Antec® Scientific

Carbohydrate Analysis

Application Notebook

High Performance Anion Exchange Chromatography with Pulsed Amperometry Detection (HPAEC-PAD)

Design: MGO-studio, Maarssen, NL
Illustrations: Antec Scientific

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Contents

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Don’t trust everything you see.
Even salt looks like sugar.

Introduction

Carbohydrates (also called saccharides) are the most abundant biomolecules in nature and play an important role in many physiological processes, e.g., metabolism, storage of energy, structure, etc. They are central to **nutrition** and are found in a wide variety of natural and processed foods.

Carbohydrate is a general term that includes sugars, starch, and cellulose. Saccharides are divided into four major groups: **monosaccharides**, **disaccharides**, **oligosaccharides**, and **polysaccharides**.

Examples of monosaccharides include **glucose** (dextrose), fructose (levulose), and galactose. Monosaccharides are the building blocks of **disaccharides** (such as sucrose and lactose) and **polysaccharides** (such as cellulose and starch). The table sugar used in everyday language is itself a disaccharide, sucrose which is a combination of of D-glucose and D-fructose.

Oligosaccharides contain a small number (typically two to ten) of monosaccharides and play an essential role in the **glycosylation** process in which an oligosaccharide is covalently attached to a protein or a lipid, creating structures such as glycoproteins and glycolipids. Other oligosaccharides such as Trans-galactooligosaccharides (TGOS) are prebiotic water-soluble carbohydrates. As a prebiotic, **TGOS** has a wide application in human and animal foods.

Polysaccharides are the most abundant carbohydrates found in food. They are long chain polymeric carbohydrates composed of monosaccharide units. Examples include storage polysaccharides such as starch, glycogen and galactogen and structural polysaccharides such as cellulose and chitin. **Inulin** is a naturally occurring polysaccharide composed of fructose, a plant-derived food that cannot be completely broken down by human digestive enzymes. The inulins belong to a class of **dietary fibers** known as **fructans** and are known for their prebiotic function.

Due to the presence of hydroxyl groups which can be oxidized, carbohydrates can be detected using pulsed amperometric detection with **pico- and femtomol sensitivity**. The analysis of carbohydrates is of interest to the **food industry** but also many fields in life sciences. One important field is **glycomics**. 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 with Pulsed Amperometric Detection (**HPAEC-PAD**) is the technique of choice for the sensitive analysis of saccharides of different origin. In the following section various applications are shown using Antec Scientific **ALEXYS™ Carbohydrate Analyzer.**

Corn is a healthy but "high carb" vegetable

Corn contains up to 19 g of total carbohydrates per 100 gram (3.5 oz), including 4 g starch (glucose polymer consisting of amylose and amylopectin), 3 gram dietary fiber (cellulose and other non-digestible oligo- and polysaccharides), 3 g sugar (disaccharide sucrose) and 0.1 g sugar alcohol (polyalcohol).

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.

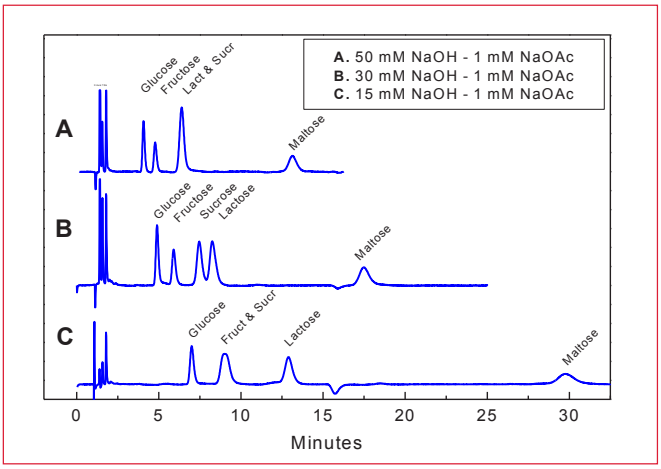

Figure 2: Retention times of common food carbohydrates as a function of sodium hydroxide concentration in the mobile phase

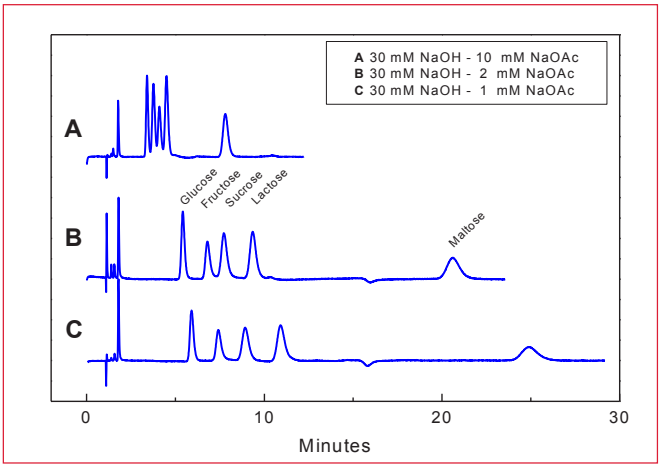

Figure 3: Retention times of common food carbohydrates as a function of the sodium acetate concentration in the mobile phase

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.

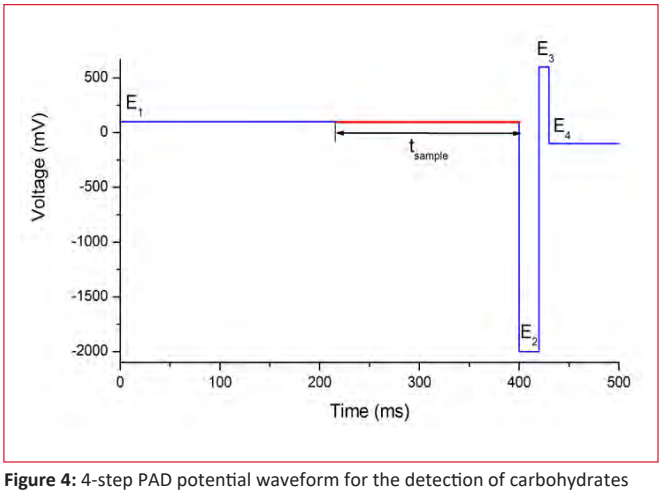

Figure 4: 4-step PAD potential waveform for the detection of carbohydrates

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.

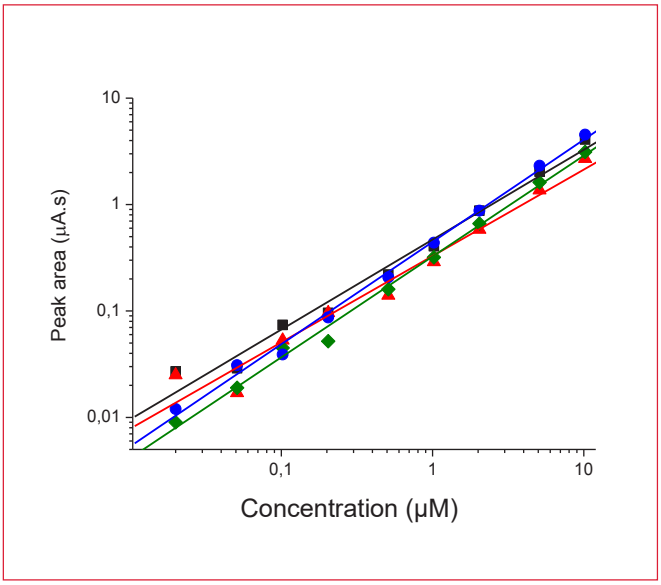

Figure 5: Calibration plots of glucose, fructose, sucrose and lactose in the concentration range 20 nM - 10 µM (n=10 per concentration). Conditions as given in Table 1.

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.

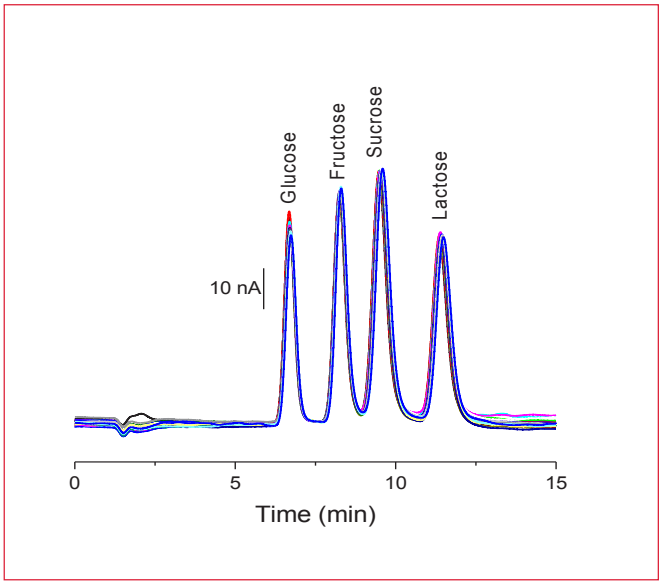

Figure 6: Overlay of 23 chromatograms of a standard mixture of 2 µM glucose, 2 µM lactose, 4 µM fructose and 4 µM sucrose in water (20 µl injected). The theoretical plate numbers for the components are 14.900, 10.900, 12.000 and 17.300 plates/meter, respectively. Conditions as given in Table 1, except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

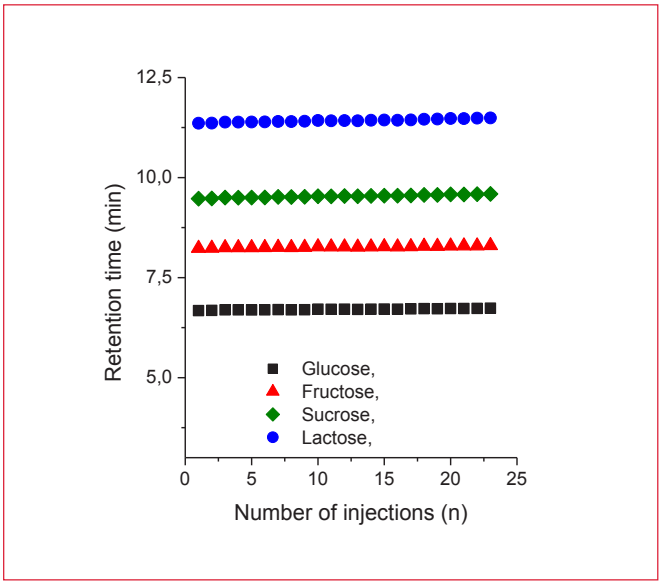

Figure 7: Retention times from 23 consecutively analyzed injection of a standard mixture of 2 µM glucose, 2 µM lactose, 4 µM fructose and 4 µM sucrose in water, 20 µL injections. Conditions as given in Table 1.

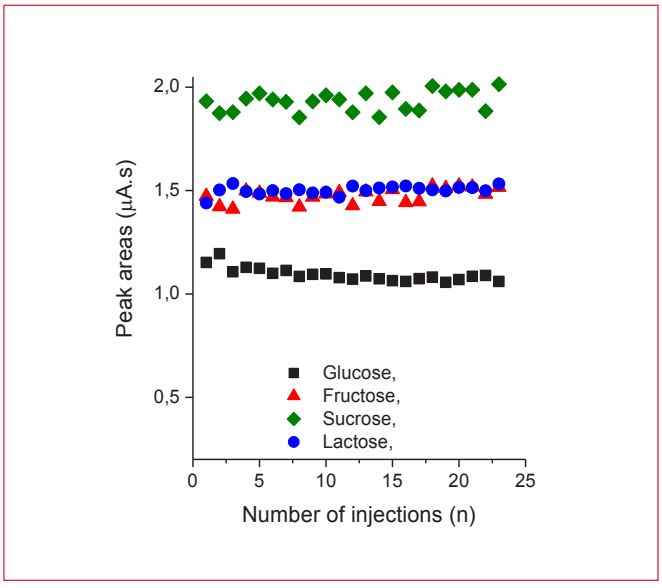

Figure 8: Peak area from 23 consecutively analyzed injection of a standard mixture of 2 µM glucose, 2 µM lactose, 4 µM fructose and 4 µM sucrose in water, 20 µL injections. Conditions as given in Table 1.

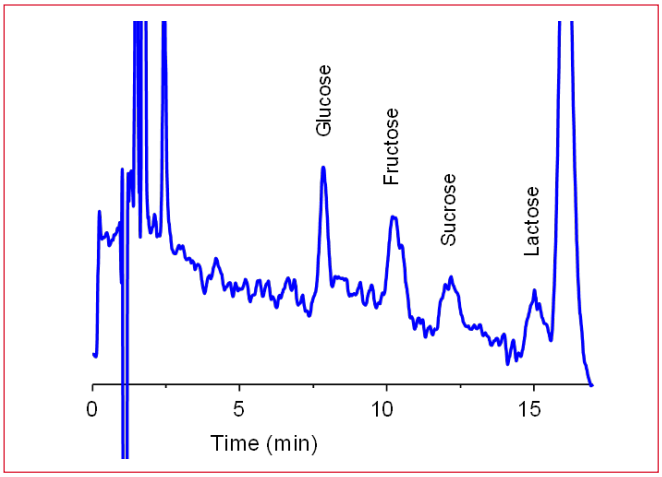

Figure 9: Chromatogram of a 20 nM glucose, lactose, fructose and sucrose in water, 20 µL injections. Conditions as given in Table 1.

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.

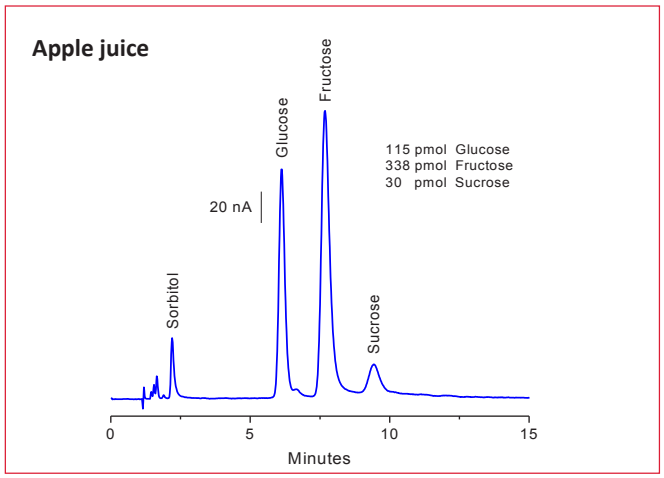

Figure 8: Chromatogram of apple juice. Sample diluted 10.000 x with water. Conditions as in Table 1, except 10 µL injection, and mobile phase: 30 mM NaOH.

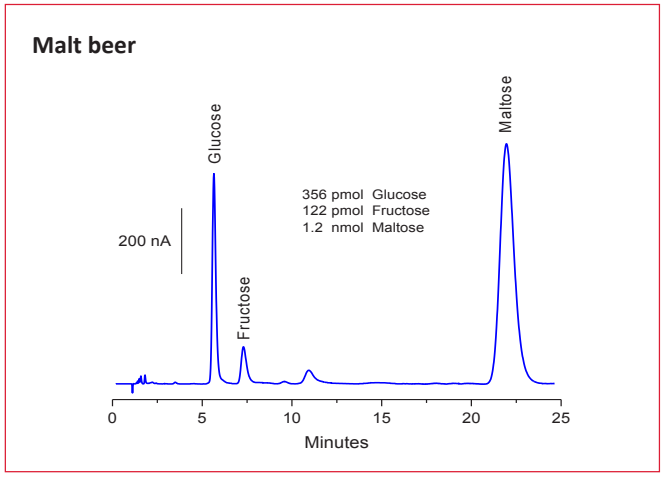

Figure 9: Chromatogram of malt beer. Sample was degassed for 10 minutes (ultrasonic bath) to remove dissolved CO_2 and diluted 1.000 x with water. Conditions as in Table 1, except 10 µL injection, and mobile phase: 30 mM NaOH, 2 mM NaOAc.

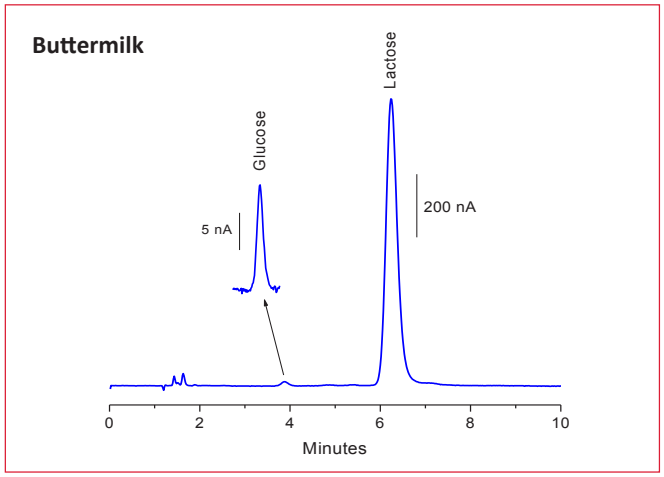

Figure 10: Chromatogram of buttermilk. Sample was diluted 1.000 x with water and filtered over a 0.2 µm membrane. Conditions as in Table 1, except 10 µL injection, and mobile phase: 30 mM NaOH.

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.

