable peaks for the three oligomers (S/N > 15 for DP9) demonstrating the sensitivity of the method.

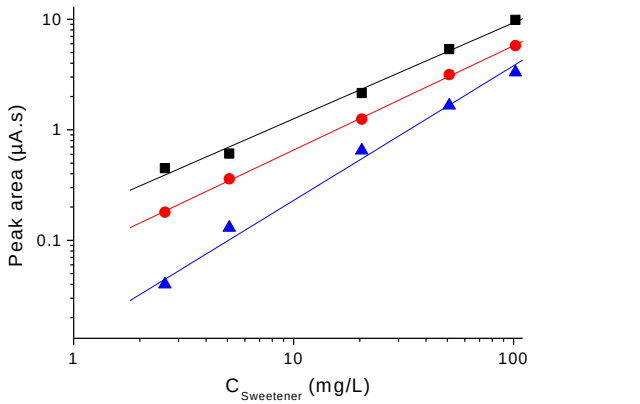

Figure 13: Peak area of DP3, DP5 and DP9 in response to the concentration of artificial sweetener in the concentration range of 2.5 - 100 mg/L. Conditions as in Table 2.

Table 7

Correlation coefficient r, determined via weighted linear regression method in the range of 2.5 - 100 mg/L artificial sweetener (Figure 13)

Component	*R*
DP3	0.9992
DP5	0.9989
DP9	0.9999

Selectivity RCX-10 column

In Table 7 the capacity factor for a series of carbohydrates are given as a function of the pH of the mobile phase. The capacity factors were determined using carbohydrate standards dissolved in water. This table can serve as a rough guideline to determine if the RCX-10 anion-exchange column has potentially enough selectivity for your specific application.

Table 8

Capacity factor k' as function of pH (NaOH concentration of the mobile phase) for a series of carbohydrates analyzed on an RCX-10 column

NaOH (mM)	*20*	*50*	*100*	*200*
pH	*12.3*	*12.7*	*13*	*13.3*
Inositol	0.38	0.33	0.32	0.30
Xylitol	0.73	0.65	0.61	0.59
Arabitol	0.85	0.7	0.68	0.63
Dulcitol	0.98	0.76	0.76	0.69
Adontiol	1.03	0.98	0.93	0.80
Sorbitol	1.14	0.93	0.86	0.79
Mannitol	1.32	1.03	0.98	0.83
Galactosamine	5.59	2.88	1.98	1.17
Fucose	5.82	3.45	2.68	1.72
Glucosamine	6.15	3.22	2.03	1.18
Arabinose	6.24	3.17	2.20	1.34
Galactose	6.53	3.49	2.42	1.43
Glucose	6.83	3.39	2.33	1.35
Mannose	7.69	3.67	2.19	1.25
Xylose	7.97	4.14	2.48	1.40
Sorbose	8.99	4.42	2.67	1.51
Fructose	9.20	4.13	2.77	1.60
Sucrose	9.50	7.43	5.40	4.43
Melibiose	9.66	5.65	3.25	1.85
Ribose	11.52	5.50	3.33	1.89
Lactose	13.48	7.92	4.45	2.18
Raffinose	13.49	10.89	7.33	3.73
Stachinose	15.05	11.06	8.10	4.35
Rhamnose		3.30	1.93	2.20
Cellobiose		11.25	6.41	3.28
Maltose		17.80	9.74	4.39
Maltotriose			11.02	

References

1. R.D. Rocklin, A. P. Clarke, M. Weitzhandler. (1998). Improved long-term reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform. Analytical chemistry, 70(8), 1496-1501.
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4. J.D. Olechno, S.R. Carter, W.T. Edwards, D.G. Gillen, Developments in the chromatographic determination of carbohydrates, Am. Biotech. Lab., 5, 38 50 (1987)
5. W.R. LaCourse, Pulsed Electrochemical Detection in High Performance Liquid Chromatography., John Wiley & Sons, New York, 1ed,1997.

Conclusion

The ALEXYS Carbohydrate Analyzer provides a reliable solution for the routine analysis of carbohydrates in food. Difficult sample matrices can be analysed using the binary gradient system. Excellent reproducibility and detection sensitivity are demonstrated.

Ordering information

Detector only	
176.0035A	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
ALEXYS analyzers	
180.0057W	ALEXYS Carbohydrate Analyzer with LPG gradient mixer
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. The ALEXYS Carbohydrates Analyzer 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.

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