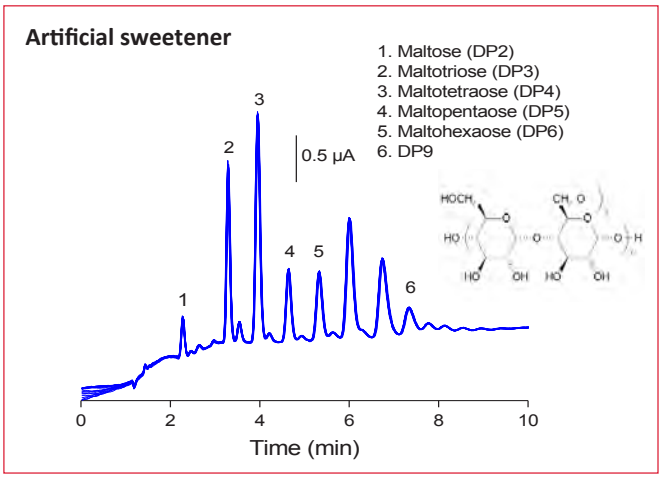

Figure 11: Overlay of 25 chromatograms of a 100 mg/L solution of artificial sweetener containing maltodextrin. Conditions as in Table 2.

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).

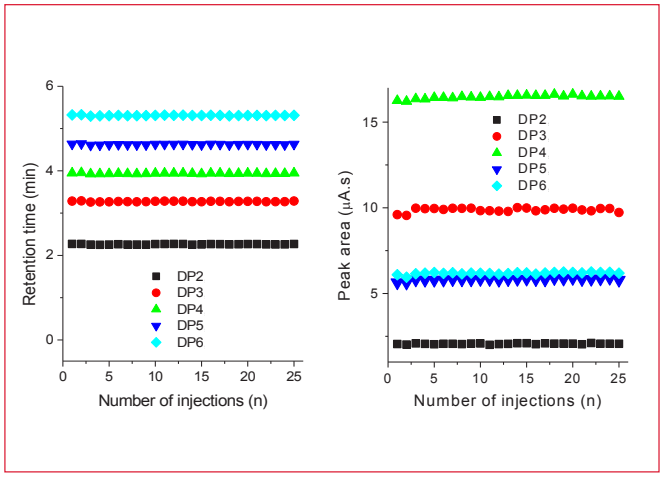

Figure 12: Retention times (left) and peak areas (right) of 25 subsequent analyses of a 100 mg/L solution of artificial sweetener containing maltodextrin. Top: retention time, Bottom: peak area. Conditions as in Table 2.

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.

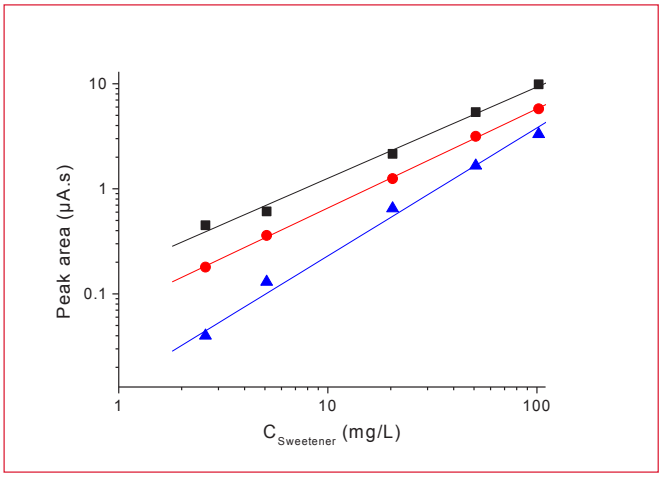

Figure 13: Peak area of DP3, DP5 and DP9 in response to the concentration of artificial sweetener in the concentration range of 2.5 - 100 mg/L. Conditions as in Table 2.

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.
2. D.C. Johnson, D. Dobberpuhl, R. Roberts, P. Vandeberg, Review: Pulsed amperometric detection of carbohydrates, amines and sulfur species in ion chromatography the current state of research, J. Chromatogr., 640, 79-96 (1993)
3. D.C. Johnson, W.R. LaCourse, LC with pulsed ECD at gold and platinum, Anal. Chem., 62, 589A – 597 A (1990)
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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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
Back pressure	About 210 bar (at start of the run)
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.

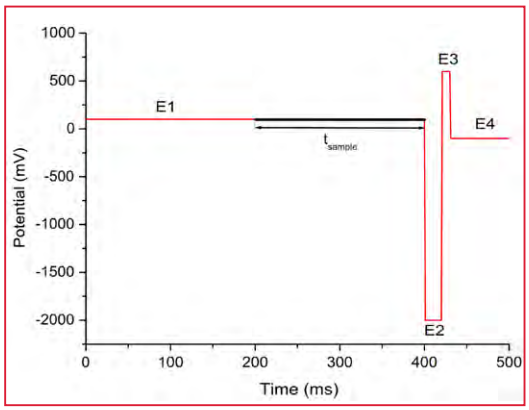

Figure 2: 4-step PAD potential waveform for the detection of carbohydrates.

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).

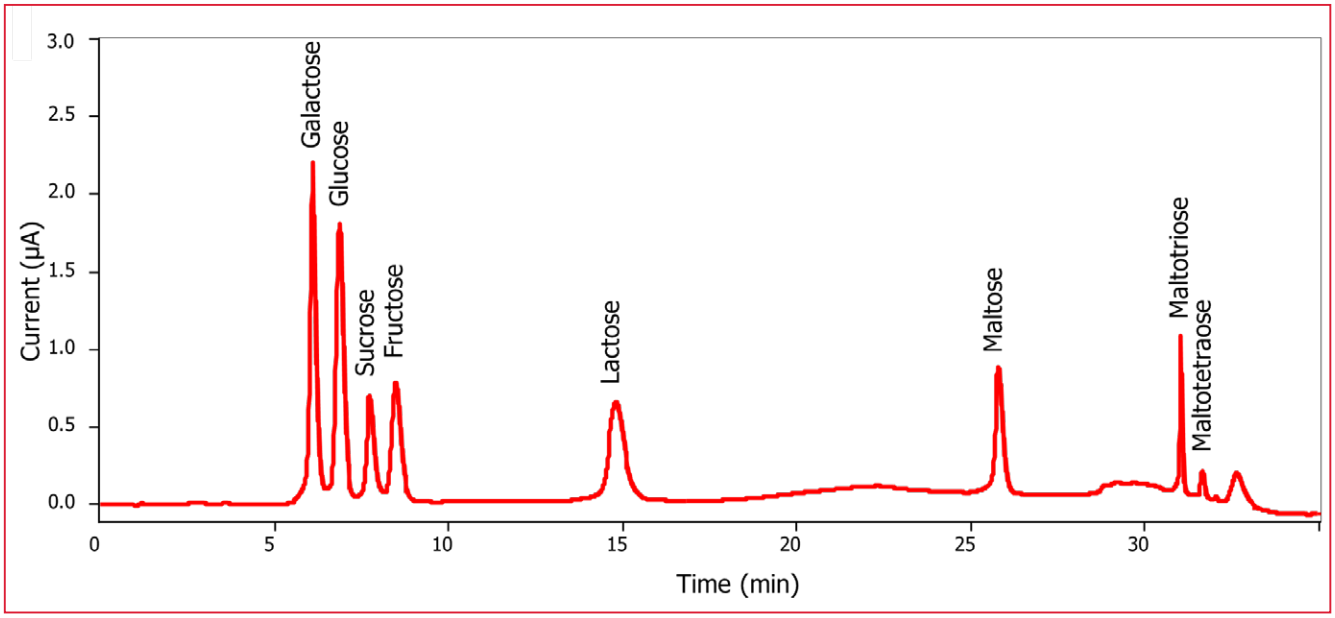

Figure 3: Chromatogram of a 25 µL injection of a 10 µg/mL standard mix of 8 saccharides in water: (1) Galactose, (2) Glucose, (3) Sucrose, (4) Fructose, (5) Lactose, (6) Maltose, (7) Maltotriose and (8) Maltotetraose.

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.

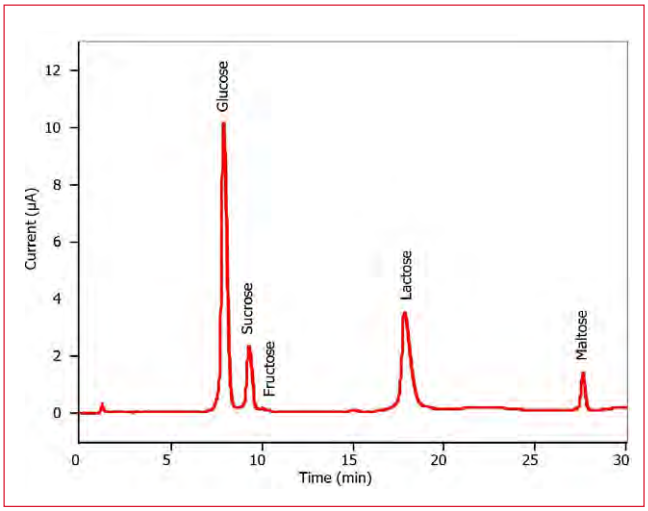

Figure 4: Chromatogram of a 25 µL injection of a 0.5 g/L infant cereal sample (red).

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

1. T. R. Vennard, A. J. Ruosch, S. M. Wejrowski, D. J. Ellingson, Sugar Profile Method by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection in Food, Dietary Supplements, Pet Food, and Animal Feeds: First Action 2018.16, Journal of AOAC International Vol. 103, 89 –102
2. A. Maton, J. Hopkins, C.W. McLaughlin, S. Johnson, M.Q. Warner, D. LaHart, J.D. Wright, Human Biology and Health, 1993, 52 - 59
3. D.C. Johnson, D. Dobberpuhl, R. Roberts, P. Vandeberg, Review: Pulsed amperometric detection of carbohydrates, amines and sulfur species in ion chromatography- the current state of research, J. Chromatogr., 640 (1993), 79 -96
4. D.C. Johnson, W.R. LaCourse, LC with pulsed ECD at gold and platinum, Anal. Chem., 62 (1990), 589A – 597
5. W.R. LaCourse, Pulsed Electrochemical Detection in High Performance Liquid Chromatography, John Wiley & Sons, New York, 1ed, 1997
5. J. Rohrer, Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection, TN70669-EN 05/17S, 2017
6. 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

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

*) 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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Carbohydrates are the major constituents of coffee beans

Carbohydrates serve various functions like binding of aroma, stabilization of foam, formation of sedimentation, and increased viscosity of the extract.
The principal low molecular weight carbohydrate is the disaccharide sucrose.
During roasting, the polysaccharide content is reduced due to degradation to low molecular weight carbohydrates (mono- and oligosaccharides).

Application Note

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A, C, D, E, and K
Iodide
Q10, ubiquinols

Carbohydrates in Instant Coffee

- **ALEXYS Carbohydrate Analyzer**
- **Method is based on HPAEC-PAD**
- **Analysis adapted from ISO Method 11292:1995**
- **Comparison of the standard method with adapted method**
- **Adapted method is 'fast and green'**

Summary

In this application note the analysis of carbohydrates in instant coffee, following the ISO 11292:1995 method, was evaluated using the ALEXYS Carbohydrates Analyzer. The method is based on separation of carbohydrates by High Performance Anion Exchange Chromatography followed by Pulsed Amperometric Detection (HPAEC-PAD) under standard bore conditions. An adapted method was evaluated, which uses narrow-bore conditions and a column with smaller particle size. In comparison with the standard method, the adapted method resulted in 50% shorter analysis times and 90% lower solvent use ('greener' solution). Both standard bore and narrow-bore methods provide excellent sensitivity, linearity and reproducibility, and are suitable for the routine analysis of carbohydrates in coffee applications using the ALEXYS Carbohydrates Analyzer.

Introduction

Carbohydrates (also called saccharides) are the most abundant biomolecules in nature and a major constituent in many food and beverage products. These molecules provide the most easily accessible energy source for our body and are highly valued for their structural properties and (sweet) taste. Measurement of their presence in food and drinks is required for product labeling purpose, but carbohydrate composition is also a very good tracer for authenticity assessment [1, 2].

Coffee is one of the most popular drinks in the world. Carbohydrates are the major constituents of coffee beans and they act as aroma binders, foam stabilizers and they increase the viscosity of the drink. Their analysis in coffee and coffee products is covered by method ISO 11292:1995 [3], issued by the International Organization for Standardization (ISO). The principle of this method is the same as Method 995.13 [4], which was issued in 1995 by the Association of Analytical Chemists (AOAC) and originally implemented as the UK national standard BS 5752-15:1995. This UK standard was withdrawn and replaced in 1997 by the unaltered text of the ISO method as BS 5752-15:1997 [5]. The text of the ISO method has further been approved by the governments as suitable for publication without deviations as the national standard method for use in India [6] and Mexico [7], which are major coffee producing countries. Germany, which is a coffee importing country, has adopted a translated version of the ISO method [8].

The principle of the ISO method for analysis of free and total carbohydrate content in soluble/instant coffee is:

- extraction of the free carbohydrates from the coffee with water, and hydrolysis of the coffee to obtain an extract of the monosaccharides for quantitation of the total carbohydrate content
- separation of the individual mono- and disaccharides with High Performance Anion Exchange Chromatography (HPAEC) using water as the mobile phase
- detection on a gold electrode using Pulsed Amperometric Detection (PAD) after post-column NaOH addition.

The ISO method was developed in a time when applying normal bore HPLC in combination with large particles size was the best available commercial option. Nowadays, commercially available HPAEC columns with a smaller internal diameter are available, which reduces the need of solvents (‘going green’). Another development has been the reduction of column particle size. The higher separation efficiencies of smaller particles can be used to reduce the analysis time. To apply these new column types, the only adaptation to the parameters given in the ISO method is to lower the given flow rate and injection volume to match the narrower column.

This note shows the applicability of Antec’s ALEXYS Carbohydrate Analyzer (Figure 1) to analyze carbohydrates using the standard ISO method. The second part of this note shows the excellent results (‘fast and green’) when using the dedicated ALEXYS Carbohydrates Analyzer and a modern narrow-bore small particle sized HPAECcolumn.

Figure 1: ALEXYS Carbohydrate Analyzer with post-column addition pump

Method

The ALEXYS Carbohydrate Analyzer is a system for HPAEC-PAD analyses and it was developed around the DECADE Elite™ electrochemical detector. The system is standard equipped with a low pressure Solvent Selection Valve (SSV) for easy switching between solutions, helium blanketing hardware (pressure resistant bottles, pressure regulator, valves and lines), autosampler and column thermostat. The system was expanded with a post-column NaOH addition pump to meet the requirements of the method. The system is standard equipped with normal bore HPLC PEEK lines, but for the method valuation of the adapted ‘fast and green’ method, a dedicated narrow-bore tubing set was installed, as well as a high pressure SSV with a 2 mL stainless steel loop (pressurized reservoir for column flush solution).

For comparison purpose, a summary of the ISO method 11292: 1995 is given in Table 1, which is by no means intended to make the use of the official text superfluous.

Table 1

Summary of method ISO 11292: 1995

HPLC	Metal free liquid chromatograph
Detector	Pulsed amperometric detector (PAD) with gold electrode
Column	High-performance anion-exchange column filled with pellicular polystyrene-divinylbenzene resin and guard column
Mobile phase (Eluent S1)	demineralized water (18 MΩ·cm)
Post-column addition and post-run flush solution (Eluent S2)	0.3 M NaOH in demineralized water
Flow rate mobile phase	1.0 mL/min
Flow rate post column addition	0.6 mL/min
Temperature	Ambient
Detector settings	Use the optimum conditions given by the manufacturer
Carbohydrate standard solutions	Arabinose, fructose, galactose, glucose, mannose, sucrose, mannitol
$V_{injection}$	20 µL
Analysis	Inject a standard solution every 4 injections to account for any changes in retention times or peak integration

Table 2

Conditions, based on ISO method 11292:1995

HPLC	ALEXYS Carbohydrate Analyzer.
Add-on parts	Pump and mixing coil for post-column NaOH addition
Mobile phase	Demineralized water (18 MΩ·cm, TOC<10ppb), blanketed with Helium 5.0
Post-column addition	0.3 M NaOH in demineralized water, blanketed with Helium 5.0
Post-run flush solution	0.3 M NaOH in demineralized water, blanketed with Helium 5.0
Temperature	32 °C*
Flow cell	SenCell™ with gold working electrode, HyREF™, AST 2
Detector settings	PAD mode (4-step): E_1, E_2, E_3, E_4: +0.1, -2.0, +0.6, -0.1 V t1, t2, t3, t4: 0.40, 0.02, 0.01, 0.07 s; ts: 200 ms
I-cell	0.2-1.0 µA
ADF	0.02 Hz
Range	20 µA/V (1 µA/V for measuring near-LOD levels)

Separation

Due to the numerous hydroxyl groups on carbohydrate molecules, they can be well separated in isocratic mode on a HPAEC separation column using water as the mobile phase. Post-column addition of NaOH is necessary to increase the pH and make the carbohydrates detectable at the gold electrode. A 15-minute post-run column clean up with 0.3 M NaOH is part of the ISO method to keep column performance in check. A re-equilibration time of 15 minutes with mobile phase makes the column ready for the next injection.

Temperature is a crucial parameter for separation, and stabile reproducible retention times are only possible using a thermostatic compartment. The ISO method, however, states the use of ambient temperature, and the analysis of a standard every 4 injections to compensate for any changes in the chromatograms. The moment-to-moment temperature changes in a laboratory without air-conditioning have a significant effect on the retention times (data not shown), and therefore we chose to apply a fixed temperature that was only a few degrees above ambient.

An HPAEC column with 4 mm ID and 10 µm particles is mentioned in the ISO method as an example of a suitable column (Table 1 and 3). An HPAEC column with narrower internal diameter and smaller particles that fits the description of the ISO method is chosen to make the 'fast and green' method (Table 1 and 4).

Traditional conditions:
The traditional wide-bore HPAEC column (and matching precolumn) was chosen for method evaluation, using conditions given in Table 2 and Table 3. A schematic representation of the system is given in Figure 2. These conditions are advised in case there is no freedom to apply the reduced flow rate as needed for the adapted method.

Adapted conditions for the 'fast and green' method:
A narrow-bore HPAEC column and matching precolumn are chosen for the 'fast and green' method, with conditions given in Table 2 and Table 4. Due to the narrow-bore dimensions of the column, a lower flow rate has to be applied to compensate for the narrower internal diameter, and the flow rate of the post-run NaOH addition is decreased accordingly.

Table 3

Conditions matching the ISO method settings	
Column (guard + analytical)	Thermo Scientific™ Dionex™ CarboPac™ PA1 4 x 50 mm, 10 µm + 4 x 250 mm, 10 µm
Mixer volume	150 µL
Flow rate	1.0 mL/min: mobile phase 0.6 mL/min: post-column addition
Back pressure LC	about 70 bar
Back pressure PC	about 95 bar (post-column addition)
Injection volume	20 µL

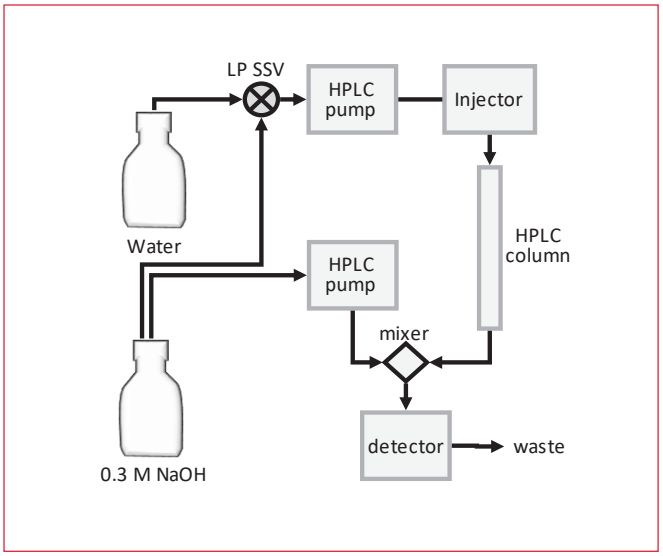

Figure 2: Schematic hardware representation of the set-up as used for testing the original conditions

Table 4

Adapted 'fast and green' method settings	
Column (guard + analytical)	Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast-4µm, 30 x 2.0 mm ID, 4 µm + 150 x 2.0 mm ID, 4 µm
Mixer volume	8 µL
Flow rate	0.15 mL/min: mobile phase 0.09 mL/min: post-column addition
Back pressure LC	about 181 bar
Back pressure PC	about 188 bar (post-run flush)
Injection volume	5 µL

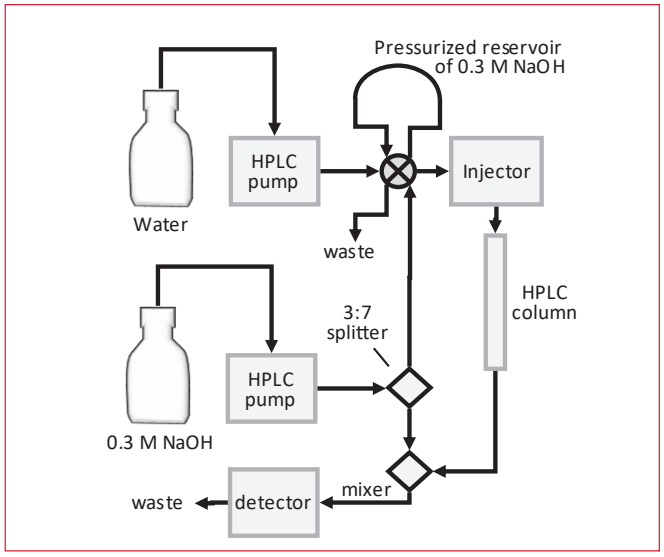

Figure 3: Schematic hardware representation of the set-up as used for testing the adapted 'fast and green' conditions

The lag time of the post-run column flush is minimizes by installing the pressurized reservoir with column flush solution right before the injector. A splitter provides a constant flow of NaOH solution to the mixer at a flow rate of 90 µL/min and to the pressurized reservoir (2 mL) at a flow rate of 210 µL/min. The solution in the pressurized solvent reservoir is redirected to the columned before the end of the run and refilled during the first part of the next run. A schematic representation of the system is given in Figure 3.

Detection

Carbohydrates need a high pH to be detectable on a gold electrode with PAD. This makes post-column NaOH addition necessary when running pure water as the mobile phase. Under the recommended mixing ratio, the acidity inside the electrochemical flow cell is above pH 13. The optimum conditions for the PAD settings are a 4-step potential waveform as shown in Figure 4. This has been claimed to have as benefit (1) a consistent long-term peak area response and (2) minimal electrode wear [9]. The DECADE Elite applies this 4-step pulse to the gold electrode of SenCell, which has a three-electrode configuration. For analysis of carbohydrates, a combination of gold working electrode (WE), HyREF (Pd/H_2) reference electrode (REF) and stainless-steel auxiliary electrode (AUX) is advised. The chromatograms were recorded using Clarity software (Data Apex), which was also used to control the system.

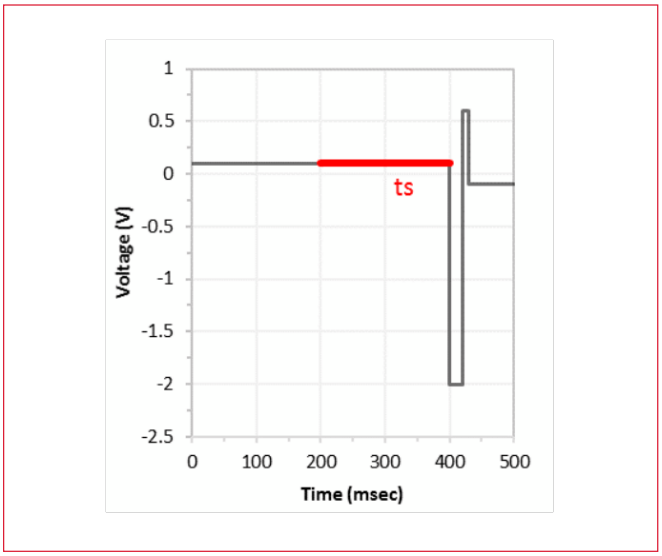

Figure 4: 4-step PAD potential waveform for the detection of carbohydrates. The duration of the sampling time (ts) is highlighted in red.

Performance evaluation

Performance of both traditional and 'fast and green' methods were evaluated using standard measurements for repeatability (n=6), linearity and Limit of Detection (LOD). The LOD levels were calculated for each carbohydrate as the concentration that would show a signal-to-noise ratio of 3, using the response of a 10 µM standards mix chromatogram recorded under a range setting of 1 µA/V and an ADF setting of 0.02 Hz. The ASTM noise level was calculated as the average from 30 subsequent 0.5 min segment peak-to-peak values.

Sample measurements

The soluble coffee sample preparation was done according to the standardized method10, which comprises (1) the extraction of 'free' carbohydrates with water, and (2) generating a hydrolyzed fraction that yields the total carbohydrate content as a mix of monosaccharides:

Sample preparation for analysis of free carbohydrates
Extract the free carbohydrates from approximately 300 mg of instant coffee with 70 mL water by shaking in a 100 mL volumetric flask until dissolution is complete. Dilute to the mark with water and pass 5-10 mL of the solution through a C18 cartridge. Discard the first few milliliters and pass the filtrate through a 0.2 µm membrane filter before injection.

Sample preparation for analysis of total carbohydrates
Hydrolyze the carbohydrates by heating approximately 300 mg of instant coffee dissolved in 70 mL 1.0 M HCl in a boiling water bath for about 2.5 h. Keep the level of the solution below that of the water and swirl every 30 min. Cool to room temperature with running tap water before diluting to the mark with water. Filter the solution through a folded filter paper and pass 3 mL of the solution through a disposable cartridge in the silver form to eliminate the chloride ions. Discard the first few milliliters and pass the filtrate through a 0.2 µm membrane filter before injection.

Standards
For quantification purpose, stock solutions of individual carbohydrates in water were mixed and diluted with water to prepare calibration standards in the range of 1-10 µM (10x higher galactose and arabinose concentrations). An additional calibrator standard mix (Table 5) was prepared with concentrations close to the levels as found in the hydrolyzed fraction (single point calibration).

Table 5

Calibrator concentrations for quantification of hydrolyzed fractions of coffee using traditional conditions as given in Table 3

Component	*µM*	*mg/L*
Mannitol	2	0.4
Arabinose	500	75
Galactose	500	90
Glucose	500	90
Sucrose	50	17
Xylose	10	1.5
Mannose	100	18
Fructose	50	9

Results - traditional conditions

Figure 5 shows the chromatogram for a standard mix of the 8 carbohydrates in water that are mentioned in the ISO method for the analysis of instant coffee, plus xylose. All the peaks are well separated (resolution ≥ 1.5), including the potentially problematic peak pair of sucrose and glucose (Table 6). The total analysis time for each sample is 80 min, which includes the post-run step gradient for column clean-up and 15 min re-equilibration time.

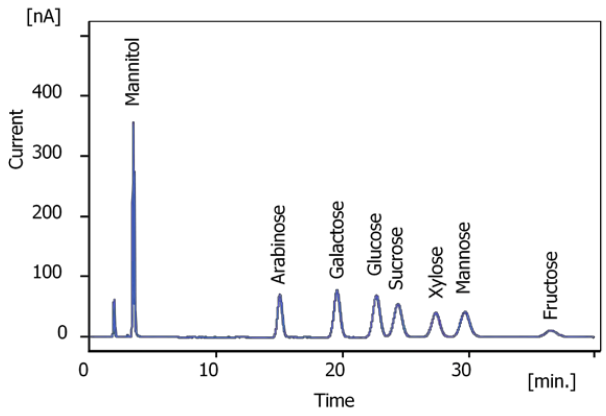

Figure 5: Chromatogram of a 20 µL injection of 10 µM standard mix of 8 carbohydrates in water, analyzed with ISO method 11292:1995 as detailed in Table 2 and Table 3. Peak parameters given in Table 6.

Table 6

Peak table of chromatogram given in Figure 5

Component	*Tr (min)*	*Area (nA.sec)*	*Height (nA)*	*Assym. (-)*	*Eff. (t.p.)*	*Eff/l (t.p./m)*	*Res. (-)*
Mannitol	3.50	3 051	356	1.14	3 301	11 005	
Arabinose	15.1	1 845	72	1.05	7 526	25 086	24.9
Galactose	19.6	2 677	78	1.02	7 018	23 392	5.6
Glucose	22.7	2 743	69	1.04	7 303	24 342	3.1
Sucrose	24.4	2 503	55	1.01	6 417	21 391	1.5
Xylose	27.4	1 847	40	0.98	7 918	26 392	2.5
Mannose	29.7	2 136	42	1.05	7 483	24 944	1.8
Fructose	36.6	686	11	1.10	7 299	24 330	4.5

Repeatability

The ISO method states the advice to analyze a standard after every 4 injections to account for any changes in retention time or response. The repeatability of retention time was >2%RSD (n=4) when applying ambient temperature (data not shown). With the use of the ALEXYS system and application of thermostatted instead of ambient temperature, excellent repeatability of chromatograms (n=6) is evident from the obtained RSD values: <0.25% for retention time and <2% for peak area for most of the carbohydrates (Table 7).

Table 7

Repeatability (n=6) using 20 µL of 10 µM carbohydrate standards mix in water, analyzed with ISO method 11292:1995 as detailed in Table 2 and Table 3

Component	*Retention time RSD (%)*	*Peak area RSD (%)*
Mannitol	0.16	0.5
Arabinose	0.15	0.8
Galactose	0.14	0.4
Glucose	0.19	0.7
Sucrose	0.24	1.0
Xylose	0.20	1.1
Mannose	0.21	1.2
Fructose	0.20	3.8

Linearity

The peak area responds linearly in the tested range of 1-10 µM standards (and 10-100 µM for galactose and arabinose) with a correlation coefficients of at least 0.998 (except 0.995 for fructose). As a double check, the carbohydrate concentrations in the calibrator solution for the hydrolyzed samples (Table 5) were quantified using the standards generated to evaluate linearity, and the outcome deviated less than 2% from the concentrations that they were supposed to represent, except for fructose (showing 10% deviation). Fructose seems to behave slightly different compared to the other tested carbohydrates, as it was also showing the highest RSD value when checking repeatability.

Detection limit

The Limit of Detection (LOD) was calculated for each carbohydrate as the concentration that would show a signal-to-noise ratio of 3, using the response and noise level in a 10 µM standards mix chromatogram. The noise level was 0.1 nA and the calculated LOD's were in the range of 0.01 0.40 µM or 0.002 - 0.07 mg/L (Table 8). Considering the ISO method prescription where 300 mg of sample is extracted with 100 mL water, the detection limit for the various carbohydrates is in the range of 0.1 2 mg/100 g product using the ALEXYS system and the traditional conditions.

Table 8

Calculated LOD (S/N = 3) for 20 µL injections using ISO method 11292:1995 as detailed in Table 2 and Table 3 (range 1 µA/V; ADF: 0.02 Hz)

Component	*µM*	*mg/L*
Mannitol	0.01	0.002
Arabinose	0.07	0.010
Galactose	0.06	0.010
Glucose	0.07	0.012
Sucrose	0.10	0.036
Xylose	0.13	0.019
Mannose	0.14	0.024
Fructose	0.41	0.074

Table 9

Concentration of carbohydrates (g/100 g) in a sample of instant coffee, analyzed using traditional version of ISO method

Component	*A - free*	*B - total*
Mannitol	0.013	0.015
Arabinose	1.0	4
Galactose	0.22	13
Glucose	0.20	19
Sucrose	0.061	-
Xylose	0.006	0.15
Mannose	0.07	5
Fructose	0.09	0.19
Total	**1.7**	**41**

Sample measurement: instant coffee

A sample of instant coffee was analyzed using the traditional conditions, and the main peaks in the chromatograms were well separated and identified (Figure 6 and 7). The concentrations of free and total carbohydrates are given in Table 9. The high level of total glucose (>>2.46%) suggests that the tested instant coffee is not pure [1, 2].

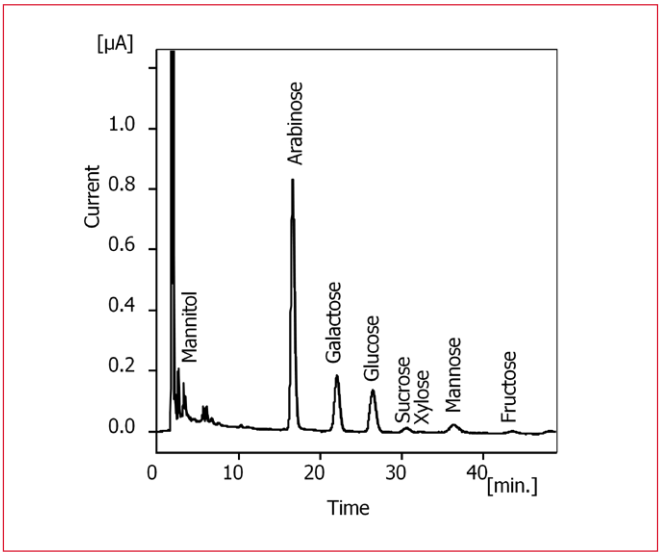

Figure 6: Chromatogram of the *free* carbohydrate fraction (non-hydrolyzed) of an instant coffee sample extracted and analyzed according to ISO method 11292:1995 with details as given in Table 2 and Table 3.

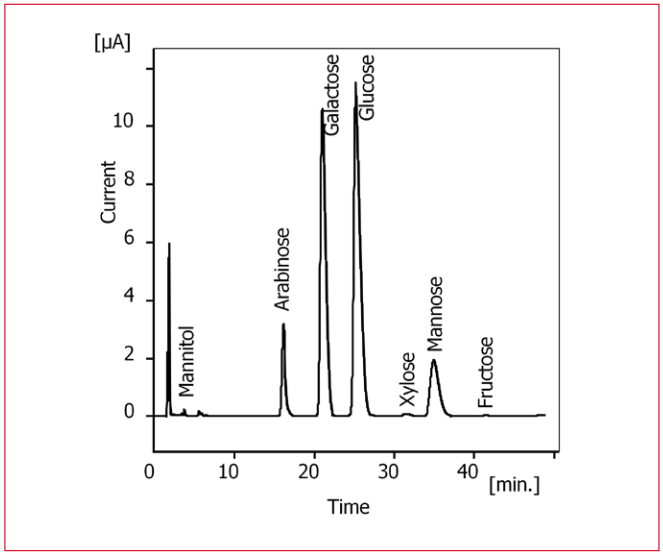

Figure 7: Chromatogram of the *total* carbohydrate fraction (hydrolyzed) of an instant coffee sample extracted and analyzed according to ISO method 11292:1995 with details as given in Table 2 and Table 3.

Results - adapted 'fast and green' method

Using the dedicated ALEXYS Carbohydrates Analyzer and the narrow-bore HPAEC column, the analysis of the standards mix resulted in the chromatogram shown in Figure 8. All the peaks are well separated (resolution ≥ 1.5) and the total analysis time for each sample is reduced to 35 min, which includes a 10 min post-run step gradient for column clean-up and 10 min re-equilibration time. The reduction in solvent use is almost 90% per analysis, compared to the traditional settings.

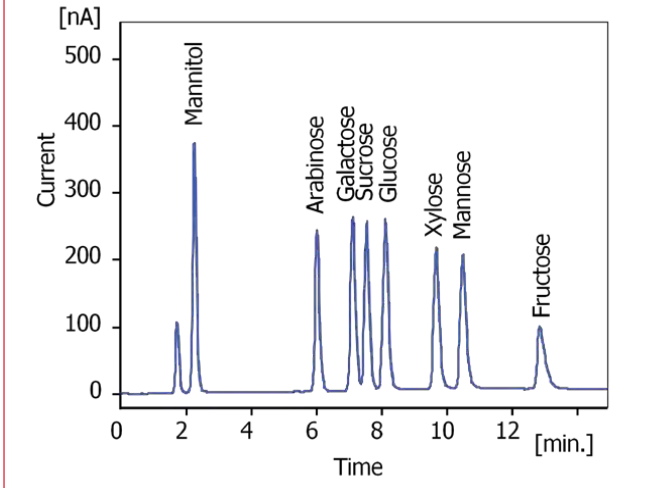

Figure 8: Chromatogram of a 5 µL injection of 10 µM standard mix of 8 carbohydrates in water, using adapted 'fast and green' version of ISO method 11292:1995 as detailed in Table 2 and Table 4. Peak details in Table 10.

Table 10

Peak table of chromatogram given in Figure 8

Component	*Tr (min)*	*Area (nA.sec)*	*Height (nA)*	*Assym. (-)*	*Eff. (t.p.)*	*Eff/l (t.p./m)*	*Res. (-)*
Mannitol	2.3	3 030	371	1.31	1 578	8 764	-
Arabinose	6.0	2 337	241	1.50	8 889	49 381	15.7
Galactose	7.1	2 760	260	1.29	9 140	50 780	4.0
Glucose	7.5	2 671	253	1.38	11 318	62 880	1.5
Sucrose	8.1	2 970	255	1.33	10 837	60 203	1.9
Xylose	9.7	2 531	211	1.39	14 092	78 288	4.9
Mannose	10.5	2 604	200	1.61	15 197	84 428	2.4
Fructose	12.8	1 573	92	2.65	12 847	71 374	6.0

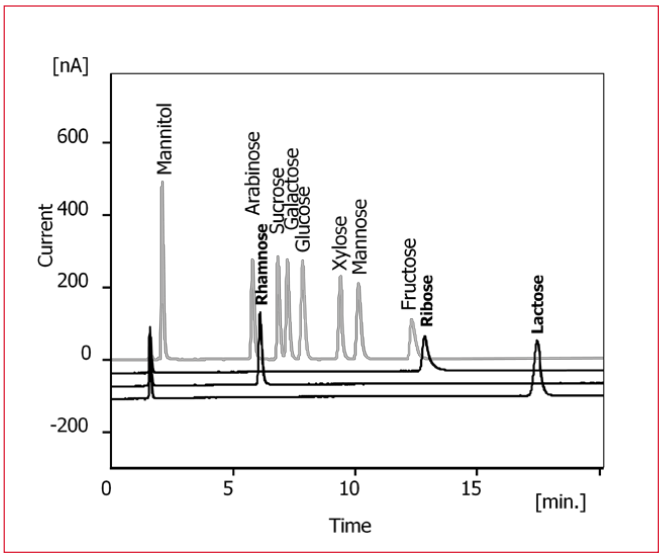

Figure 9: Chromatogram overlay of 5 µL injections of 10 µM standard mix of different carbohydrates in water, analyzed with adapted 'fast and green' version of ISO method 11292:1995 as detailed in Table 2 and Table 4. The light grey trace is the same standard mix of 8 carbohydrates as presented in Figure 8 for comparison purpose.

According to the ISO method, rhamnose and arabinose are a potentially problematic pair to separate, and the advice is not to add rhamnose in case they co-elute. Figure 9 shows a chromatogram overlay that indicates that rhamnose, ribose and lactose (relevant for other applications) are not co-eluting with the other peaks.

Repeatability

The excellent repeatability of chromatograms using the ALEXYS system is evident from the obtained RSD values, which are <0.4% for retention time and <0.3% for peak area, except for fructose (Table 11).

Linearity

The peak area responds linear in the tested range of 0.2 - 10 µM and 10 - 250 µM with a correlation coefficients of 0.999 for all carbohydrates, except sucrose. Concentrations higher than 250 µM result in increased peaks asymmetry and relatively lower area response. For sucrose, the response stops being linear above 50 µM (=17 mg/L).

Table 11

Repeatability (n=6) using 5 µL of 10 µM carbohydrate standards mix in water, analyzed with adapted 'fast and green' version of ISO method 11292:1995 as detailed in Table 2 and Table 4

Component	*Retention time RSD (%)*	*Peak area RSD (%)*
Mannitol	0.29	0.1
Arabinose	0.18	0.1
Galactose	0.18	0.2
Glucose	0.15	0.2
Sucrose	0.17	0.2
Xylose	0.15	0.2
Mannose	0.12	0.3
Fructose	0.37	1.8

Table 12

Calculated LOD (S/N = 3) for 5 µL injections using adapted 'fast and green' version of ISO method 11292:1995 as detailed in Table 2 and Table 4 (Range 1 µA/V; ADF: 0.02 Hz)

Component	*nM*	*µg/L*
Mannitol	17	3
Arabinose	25	4
Galactose	24	4
Glucose	24	4
Sucrose	24	8
Xylose	29	4
Mannose	31	6
Fructose	61	11

Detection limit

With a noise level of 0.2 nA, the calculated LOD's were better than 0.03 µM for most tested carbohydrates, except for fructose (Table 12). Comparing the LOD values with those reported using the already highly sensitive traditional conditions, about 4x lower LOD levels are found for the adapted 'fast and green' method. Note that the injection volume was also a factor 4 lower (20 vs 5 µL), giving a total on-column detection sensitivity improvement of a factor around 16 (with some deviations among components).

Sample measurement: instant coffee

Both hydrolyzed and non-hydrolyzed samples of the same instant coffee were re-analyzed using the adapted 'fast and green' method, resulting in the chromatograms shown in Figure 10, Figure 11 and the carbohydrate composition given in Table 13.

The hydrolyzed sample had to be diluted 10x with water before analysis, as undiluted sample showed tailing peaks with an asymmetry factor of 3, indicating column saturation

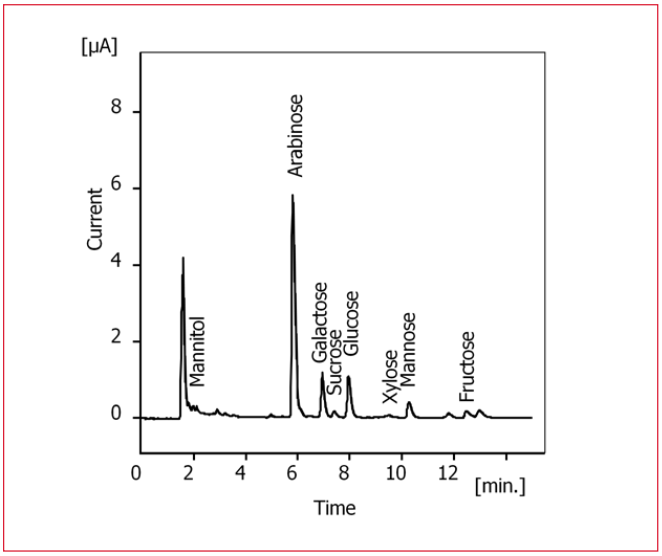

Figure 10: Chromatogram of the free carbohydrate fraction (non-hydrolyzed) of an instant coffee sample extracted according to ISO method 11292:1995 and analyzed using a narrow bore HPAEC column (adapted 'fast and green' conditions) as detailed in Table 2 and Table 4.

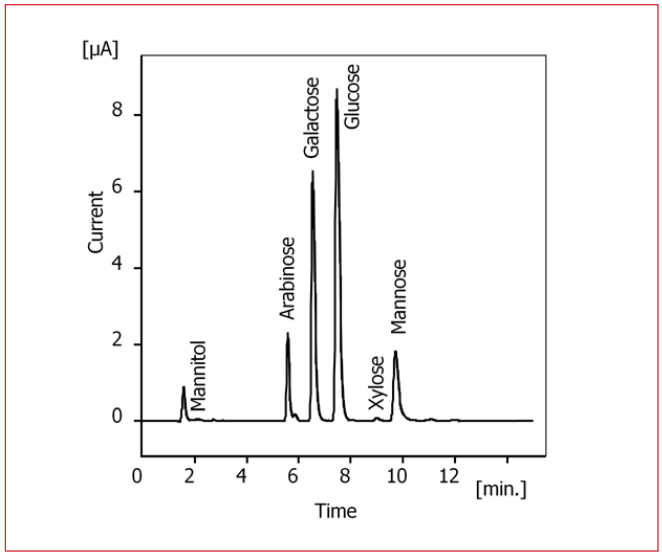

Figure 11: Chromatogram of the total carbohydrate fraction (hydrolyzed) of an instant coffee sample extracted according to ISO method 11292:1995 and analyzed using a narrow bore HPAEC column (adapted 'fast and green' conditions) as detailed in Table 2 and Table 4.

Table 13

Concentration of carbohydrates (g/100 g) in a sample of instant coffee, analyzed using adapted 'fast and green' version of ISO method 11292:1995 as given in Table 2 and Table 4.

Component	*A - free*	*B - total*
Mannitol	0.012	0.045
Arabinose	1.1	4
Galactose	0.24	13
Glucose	0.23	19
Sucrose	0.07	-
Xylose	0.020	0.16
Mannose	0.12	5
Fructose	0.09	-
Total	**1.9**	**42**

(chromatogram not shown). The levels of the most abundant carbohydrates in the hydrolyzed fraction were quantified to reach up to 3 mM, which far exceeded the linear response range for the adapted method. An additional sample dilution step is therefore advised for the hydrolyzed fraction when using the narrow-bore HPAEC column and adapted conditions.

When comparing the sample composition tables and chromatograms as obtained with the traditional and adapted 'fast and green' conditions, it can be observed that the data are comparable among methods. The small differences in quantification data may have developed during the storage of the samples before re-analysis.

Sample measurement: breakfast cereal

To show that this detection method is more generally applicable, a chromatogram was made from an extract of breakfast cereal (Figure 12). According to the labelling information the sugar content was 15 g/100 g product. The main 'free' carbohydrate was found to be sucrose, at the concentration stated on the label. An alternative sweetener to sucrose is high glucose-fructose corn syrup, but in the tested product the main sweetener is clearly sucrose.

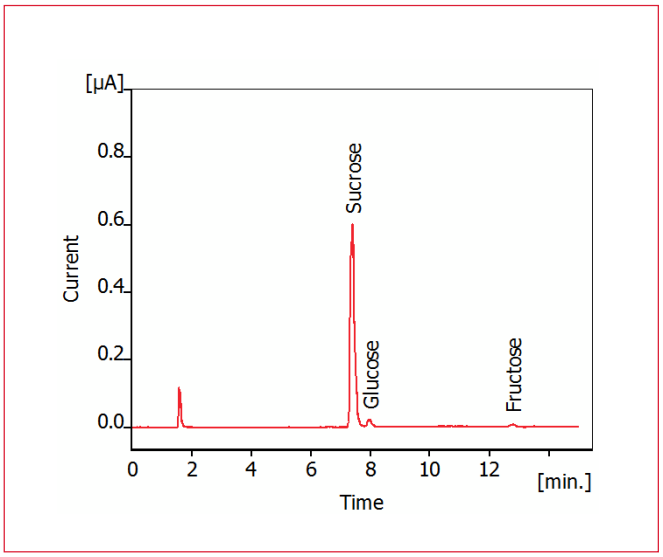

Figure 12: Chromatogram of an extract of 'Special flakes' breakfast cereal from Albert Heijn. About 10 g of product was extracted with 200 mL water for 10 min, centrifuged, filtered through 0.2 µm filter and 1000x diluted with water before injection (5 µL). Chromatogram recorded with conditions as detailed in Table 2 and Table 4. Sucrose, glucose and fructose contents were quantified as respectively 15.4, 0.3 and 0.2 g/100g product.

References

1. P. Girard, P. Stöber, M. Blanc, J. Prodolliet, Carbohydrate specification limits for the authenticity assessment of soluble (instant) coffee: statistical approach. J. AOAC Int., 89 (2006), 999-1003.
2. ISO24114:2011(en). Instant coffee Criteria for authenticity
3. ISO 11292 (1996). Instant coffee – determination of free and total carbohydrate contents – method using High performance anion-exchange chromatography. International Organization for Standardization
4. AOAC Official Method 995.13, Carbohydrates in soluble (instant) coffee. Association of Analytical Chemists
5. BS 5752-15:1997. Methods of test for Coffee and coffee products – Part 15: Instant coffee: determination of free and total carbohydrate contents by high performance anion exchange chromatography. British Standard
6. IS 16031 (2012). Instant coffee – determination of free and total carbohydrate contents – method using Highperformance anion-exchange chromatography. Bureau of Indian Standards
7. PROY-NMX-F-000-SCFI-2016 (2016). Café soluble – Determinación de contenido de carbohidratos libres y totals – Método usando cromatographía de alta resolución de intercambio anicónico. Proyecto de Norma Mexicana
8. DIN 10780:2003-02. Kaffee-Extrakt Bestimmung der freien und Gesamt-Kohlenhydrate Verfahren mit Hochleistungs-Anionenaustausch-Chromatographie. Deutsches Institut für Normung
9. 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

Conclusion

The ALEXYS Carbohydrate Analyzer with DECADE Elite detector and SenCell flow cell can be used to run the ISO method 11292:19956 for the analysis of carbohydrates in instant coffee. The use of the dedicated hardware additions, the narrow-bore column with smaller particle size, and an adapted lower flow rate results in a significant increase in sensitivity, a 50% decrease in analysis time and a 90% reduction of solvents-use compared to the traditional conditions.

Ordering information

ALEXYS analyzer	
180.0055W	ALEXYS Carbohydrate Analyzer with SSV and degasser
180.0606	Add-on parts for post-column addition, 375 µL (4 mm ID columns)
180.0608	Add-on parts for post-column addition, 8 µL (2 mm ID columns)
116.4321	SenCell 2 mm Au HyREF
Software#	
195.0035	Clarity CDS single instr. incl LC, AS module

#) The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

Figure 13: ALEXYS Carbohydrate Analyzer with add-on parts for post-column addition for demanding separations
The ALEXYS Carbohydrate Analyzer can be operated under DataApex™ Clarity™ CDS (version 8.3 and up) or Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software (version 7.2 SR 5 and up).

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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Summary

The monosaccharides glucose and fructose along with the disaccharides sucrose, maltose, and lactose are commonly added to flavor or preserve processed meats. Processed meat and fish products are generally not associated with high sugar content and consumers may not be aware of of the amount of added sugars in these products. To increase awareness of sugar intake and to comply with current FDA food labeling regulation, it is required to provide accurate information about added sugar content on food product labels [1,2].

In this publication the analysis of mono– and disaccharides in processed meat and fish samples is demonstrated using an ALEXYS® Carbohydrates Analyzer equipped with a DECADE Elite electro-chemical detector. The method is based on separation by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a high-resolution IC column with 4 µm particle size. The combination of isocratic elution with a step gradient resulted in fast separation and sensitive detection of the relevant mono- and disaccharides in meat & fish samples.

Introduction

Meat, poultry, and fish products do not contain a lot of naturally occurring sugars, however simple carbohydrates can be used in large quantities during processing of these products, such as during curing, drying or smoking. Monosaccharides like glucose, fructose, along with the disaccharides sucrose, maltose, and lactose are often added during processing for varying reasons.

Historically, sugar has been used along with salts as a dehydrating agent to preserve meats. At the other hand, in some products sugars helps retain moisture throughout processing and storage. For example, in deli meats sugars are added to stabilize the emulsion of moisture, fat and protein. Simple sugars also assist in the the Maillard reaction, which provides desirable color and flavor formation [3]. Although sugars play an important role in processed meat products, they are often not present in sufficient amounts to impart a sweet taste [4] and could therefore be a hidden source of sugar intake.

WHO guideline recommends adults and children to limit their daily intake of free sugars to less than 10% of their total energy intake [5]. Likewise, avoiding too much sugar is one of the most common accepted dietary guidance throughout the world. In order to reduce excessive discretionary calorie intake from added sugars, the FDA introduced the mandatory declaration of added sugars on the nutrition facts label [1,2]. In the EU, the vast majority of pre-packed foods are required to bear declaration of nutrition value including total sugar content [6]. To determine the (added) sugar content in meat products there is a need robust and sensitive analytical method.

High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) is the method of choice. It combines superior selectivity with sensitive detection. Due to the presence of hydroxyl groups which can be oxidized, carbohydrates can be detected without derivatization using pulsed amperometric detection with pico- and femtomol sensitivity [7]. The use of a HPAEC column with 4 µm particle size in combination with a step gradient allows for fast separation of the relevant mono- and disaccharides, resulting in a fast, sensitive, and selective method.

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.

Method

The analysis was performed using the ALEXYS Carbohydrates Analyzer as shown in figure 1. The HPAEC-PAD system consists of a P6.1L pump with integrated Solvent Switch Valve (SSV) capable of running step gradients, AS6.1L autosampler, ET 210 eluent tray for Helium blanketing, CT 2.1 column oven and the DECADE Elite electrochemical detector. The SenCell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the sugars. The system was operated under the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 7.2.10.

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.

Table 1

Conditions	
HPLC system	ALEXYS Carbohydrates Analyzer
Detector	DECADE Elite electrochemical detector
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast-4µm guard column, 30 x 4.0 mm ID Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast-4µm analytical column, 150 x 4.0 mm ID
Mobile phase (MP)	A: 15 mM NaOH B: 100 mM NaOH C: 100 mM NaOH, 100 mM NaOAc Eluents prepared & blanketed with Helium 5.0
Flow rate	0.8 mL/min
Back pressure	about 300 bar
Injection	10 µL
Temperature	30 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF RE, 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 0.2— 0.4 µA
ADF	0.05 Hz
Range	10 µA/V

Table 2

Step-gradient program		
Time (min)	*Mobile phase*	*Description*
0 - 10	15 mM NaOH	Elution & detection
10-15	100 mM NaOH	
15 - 20	100 mM NaOH, 100mM NaOAc	Column clean-up and regeneration
20 – 40	15 mM NaOH	Equilibration, starting conditions

The retention time of carbohydrates is inversely correlated with pKa value and depends on molecular weight and structural features such as linkage isomerism. For the separation of the mono– and disaccharides an anion-exchange column with with 4 µm particle size was chosen. This type of column enables fast and high resolution LC separations of sugars with short analysis time.

The temperature for separation was set at 30 °C using the CT 2.1 column thermostat. The analysis is based on isocratic elution at 15 mM NaOH combined with a step gradient of 100 mM NaOH to elute Maltose, which is more strongly retained on the column. A strong column clean-up and regeneration step is executed at t =15 min in every run to elute late eluting components and to remove carbonate ions (CO_3^{2-}) build up on the column. After the clean-up step the column is equilibrated for 15 minutes at the starting conditions, resulting in a total run time of 40 minutes.

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 and electrochemical grade sodium acetate salt (all commercially available). The diluent was DI water (resistivity >18 MΩ.cm), which was sparged with Helium 5.0 using the sparging function of the ET 210 Eluent tray. During analysis the eluent tray is used to pressurize the head space above the mobile phase with Helium gas (0.2—0.4 bar He overpressure). The inert gas atmosphere will minimizes the introduction of CO_2 in the mobile phase and the subsequent formation of CO_3^{2-} ions, ensuring reproducible analysis.

Detection

For the pulsed amperometric detection of simple sugars the Antec SenCell electrochemical flow cell is used. This flow cell [8] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/ H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform was applied as shown in figure 2. The temperature for detection was set to 35°C. The cell current was typical about 0.2—0.4 µA using 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

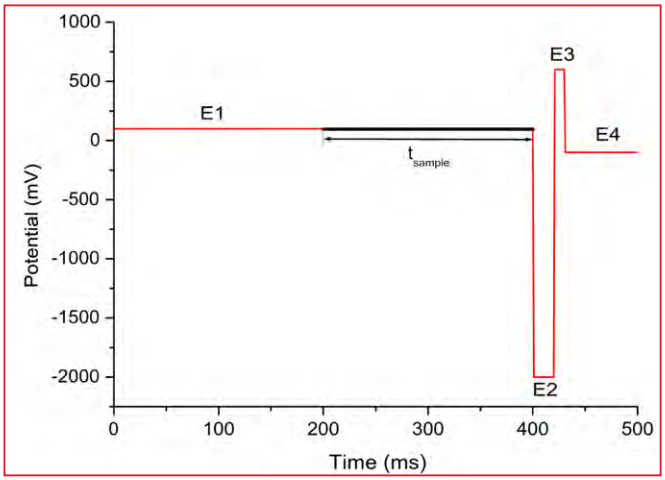

Figure 2: 4-step PAD potential waveform for the detection of carbohydrates.

electrode wear [9], resulting in less flow cell maintenance and system down time.

Preparation of standards, reagents and samples

Standards: 5 g/L stock standards of the individual sugars were prepared in 95/5 (v/v%) water/acetonitrile to increase the storage life. Stock standards under these conditions are approximately stabile for more than a month in the fridge at 4° C. Working standards in the concentration range of 0.25 - 7.5 mg/L were prepared by dilution of the stock standards with DI water.

Carrez reagents: a Carrez clarification procedure is used for deproteination of the dairy samples. By removing all proteins also the enzymatic activity will be quenched, eliminating any unwanted conversion of the sugars inside the sample during the analysis process. The Carrez I solution was prepared by dissolving 15.0 g potassium hexacyanoferrate(II) trihydrate in 100 mL of DI water in a volumetric flask. The Carrez II solution was prepared by dissolving 30.0 g zinc sulfate heptahydrate in 100 mL of water in a volumetric flask. Both Carrez solutions were filtered over a qualitative filter paper (Whatman™ 590/1) prior to use.

Sample preparation meat products: Two proficiency test samples with a known concentration of glucose, sucrose, fructose, lactose, and maltose were prepared and analyzed using the procedure below.

Procedure:

1. The sample was homogenized using a blender.
2. 1 gram of sample was weighted in a 100 mL volumetric flask and 50 mL DI water added.
3. the sugars were extracted by placing the flask in a hot shaking water bath (60 °C) for 20 minutes
4. Subsequently, 100 µL Carrez I and 100 µL Carrez II reagent was added. Followed by addition of DI water up to a total volume of 100 mL.
5. The solution was allowed to stand for 30 minutes and vortexed again to obtain a homogeneously turbid solution.
6. The turbid sample solution was centrifuged 10 min at 4000 xG
7. The supernatant was collected, diluted 50 times, and filtered over a 0.2 µm Polyethersulfone (PES) syringe filter (25 mm Ø FFL/MLS).
8. 10 µL of the filtered supernatant was injected into the LC system and analyzed.

Results

In figure 3 a typical chromatogram of the sugar standard mix is shown. The standard consist of 7.5 µg/L glucose, fructose sucrose, lactose, and maltose DI water. All saccharides except maltose elute within 10 minutes during the isocratic elution step with 15 mM NaOH. After 10 minutes the hydroxide concentration is increased from 15 to 100 mM, resulting in a temporal elevation of the background current (baseline). The signal quickly stabilizes within 2 minutes allowing sensitive detection of Maltose. All compounds of interest eluted within 16 minutes, and the total run cycle time is 40 minutes due to the wash and equilibration step. The peak efficiencies found for the sugars ranged from 50.000 to 65.000 theoretical plates per meter with the exception of Maltose, which has a peak efficiency of 300.000 theoretical plates per meter. All peak tailing factors ranged from 1.0 to 1.5.

In addition to the 5 sugars in the standard mix, other saccharides, like galactose can be present in processed meat samples. Due to the structural similarity of galactose and glucose, the separation of these monosaccharides can be challenging.

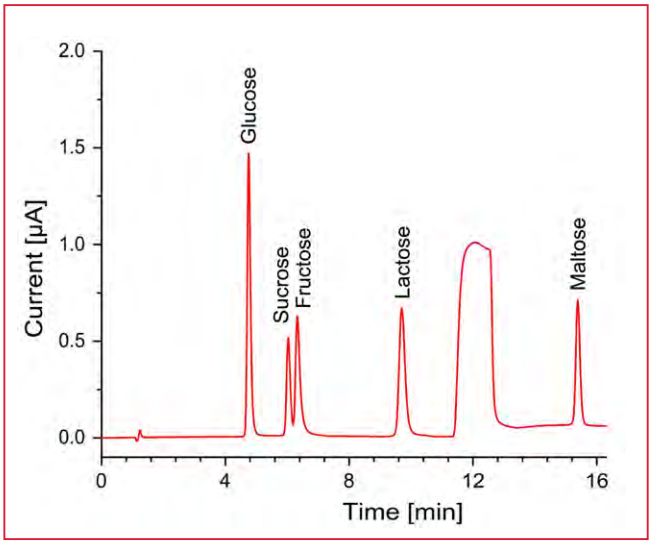

Figure 3: An example chromatogram obtained with a 10 µL injection of a 7.5 µg/L standard mix of glucose, sucrose, fructose, lactose and maltose in DI water.

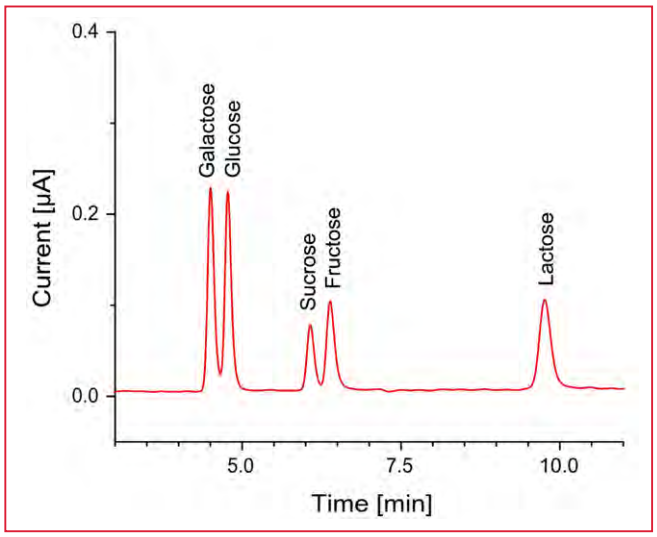

Figure 4: Zoom of the chromatogram (isocratic elution at 15 mM NaOH) obtained with a 10 µL injection of a 1 µg/L standard mix of sugars in DI water including galactose.

In figure 4 an example chromatogram is shown of a 10 µL injection of a 1 µg/L standard mix including galactose. Under the isocratic separation conditions (15 mM NaOH) the resolution of Galactose and glucose is 1.5, allowing for accurate quantification of both monosaccharides if required.

Linearity

The linearity was investigated with standards dissolved in DI water in the concentration range of 0.25 - 7.5 mg/L . The obtained calibration curves are shown in figure 3. Real samples

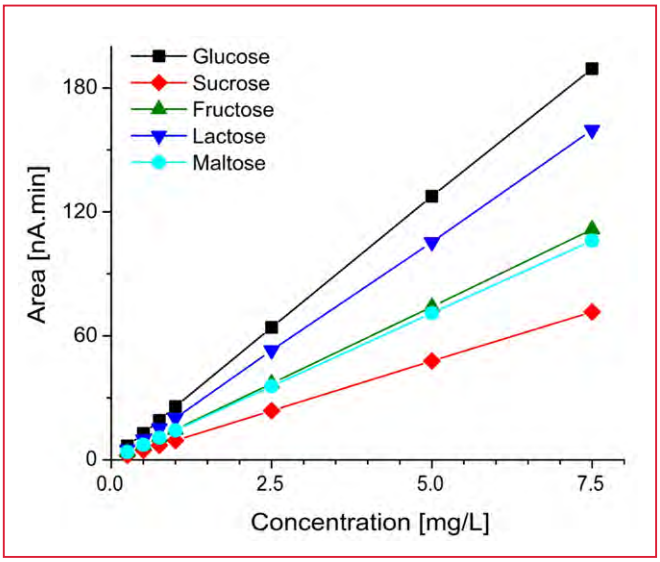

Figure 5: Calibration curve of glucose, fructose, sucrose, lactose and maltose in the concentration range of 0.25 - 7.5 mg/L

are diluted 50 times during sample preparation, so this calibration range corresponding to a sugar contents of 0.125 g—3.75 g per 100g product in samples. The linearity is excellent in this concentration range with correlation coefficients for peak area better than 0.9999 for all 5 sugars. The galactose concentration was not quantified in the proficiency samples because no acceptance criteria were specified by the supplier.

Repeatability

The relative standard deviation (RSD) of the retention time and peak area were determined for 10 repetitive injections with a 0.1 and 1 mg/L standard, which corresponds to 0.55 and 5.5 µM for the monosaccharides and 0.29 and 2.9 µM for the disaccharides, respectively. Retention times were stable, with RSD values in the range of 0.06 - 0.15% for all analytes. The RSD for peak areas for all sugars was < 2% for the 0.1 mg/L standard and < 1% for the 1 mg/L standard. These data demonstrate that with this method reproducible analysis of all the analytes of interest can be achieved using the ALEXYS Carbohydrates

Table 3

Repeatability of 10 µL injections of a 0.1 and 1 and mg/L sugar standard mix in DI water (n=10)

	RSD's (%) *1 mg/L*		*RSD's (%)* *0.1 mg/L*	
Compound	t_R	*Area*	t_R	*Area*
Glucose	0.10	0.67	0.14	0.92
Sucrose	0.06	0.53	0.13	0.84
Fructose	0.15	0.85	0.15	1.34
Lactose	0.09	0.36	0.08	1.36
Maltose	0.07	0.57	0.10	1.78

analyzer.

Limit of detection (LOD)

The LOD and limit of quantification (LOQ) for all sugars are shown in table 4. The LOD's and LOQ's were calculated as the analyte response corresponding to 3x and 10x the ASTM noise (average peak-to-peak baseline noise of 10 segments of 0.5 min), respectively. The noise was calculated based on a 5 minute section of the baseline close to the peaks of interest. The average response based on 10 replicate injections obtained with the 0.1 mg/L standard were used to calculate the LOD and LOQ.

Table 4

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

	Limit of detection		*Limit of Quantification*	
Compound	*nM*	*µg/L*	*nM*	*µg/L*
Glucose	11	2.1	38	6.8
Sucrose	16	5.6	55	19
Fructose	27	4.9	91	16
Lactose	15	5.0	49	17
Maltose	16	5.6	55	19

The calculated LODs ranging from 2.1 to 5.6 µg/L demonstrate the excellent sensitivity of the method.

Sample analysis

Two proficiency test samples with a known concentration of glucose, sucrose, fructose, lactose and maltose were prepared and analyzed using the presented method. An overlay of the chromatograms of the fish sample (black) and a 1 mg/L standard + galactose (red) is shown in figure 6. In addition to the five sugars, galactose was also present in the fish sample. The large baseline disturbance between 11 - 13 minutes is due to the onset of the step gradient (15 —> 100 mM NaOH), as explained on page 4.

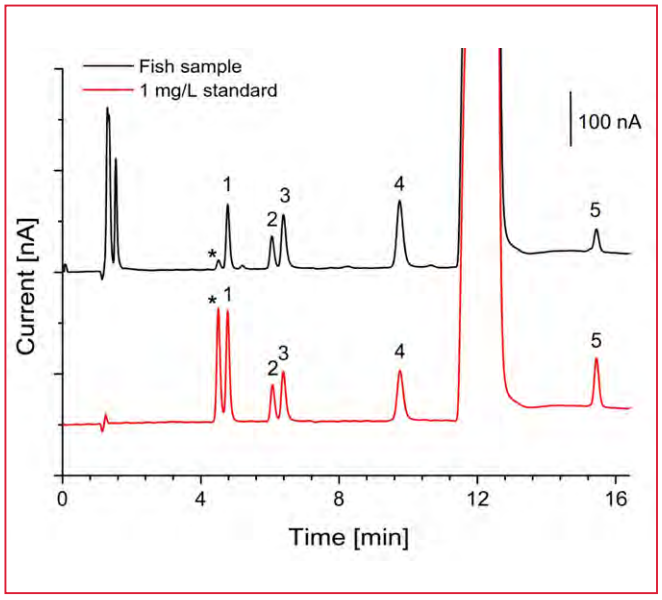

Figure 6: Chromatogram of the fish sample (black) and a 1 mg/L standard (red, -35% offset). Labeled peaks: * Galactose, 1. Glucose, 2. Sucrose, 3. Fructose, 4. Lactose, 5. Maltose.

Table 5

Sugar content fish sample [g/100g product]

Compound	*Measured*	*Target value * proficiency sample*	*Acceptance criteria * proficiency sample*
Glucose	0.30	0.31	0.266 – 0.354
Sucrose	0.54	0.57	0.509 – 0.631
Fructose	0.45	0.47	0.372 – 0.568
Lactose	0.70	0.70	0.549 – 0.851
Maltose	0.23	0.23	0.189 – 0.271

*) data provided by the supplier of the proficiency samples.

The sugar content of the fish sample is summarized in table 5. The measured concentrations listed in the table were calculated using a calibration curve based on standards in the range of 0.25—7.5 mg/L. The calculated contents for all sugars are within the limits of the acceptance criteria and are close to the target values reported by the supplier.

The overlay of the chromatograms of the meatball sample (black) and 1 mg/L standard +galactose (red) is shown in figure 7. Also in this sample a small amount of galactose is present. It is unknown if galactose was present as a naturally occurring sugar, added sugar or a degradation product. However, the presence of galactose in both analyzed samples highlights the need of the separation of galactose and glucose in order to accurately quantify the amount of glucose.

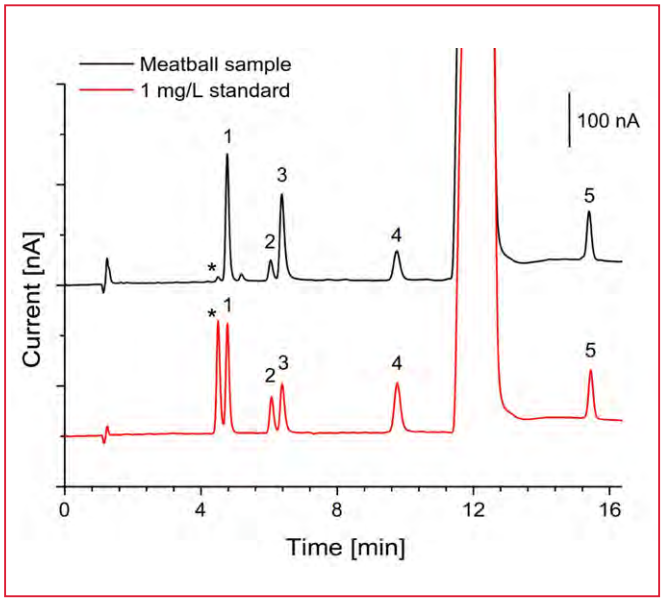

Figure 7: Chromatograms of the meatball sample (black) and a 1 mg/L standard (red, -35% offset). Labeled peaks: * Galactose, 1. Glucose, 2. Sucrose, 3. Fructose, 4. Lactose, 5. Maltose.

Table 6

Sugar content of the meatball sample [g/100g product]

Compound	*Measured*	*Target value proficiency sample*	*Acceptance criteria proficiency sample*
Glucose	0.59	0.61	0.540–0.680
Sucrose	0.92	0.93	0.826–1.030
Fructose	0.30	0.28	0.200–0.368
Lactose	0.30	0.32	0.282–0.362
Maltose	0.52	0.66	0.450–0.870

The sugar content of the meatball sample, calculated using the external calibration curve, is shown in table 6. Also for this sample the measured contents match well with the target values and meet the acceptance criteria.

Ordering information

System	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quarternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT 2.1 Column thermostat
Software*	
195.0035	Clarity CDS single instr. incl LC, AS module

*) The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

References

1. Goldfein, Kara R., and Joanne L. Slavin. "Why sugar is added to food: food science 101." Comprehensive Reviews in Food Science and Food Safety 14.5 (2015): 644-656.
2. Revision of the Nutrition and Supplement Facts Labels, Food and Drug Administration, May 27, 2016 CFR: 21 CFR Part 101.
3. Danehy J, Wolnak B. 1983. Maillard technology: manufacturing applications in food products. In: Waller G, editor. The Maillard reaction in foods and nutrition. Washington, DC: American Chemical Society. p. 303–15
4. H. R. Kraybill, Sugar and other carbohydrates in meat processing, Advances in Chemistry, 12 (1955), 83–88
5. WHO, Guideline: sugars intake for adults and children. World Health Organization, (2015), https://www.who.int/publications/
6. Regulation (EU) No 1169/2011, Food information to consumers, (2011), https://eur-lex.europa.eu/
7. W.R. LaCourse, Pulsed Electrochemical Detection in High Performance Liquid Chromatography, John Wiley & Sons, New York, 1ed, 1997
8. Louw, H.J. Brouwer, N. Reinhoud, Electrochemical flowcell, (2016) US patent 9310330
9. 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

Conclusion

The ALEXYS Carbohydrates Analyzer, in combination with the SenCell flow cell offers a dedicated and optimized analysis solution for the fast and sensitive quantification of sugars in processed meat and fish samples.The use of a high-resolution HPAEC column with 4 µm particle size in combination with a step gradient allows for fast separation of all relevant mono- and disaccharides. All sugars of interest eluted within 16 minutes. The total runtime including column clean-up and equilibration is 40 min. The results obtained with the proficiency test samples demonstrate that the sugar content in such processed meat and fish products can be reliably quantified.

With 82%, carbohydrates comprise the major portion of honey

The carbohydrates present are the monosaccharides fructose (38.2%) and glucose (31%); and disaccharides (9%) such as sucrose, maltose, isomaltose, etc. There are also some oligosaccharides present (4.2%), formed from incomplete breakdown of the higher saccharides.

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.

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.

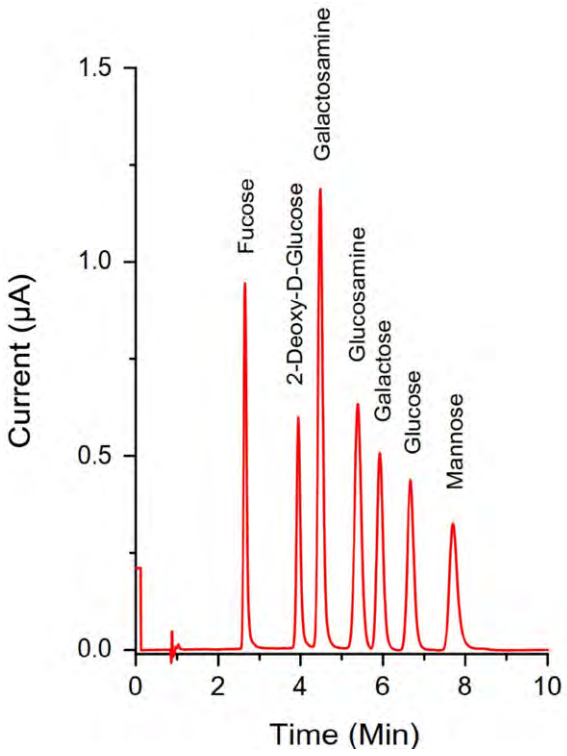

Figure 1: Chromatogram of a 10 µL injection of a 10 µM standard mix of 7 monosaccharides in water: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and

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
Back pressure	About 180 bar
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

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

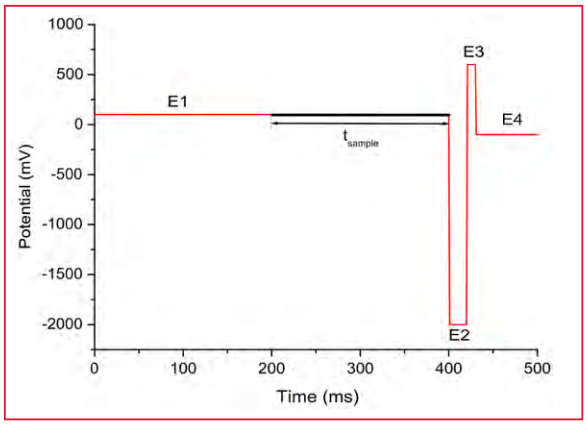

Figure 2: 4-step PAD potential waveform for the detection mono-saccharides and other carbohydrates.

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 ≥ 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. 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.

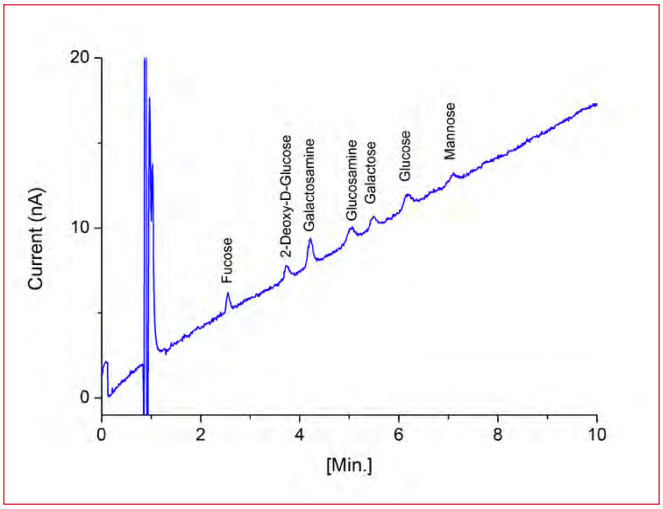

Figure 3: Chromatogram of a 10 µL injection of a 10 nM standard mix of 7 monosaccharides in water: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and (7) Mannose.

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.

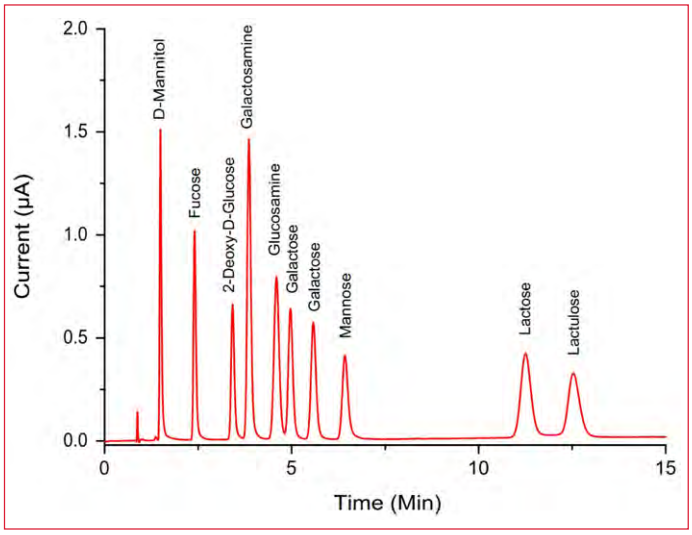

Figure 4: Chromatogram of a 10 µL injection of a 10 nM standard mix of 10 mono- and disaccharides in water: (1) Mannitol, (2) Fucose, (3) 2-Deoxy-DGlucose, (4) Arabinose, (5) Glucosamine, (6) Sucrose, (7) Xylose, (8) Fructose, (9) Lactose and (10) Lactulose.

References

1. K.F. Aoki-Kinoshita, An introduction to Bioinformatics for Glycomics, PLoS Comput Biol, vol 4 issue 5 (2008), 1– 6
2. D.C. Johnson, D. Dobberpuhl, R. Roberts, P. Vandeberg, Review: Pulsed amperometric detection of carbohydrates, amines and sulfur species in ion chromatography- the current state of research, J. Chromatogr., 640 (1993), 79 - 96
3. D.C. Johnson, W.R. LaCourse, LC with pulsed ECD at gold and platinum, Anal. Chem., 62 (1990), 589A – 597
4. W.R. LaCourse, Pulsed Electrochemical Detection in High Performance Liquid Chromatography, John Wiley & Sons, New York, 1ed, 1997

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
6. Antec, Carbohydrates in food, Application note, 220.002 rev. 04
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

#) Antec ECD drivers are available for Chromeleon CDS, OpenLAB CDS and OpenLAB Chemstation 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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Trans-galactooligosaccharides in Food Products

- **ALEXYS Carbohydrate Analyzer**
- **Improvements on AOAC 2001.02**
- **High-resolution isocratic HPAEC-PAD separation**
- **Galactose, glucose, lactose & isomers**
- **'Fast and green' method**

Summary

Prebiotics are nondigestible food ingredients that provide numerous health benefits and help maintain a healthy and balanced gut microflora. They beneficially affect the host by selectively stimulating the growth or activity of health promoting bacteria in the colon, such as bifidobacteria [1]. Trans-galactooligosaccharides (TGOS) are prebiotic water-soluble carbohydrates consisting of a chain of 1-7 galactose units with a terminal glucose. TGOS are not broken down by human digestive enzymes and are therefore available as a nutrient for beneficial bacteria. As a prebiotic, TGOS has a wide application in human and animal foods.

One of the most commonly-used method to quantify TGOS is the AOAC 2001.02 method [2]. This AOAC approved method is based on enzymatic hydrolysis of TGOS and subsequent analysis of the reaction products (galactose & glucose) with High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). In this application note an adapted method was evaluated, which uses a high-resolution IC column with 4 µm particle size. The adapted method resulted in better separation under isocratic conditions and a shorter analysis time. Both the original and adapted methods provide excellent sensitivity, linearity and reproducibility, and are suitable for the routine analysis of TGOS in food and feed using the ALEXYS Carbohydrates Analyzer.

Introduction

Trans-galactooligosaccharides (TGOS) are water-soluble oligosaccharides consisting of a chain of galactose units with a terminal glucose and typically have a degree of polymerization (DP) between 2 and 8. TGOS are not digested in the small intestine but are fermented in the colon by the bacterial flora. By stimulating the growth of the beneficial bacteria, the consumption of TGOS as a prebiotic supplement may result in a broad range of positive health effects.

TGOS are produced by the trans-galactosylation activity of β-galactosidase enzymes, using lactose as both donor and acceptor substrate. During the synthesis, the glycosyl group of one or more D-galactosyl units is transferred onto the D-galactose moiety of lactose. The yield depends on the enzymatic source and reaction condition, and TGOS are produced as a complex mixture of branched and linear oligosaccharides with varying chain length and glycosidic linkages [3]. For example, up to 8 different disaccharides consisting of galactose and glucose with different β-glycoside bonds can be present in a TGOS mix [4]. All galactose-glucose based disaccharides except lactose are not susceptible to decomposition by human digestive enzymes and are therefore considered TGOS [5].

The principle of the AOAC 2001.02 method to determine the total content of TGOS is based on the enzymatic treatment of a sample with a β-galactosidase enzyme and subsequent analysis of the reaction products using High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). β-galactosidase catalyzes the hydrolysis of glycosidic bonds in the galactooligosaccharides, resulting in the release of the monosaccharides glucose and galactose. The galactose release is subsequently quantified in the AOAC method and the TGOS content is then calculated by multiplying the amount of released galactose by the so-called 'k-factor' to correct for the terminal glucose units in TGOS. The k-factor is based on the ratio glucose and galactose in the TGOS ingredient and can be calculated using the average degree of polymerization.

So, a boundary condition to analyze TGOS in a sample is that the k-factor or average degree of polymerization must be provided by the manufacturer of the GOS ingredient to be able to accurately quantify the total TGOS contents by the AOAC 2001.02. Alternatively, the total TGOS content can be determined without the use of the k-factor, by taking into account the quantification of the released glucose in the analysis [6].

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.

Such approach could be employed in case the k-factor is not known of the GOS ingredient.

In this application note an improved separation method for the analysis of TGOS in food products is presented using the ALEXYS carbohydrates analyzer in combination with a high-resolution IC column, and compared to the performance using the original AOAC 2001.02 method. The adapted method is based on simple isocratic elution and resulted in shorter run times, less mobile phase consumption and better separation of lactose-allolactose and other lactose isomers. Furthermore, in the presented method the quantification of the glucose release is also included in the analysis to determine the amount of TGOS in test samples, both with and without the use of the k-factor.

Method

The original and adapted AOAC method for total TGOS analysis was evaluated using the ALEXYS Carbohydrates Analyzer as shown in figure 1. The HPAEC-PAD system consists of a P6.1L pump with integrated Solvent Switch Valve (SSV) capable of running step gradients, AS6.1L autosampler, ET 210 eluent tray for Helium blanketing, CT 2.1 column oven and the DECADE Elite electrochemical detector. The SenCell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the sugars. The system was operated under the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 7.2.10.

Table 1

Original AOAC 2001.02 method

HPLC system	ALEXYS Carbohydrates Analyzer
Detector	DECADE Elite electrochemical detector
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA1 guard column, 50 x 4.0 mm ID Thermo Scientific™ Dionex™ CarboPac™ PA1 analytical column, 250 x 4.0 mm ID
Mobile phase (MP)	A: 12.5 mM NaOH B: 125 mM NaOH C: 125 mM NaOH, 500 mM NaOAc Eluents prepared & blanketed with Helium 5.0
Flow rate	1.0 mL/min
Back pressure	about 110 bar
Injection	10 µL
Temperature	25 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF RE, 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 0.2— 0.4 µA
ADF	0.05 Hz
Range	10 µA/V

Table 2

Gradient program

Time (min)	*Mobile phase*	*Description*
0 - 24	18.75 mM NaOH	Elution & detection
24 – 39	18.75 mM - 125 mm NaOH	
39 - 40	125 mm NaOH	
40 - 50	125 mm NaOH, 500 mM NaOAc	Column clean-up and regeneration
50 - 65	18. 75 mM NaOH	Equilibration, starting conditions

Table 3

Adapted AOAC 2001.02 method

HPLC system	ALEXYS Carbohydrates Analyzer
Detector	DECADE Elite electrochemical detector
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast-4µm guard column, 30 x 4.0 mm ID Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast-4µm analytical column, 150 x 4.0 mm ID
Mobile phase (MP)	A: 10 mM NaOH B: 100 mM NaOH, 100 mM NaOAc Eluents prepared & blanketed with Helium 5.0
Flow rate	0.8 mL/min
Back pressure	about 300 bar
Injection	2 µL
Temperature	30 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF RE, 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 0.2— 0.4 µA
ADF	0.05 Hz
Range	10 µA/V

Table 4

Step-gradient program

Time (min)	*Mobile phase*	*Description*
0 - 15	10 mM NaOH	Elution & detection
15 - 20	100 mM NaOH, 100mM NaOAc	Column clean-up and regeneration
20 – 40	10 mM NaOH	Equilibration, starting conditions

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.

AOAC 2001.02 method: The recommended HPAEC column with 10 µm particles size and quaternary ammonium functionalized latex as mentioned in the AOAC method was chosen for method evaluation, using the conditions given in Table 1 and Table 2. The temperature for separation was set at 25 °C using the CT 2.1 column thermostat. The separation is based on isocratic elution of galactose followed by a gradient for the elution of lactose. A strong column clean-up and regeneration step is executed at t = 40 min in every run to elute late eluting components. After the 10 min clean-up step the column is equilibrated for 15 minutes at the starting conditions, resulting in a total run time of 65 minutes.

Adapted method: To improve the separation of the mono– and disaccharides an anion-exchange column with 4 µm particle size was chosen. This type of column enables fast and high resolution LC separations of sugars with short analysis time. The conditions are shown in Table 3 and Table 4. The CT 2.1 column thermostat was used to perform the separation at 30°C. The use of a fast-4µm IC column with its higher column efficiency resulted in significantly sharper peaks and thus a larger response (peak height). Therefore, a smaller injection volume of 2 µL was chosen to ensure that all analyte signals fall within the linear range of the detector and to avoid any additional dilution steps, when measuring the exact same samples and calibration standards. All analytes of interest (galactose, glucose, allolactose and lactose) are separated under isocratic elution. A strong column clean-up and regeneration step is executed at t =15 min to elute strongly retaining components and to remove carbonate ions (CO_3^{2-}) build up on the column. After the clean-up step the column is equilibrated for 20 minutes at the starting conditions, resulting in a total run time of 40 minutes.

Detection

For the pulsed amperometric detection of simple sugars the Antec SenCell electrochemical flow cell is used. This flow cell [6] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/ H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). The AOAC method mentions the use of a 3-step waveform. However, a 4-step waveform yields significantly improved long-term peak area reproducibility compared the described 3-step waveform while yielding equivalent results [7, 8], and is therefore the preferred waveform. Hence, the 4-step potential waveform was used for both methods. The temperature for detection was set to 35°C. The cell current was typical about 0.2—0.4 µA using these PAD settings under the specified conditions.

Preparation of standards and reagents

Standards: 5 g/L stock standards of the individual sugars were prepared in 95/5 (v/v%) water/acetonitrile to increase the storage life. Stock standards under these conditions are approximately stabile for more than a month in the fridge at 4° C. Working standards in the concentration range of 0.5 - 48 mg/L were prepared by dilution of the stock standards with DI water.

Carrez reagents: a Carrez clarification procedure is used for deproteination of the dairy samples. By removing all proteins also the enzymatic activity will be quenched, eliminating any unwanted conversion of the sugars inside the sample during the analysis process. The Carrez I solution was prepared by dissolving 15.0 g potassium hexacyanoferrate(II) trihydrate in 100 mL of DI water in a volumetric flask. The Carrez II solution was prepared by dissolving 30.0 g zinc sulfate heptahydrate in 100 mL of water in a volumetric flask. Both Carrez solutions were filtered over a qualitative filter paper (Whatman™ 590/1) prior to use.

Buffers: A phosphate buffer (200 mM, pH 6) was prepared by dissolving 11.0 g KH_2PO_4 and 2.3 g K_2HPO_4 in 0.5 L of DI water. The buffer was sterilized by filtration over a 0.2 µm Polyethersulfone (PES) syringe filter (25 mm Ø FFL/MLS) and kept at 4°C until use.

Enzyme solution: β-galactosidase (EC 3.2.1.23, Megazyme) was diluted with phosphate buffer to obtain a final activity of 2,000 U/mL. and kept at 4°C until use.

Sample preparation

Two TGOS ingredient were obtained for this study. Vivinal® GOS Powder (>68% GOS content , TGOS A) was kindly provided by FrieslandCampina Ingredients and TGOS powder (>70% GOS content, TGOS B) was purchased from Carbosynth (Compton, United Kingdom). Two test samples were prepared based on the TGOS powders by dissolving 0.5 g of TGOS in 9.5 g of DI water. Additionally, 2 commercial food products, an yoghurt drink and semi-skimmed milk, were obtained from the local supermarket. To simulate a TGOS supplemented food product, 9.5 g of dairy product was spiked with 0.5 g TGOS A.

Four samples were prepared according to AOAC Method 2001.02 followed by a Carrez clarification for the dairy samples as summarized below. An extensive description can be found in the method [2]:

- TGOS A in DI water
- TGOS B in DI water
- Yoghurt drink spiked with TGOS A (sample S1)
- Semi-skimmed milk spiked with TGOS A (sample S2)

Extraction: 40 mL of hot phosphate buffer was added to 2g of sample. The mixture was incubated for 60 min at 80 °C and subsequently cooled down to room temperature in an ice bath. The pH of the extract was verified to be pH 6 and subsequently the extract was diluted to a the total volume of 50 mL with phosphate buffer.

Enzymatic Hydrolysis: For each samples 2 separate extracts were prepared. Assay 1 for the determination of the initial concentrations of the free lactose , glucose, and galactose present in the sample. Assay 2 for the determination of the final concentration of galactose and glucose after hydrolysis of the TGOS present in the sample.

Assay 1 (initial test solution A1): 250 µL of phosphate buffer was added to 250 µL of β-galactosidase suspension. The enzyme was deactivated by incubating the suspension at 100 °C for 10 minutes. The deactivated enzyme solution was cooled down to room temperature and 5 g of extract solution was added. The mixture was incubated at 60 °C for 30 minutes under gentle agitation, and cooled down to room temperature in an ice bath. Subsequently, 1 mL of 20% acetonitrile was added.

Assay 2 (hydrolyzed sample solution A2): 5 g of extract solution was added to 250 µL of enzyme solution. The mixture was incubated at 60°C for 30 minutes under gentle agitation, cooled down to room temperature in an ice bath, and 1.25 mL of 20% acetonitrile was added.

Carrez precipitation: The dairy-based samples were clarified using a carrez precipitation. For both assays 50 µL Carrez I and 50 µL carrez II reagent was added, followed by an addition 3% acetonitrile up to a total volume of 50 mL. The solution was incubated for 30 minutes at room temperature and centrifuged 10 min at 4000 xG to obtain a clear supernatant.

All samples were further diluted 5 times with 3% acetonitrile and filtered over a 0.2 µm Polyethersulfone (PES) syringe filter (25 mm Ø FFL/MLS) prior to injection.

Calculations

Calculation TGOS concentration AOAC Method 2001.02

The initial concentration of free galactose ($Gal_{initial}$, in mg/L) and lactose ($Lac_{initial}$, in mg/L) are determined directly in assay 1. The final concentration galactose (released + free) in the hydrolyzed solution (Gal_{total}, in mg/L) is determined in assay 2. The galactose released from lactose ($Gal_{lactose}$, in mg/L) in assay 2 can be calculated using the following formula:

$$Gal_{lactose} = \frac{180}{342} x\ Lac_{initial} = \frac{Lac_{initial}}{1.9}$$

Where, 180/342 is a factor based on the molar masses of Galactose and Lactose. The total amount of galactose released from TGOS (Gal_{TGOS}, in mg/L) is calculated by subtracting the initial galactose and the galactose released from lactose from the total galactose:

$$Gal_{TGOS} = Gal_{total} - Gal_{initial} - Gal_{lactose}$$

The concentration of TGOS in mg/L is then calculated by multiplying the galactose released from TGOS by the k-factor [2]:

$$TGOS = k\ x\ Gal_{TGOS}$$

The k-factor can be calculated using:

$$k = \frac{180 + 162n}{180n}$$

With n being the average number of galactose moieties in the TGOS molecules (i.e. the average degree of polymerization -1). For example, if n = 2, k is 1.4. An accurate estimation of the n factor, and consequently the k factor is needed to determine the TGOS contents following the AOAC 2001.02 method. The k-factor may vary among GOS manufacturers and even between GOS batches. Ideally, each manufacturers should determine the k factor for each batch and provide it to their customers.

Calculation TGOS concentration without k-factor

Alternatively, the TGOS concentration can be calculated without the k-factor by including the released glucose in the calculations [10]. The initial concentration glucose ($Glu_{initial}$, in mg/L) and glucose in the hydrolyzed solution (Glu_{total} , in mg/L) are determined directly in assay 1 and 2, respectively. The glucose in mg/L released from lactose can be calculated using the following formula:

$$Glu_{lactose} = \frac{180}{342} x\ Lac_{initial} = \frac{Lac_{initial}}{1.9}$$

The released amount of glucose is calculated in a similar way as galactose, with:

$$Glu_{TGOS} = Glu_{total} - Glu_{initial} - Glu_{lactose}$$

Subsequently, the total concentration of TGOS is calculated by adding the glucose released from TGOS to the galactose released from TGOS:

$$TGOS = \frac{162}{180} x\ Gal_{TGOS} + Glu_{TGOS} = 0.9\ x\ Gal_{TGOS} + Glu_{TGOS}$$

Results - Original AOAC Method

In figure 2 a typical chromatogram of the sugar standard mix is shown in black. The standard consist of 12 mg/L galactose, glucose and lactose in DI water. For reference a chromatogram of an injection of allolactose in DI water is shown as a red overlay. The resolution of galactose and glucose is 1.4, allowing quantification of both monosaccharides if required. However, allolactose and lactose are not well-separated, which could lead to the incorrect quantification of lactose if allolactose is present in the sample. Moreover, the increasing NaOH concentration during the gradient (from 18.75 mM at t = 24 min to 125 mM at t = 39 min) resulting in a gradually rising background current (baseline), which could potentially complicate integration of the lactose peak. The peak efficiencies found for the sugars ranged from 15.000 theoretical plates per meter for galactose and glucose to 75.000 theoretical plates per meter for lactose. All peak tailing factors ranged from 1.0 to 1.1.

Linearity

The linearity was investigated with standards dissolved in DI water in the concentration range of 0.5 - 48 mg/L. This concentration range is much larger than described in the AOAC method. The obtained calibration curves are shown in figure 3. Linearity is excellent with correlation coefficients for peak area of 1.0000, 0.9997 and 1.0000 for galactose, glucose and lactose, respectively. The larger concentration range of the calibration curve enabled that all samples (hydrolyzed and non hydrolyzed extracts) could be measured and quantified with the same dilution factor, and no additional sample specific dilution steps were required.

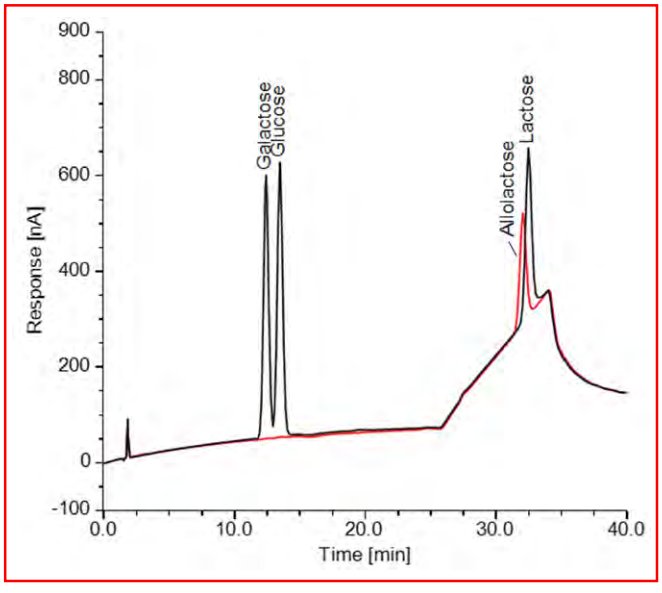

Figure 2: Overlay of a chromatogram of a 10 µL injection of 12 mg/L sugar standard mix (black) in DI water and a chromatogram of a 10 µL injection of 10 mg/L allolactose in DI water (red).

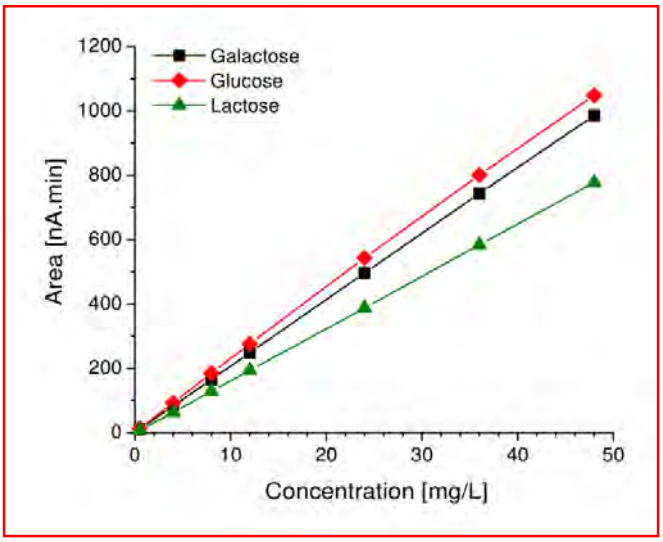

Figure 3: Calibration curves of galactose, glucose and lactose in the concentration range of 0.5 - 48 mg/L (standards in DI water).

Repeatability

The relative standard deviation (RSD) of the retention time and peak area were determined for 10 repetitive injections with a 0.5 and 4 mg/L standard (table 5). The RSD for peak areas for all sugars was < 1.5% for the 0.5 mg/L standard and < 0.6% for the 4 mg/L standard. These data demonstrate that with this method reproducible analysis of all the analytes of interest can be achieved using the ALEXYS Carbohydrates analyzer.

Detection limit

The LOD was calculated as the analyte response corresponding to 3x the ASTM noise determined on a 5-minute section of the baseline (average peak-to-peak baseline noise of 10 segments of 0.5 min). The noise level was 0.19 nA and the calculated LOD's were in the range of 10 - 22 µg/L or 120 - 220 pg on-column (Table 6).

Table 5

Repeatability of 10 µL injections of a 0.5 and 4 mg/L sugar standard mix in DI water (n=10)

	RSD's (%) 4 mg/L		*RSD's (%)* 0.5 mg/L	
Compound	t_R	*Area*	t_R	*Area*
Galactose	0.14	0.27	0.27	0.83
Glucose	0.13	0.32	0.30	0.75
Lactose	0.06	0.56	0.17	1.41

Table 6

Calculated Limit of Detection (LOD)

	Concentration		On-column amount	
Compound	*nM*	*µg/L*	*pmol*	*pg*
Galactose	69	12	0.69	120
Glucose	62	11	0.62	110
Lactose	64	22	0.64	220

Sample analysis

Two TGOS ingredients (TGOS A and B) and two dairy samples spiked with TGOS A (S1: Yoghurt drink, S2: Semi-skimmed milk) were prepared and analyzed using the AOAC Method 2001.02. An overlay of the initial test solutions (assay 1) of the TGOS ingredients is shown in figure 4. The TGOS A sample (red curve) contains a small amount of free glucose and almost no galactose. In the TGOS B sample (black curve) a small amount of galactose is present and almost no glucose. Both TGOS samples contain a significant amounts of lactose. In addition, the peak shape of lactose (peak 3) does not look symmetrical and in case of TGOS B a clear shoulder peak is visible, which is likely caused by coelution of allolactose present in the TGOS ingredients.

An overlay of the chromatograms of the yoghurt drink sample spiked with TGOS A (sample S1) is shown in figure 5. The initial test solution (assay 1) is shown in red, the hydrolyzed sample (assay 2) is shown in black. The chromatogram of assay 1 represents the carbohydrate profile before hydrolysis. In addition to the expected mono- and disaccharides some other small unidentified peaks are present in the sample. The absence of those peaks in the hydrolyzed sample indicates that these peaks represent carbohydrates with glycosidic linkages cleavable by β-galactosidase. No detectable amount of lactose is present in the assay 2 sample, which confirms that the sample is fully hydrolyzed.

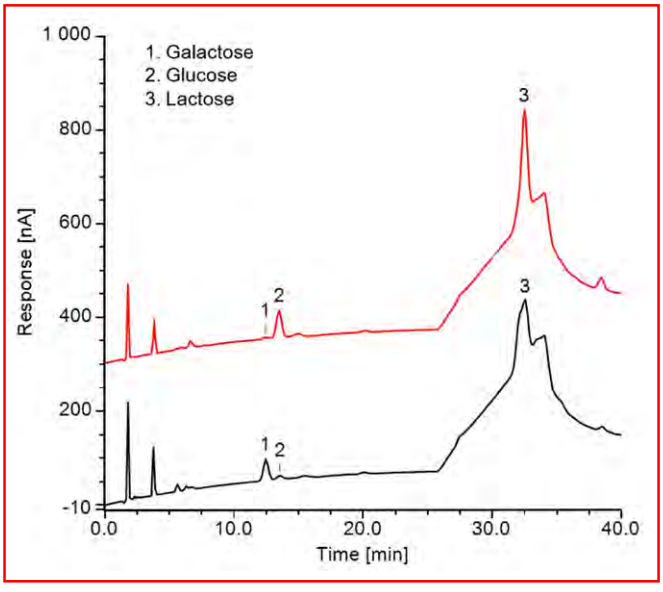

Figure 4: Overlay of the chromatograms obtained with the non hydrolyzed initial test solutions (assay 1) of TGOS A (red, +35% offset) and TGOS B (black).

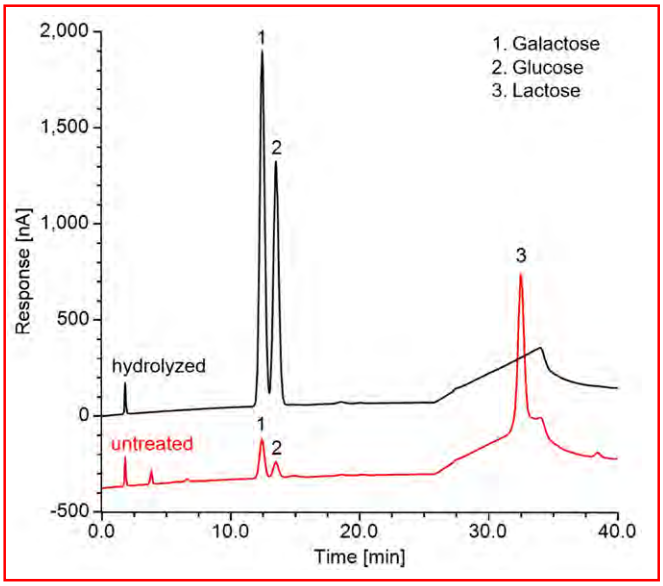

Figure 5: Chromatogram of Sample S1. The initial test solution, assay 1, is shown in red (-35% offset). The hydrolyzed sample solution, assay 2, is shown in black.

The results for all samples are summarized in table 7. The TGOS content calculated using the k-factor and based on the released amount of glucose and galactose are both listed in the table. The difference between the calculated TGOS contents with both methods is not more than 2% for all samples. Note that a k-factor of k = 1.4, corresponding with an average degree of polymerization of n = 2, was used for all samples. The actual degree of polymerisation and k-factor of the products might differ.

Table 7

TGOS content (g/100g sample)

Sample	Gal release from TGOS	Glu from TGOS	TGOS (0.9 x Gal + Glu)	TGOS (k x Gal)
TGOS A	2.66	1.37	3.76	3.72
Sample S1	2.69	1.40	3.83	3.77
Sample S2	2.78	1.34	3.84	3.89
TGOS B	2.82	1.34	3.87	3.94

Results - Adapted AOAC method

A typical chromatogram of the sugar standard mix obtained with the adapted method is shown in figure 6. The 12 mg/L calibration standard in DI water is shown in black and an 2.5 µL injection of 10 mg/L allolactose is shown as an overlay in red. The separation is significantly improved, and both lactose-allolactose and galactose-glucose are well-separated (R ≥ 1.9). Due to the isocratic separation the background current is constant resulting in a flat baseline. All analytes of interest elute within 12 minutes. The total analysis time for each sample is reduced to 40 min, which includes a 5 min post-run step gradient for column clean-up and 15 min re-equilibration time. The peak efficiencies found for the sugars are in the range of 50 000 - 70 000 theoretical plates per meter. All peak tailing factors are in the range of 1.1 to 1.2.

Linearity

The linearity was investigated with standards dissolved in DI water in the concentration range of 0.5 - 48 mg/L . The obtained calibration curves are shown in figure 7. Also with this improved method, the linearity is excellent with correlation coefficients for peak area of 1.0000, 0.9999 and 1.0000 for galactose, glucose and lactose, respectively. All samples (hydrolyzed and non hydrolyzed extracts) could be measured and quantified with the same dilution factor, and no additional sample specific dilution steps were required.

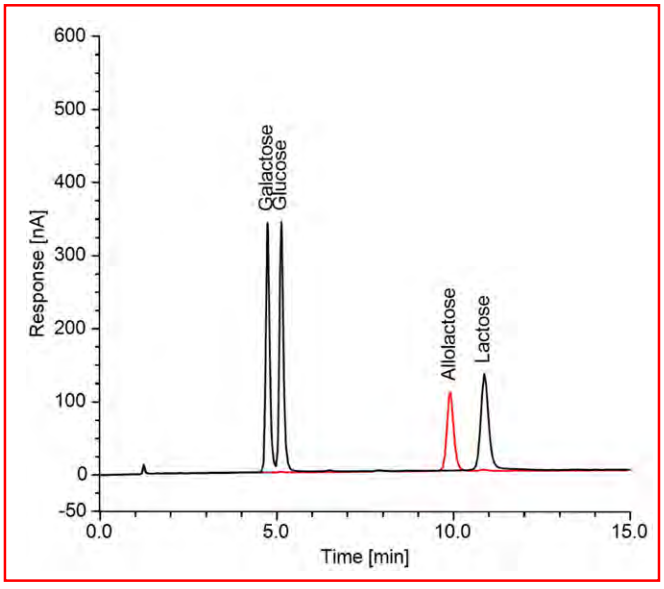

Figure 6: Overlay of a chromatogram of 2.5 µL injection 12 mg/L sugar standard (black) and a chromatogram of 2.5 µL injection 10 mg/L allolactose solution (red).

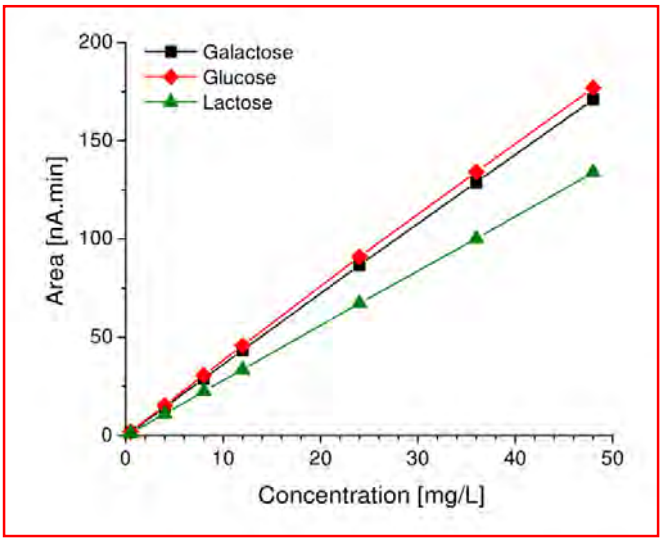

Figure 7: Calibration curve of galactose, glucose and lactose maltose in the concentration range of 0.5 - 48 mg/L

Repeatability

The relative standard deviation (RSD) of the retention time and peak area for 10 repetitive injections are summarized in table 8 for a 0.5 and 4 mg/L standard in DI water. Retention times were stable, with RSD values in the range of 0.11 - 0.17% for all analytes. The RSD for peak areas for all sugars was < 1% for the 0.5 mg/L standard and < 0.25% for the 4 mg/L standard. These data demonstrate that reproducible analysis of all the analytes of interest can be achieved with the ALEXYS carbohydrates analyzer using the improved method.

Detection limit

The LOD was calculated as the analyte response corresponding to 3x the ASTM noise determined on a 5-minute section of the baseline (average peak-to-peak baseline noise of 10 segments of 0.5 min). The ASTM noise was 0.14 nA and the calculated concentration LOD's were in the range of 15 - 40 µg/L.

Table 8

Repeatability of 2.5 µL injections of a 0.5 and 4 and mg/L sugar standard mix in DI water (n=10)

	RSD's (%) 4 mg/L		*RSD's (%) 0.5 mg/L*	
Compound	t_R	*Area*	t_R	*Area*
Galactose	0.17	0.25	0.15	0.78
Glucose	0.16	0.24	0.16	0.80
Lactose	0.14	0.22	0.11	0.94

Table 9

Calculated limit of Detection (S/N =3)

	Concentration		*On-column load*	
Compound	*nM*	*µg/L*	*pmol*	*pg*
Galactose	81	15	0.16	29
Glucose	81	15	0.16	29
Lactose	113	39	0.23	77

It is evident that the concentration sensitivity is slightly lower compared to the results reported for the conventional column due to the choice of a 2 µL injection volume. However, the on-column LOD is significantly improved with the adapted method based on the fast-4 µm column and ranges from 29 to 77 pg.

Sample analysis

The exact same samples were analyzed using the improved method. An overlay of the initial test solution of the two TGOS ingredients (assay 1) is shown in figure 8. Allolactose was found to be present in both samples, particularly in the TGOS B sample the response of allolactose is significant and as large as the response of lactose, indicating that the isomers may be present in equimolar concentrations. Also, small amounts of the isomers lactulose and epilactose are identified in both samples. In addition to the identified peaks, numerous putative TGOS component are present in the chromatogram, but they are all well-separated and do not interfere with the quantification of galactose, glucose and lactose (see figure 9).

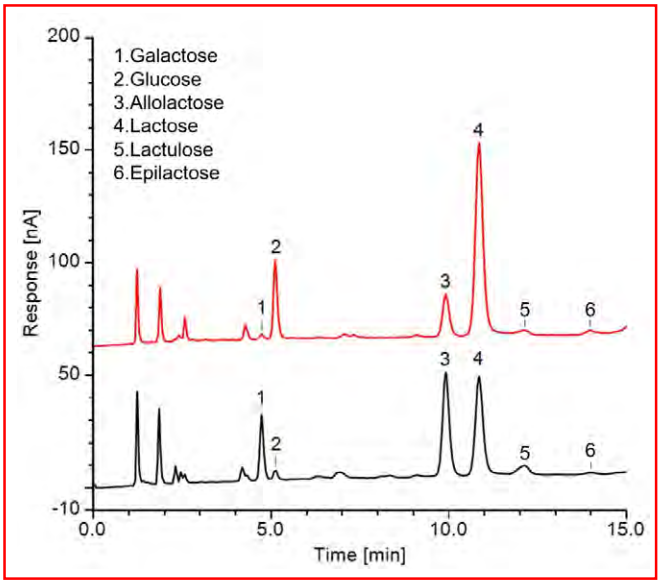

Figure 8: Overlay of the chromatograms obtained with the non hydrolyzed initial test solutions (assay 1) of TGOS A (red, +35% offset) and TGOS B (black).

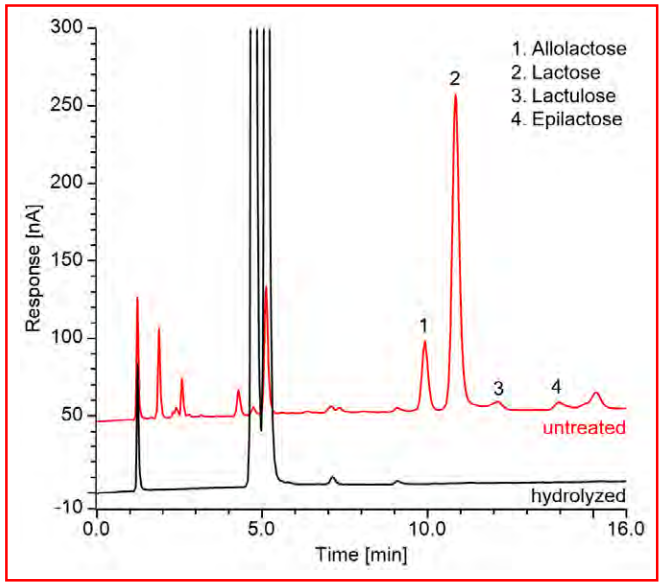

Figure 9: Overlay (zoomed in) of chromatograms obtained with the TGOS A sample. The initial test solution (assay 1) is shown in red (-35% offset). The hydrolyzed sample (assay 2) is shown in black.

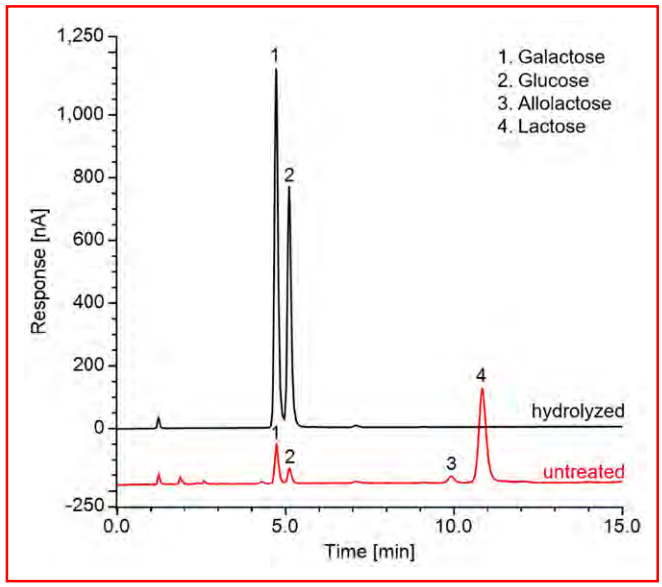

Figure 10: Overlay of chromatograms obtained with the TGOS spiked yoghurt sample (S1). The initial test solution (assay 1 is shown in red (-35% offset). The hydrolyzed sample (assay 2) is shown in black.

Table 10

TGOS content (g/100g sample)

Sample	Galactose from TGOS	Glucose from TGOS	TGOS (0.9 x Gal + Glu)	TGOS (k x Gal)
TGOS A	2.79	1.45	3.96	3.91
Sample S1	2.80	1.49	4.00	3.91
Sample S2	2.88	1.42	4.01	4.03
TGOS B	3.04	1.54	4.27	4.25

The absence of the majority of these peaks in the hydrolyzed TGOS A sample (figure 9) indicates that they indeed represent carbohydrates with glycosidic linkages cleavable by β-galactosidase. No detectable amount of lactose is present in the assay 2 sample, which confirms that the sample is fully hydrolyzed. The results for all samples are summarized in table 10. The TGOS content calculated using the k-factor and based on the released amount of glucose and galactose are both listed in the table. The difference between the TGOS contents obtained with both calculation methods is not more than 2% for all samples. The TGOS content of the TGOS A and B powders used as ingredient in the test samples, was calculated based on the values reported in table 10, taking into account the initial dilution to a 5% test solution in DI water. For TGOS A and B the TGOS content was 77.4% and 84.4%, respectively. These values are in accordance with the minimal GOS contents as specified on the product labels (>68% and >70% for TGOS A and B, respectively).

Comparison of methods

It can be concluded that the TGOS content obtained with the original AOAC method and the adapted method with improved separation (isocratic elution on a fast-4µm column) are in accordance with the product labeling information. However, the resulting TGOS values determined based on the improved separation method are significantly higher (approximately 4% for TGOS A and 8 % for TGOS B). This is related to the presence of allolactose in both TGOS powders and the lack of separation between lactose and allolactose using the original method with a conventional anion-exchange column with a 10 µm particle size* (see foot note). This is evident if one compares figure 4 and 8. So in case of insufficient separation both allolactose and lactose will coelute and this will result in an overestimation of the lactose contents. Subsequently, the TGOS contents will be underestimated, because of the larger $Gal_{lactose}$ contribution in the calculation (galactose released from lactose). In the case of TGOS B, which contains a much larger amount of allolactose, evidently the difference is more significant. So, besides a good separation of glucose and galactose from TGOS constituents and other sugars, a good separation of lactose from its relevant isomers is crucial for this analysis. The high-resolution anion-exchange column with 4 µm particle size is well suited for fast isocratic separation of lactose and isomers, as demonstrated in the application note "Analysis of Lactose and Isomers in 'Lactose-free' Labelled Products" [9]. Therefore, the application of this column for the determination of TGOS following the AOAC 2001.02 leads to a simpler, faster and significantly better separation. Furthermore, the shorter run time in combination with a lower flow rate resulted in a 50% reduction in mobile phase consumption and thus less waste ('green' method).

So to summarize the improvements on the AOAC (2001.02):

- Simple isocratic separation (within 15 minutes)
- Stabile baseline during separation (no NaOH gradient)
- Shorter total run time (40 min)
- Less mobile phase consumption (50% less)
- Separation of lactose from all relevant isomers (R_s > 2.5)
- Better on-column LOD's (almost 3 - 5 x better)

The excellent sensitivity and linear range of detection of the DECADE Elite in combination with the SenCell allowed calibration over a larger concentration range of 0.5 - 48 mg/L (correlation coefficients > 0.9999 for all sugars) then specified in the AOAC 2001.02. The larger calibration range may help to simplify and reduce the large number of sample-specific dilution factors advised in the 2001.02, into a fixed set of dilution factors for the majority of food samples.

AOAC 2001.02 limitations

It should be mentioned in general that the AOAC 2001.02 method has some limitations with respect to applicability to certain food samples [11]. The method works well for samples with a high TGOS content and/or if the lactose contents is low. For samples like infant formula the analysis is challenging and not accurate enough, because such samples contain a high concentration of lactose and a low TGOS-to-lactose ratio (TGOS content typically below 10g/100g). For such samples with high levels of lactose the upcoming AOAC 2021.01 method (first action) based on HPLC-FLD is a more suitable approach [12]. Furthermore, it is anticipated that when quantifying the TGOS contents based on the release of both galactose and glucose, after enzymatic conversion (so without using the k-factor), this will also become challenging in samples with high levels of glucose and a low TGOS contents.

*) Note that the shown separation of allolactose and lactose with the conventional anion-exchange column with a 10 µm particle size was not optimized. With the specified gradient conditions in the AOAC we were not able to separate lactose and allolactose sufficiently with our specific column. The AOAC 2001. 02 allows adjustment of parameters of the gradient profile to optimize the separation between the relevant analytes.

References

1. R.J. Boesten et al., 'Interactomics in the human intestine: Lactobacilli and Bifidobacteria make a difference.', *J Clin Gastroenterol* , 42(2008), p S163–S167
2. AOAC Official Method 2001.02: trans-Galactooligosaccharides (TGOS) in Selected Food Products
3. D.O. Otieno, "Synthesis of β-galactooligosaccharides from lactose using microbial β-galactosidases." *Comprehensive Reviews in Food Science and Food Safety* 9.5 (2010), p471-482
4. L. Coulier et al. "In-depth characterization of prebiotic galacto-oligosaccharides by a combination of analytical techniques." *Journal of agricultural and food chemistry* 57.18 (2009), p8488-8495.
5. J. de Slegte et al., "Determination of trans-galactooligosaccharides in selected food products by ion-exchange chromatography: collaborative study." *Journal of AOAC international,* 85 (2002), p417 -423.
6. Louw, H.J. Brouwer, N. Reinhoud, Electrochemical flow cell, (2016) US patent 9310330
7. R.D. Rocklin et al., "Improved long-term reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform", *Anal. Chem*, 70 (1998), p1496 – 1501
8. Dionex Corporation, application note 155, "Determination of Trans-Galactooligosaccharides in Foods by AOAC Method 2001.02" (2016)
9. Antec Scientific, Analysis of Lactose and Isomers in 'Lactose-free' Labelled Products, Application note, 220_009
10. H. Lin et al., "Simultaneous determination of galactose, glucose, lactose and galactooligo-saccharides in galactooligosaccharides raw materials by high performance anion-exchange chromatography with pulsed amperometric detection", *Food Chemistry*, 263 (2018), p29-36
11. S. Austin et al., "Determination of β-Galactooligo-saccharides by Liquid Chromatography", *Int. J. Anal. Chem*, 2014, p1-10
12. D. Cuany et al., "Determination of β-Galactooligo-saccharides (GOS) in Infant Formula and Adult Nutritionals: Single-Laboratory Validation, First Action 2021.01", *Journal of AOAC international,* 105(1), (2022), p142-158

Ordering information

System	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT 2.1 Column thermostat
Software*	
195.0035	Clarity CDS single instr. incl LC, AS module

*) The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

Conclusion

The ALEXYS Carbohydrate Analyzer with DECADE Elite detector and SenCell provides a fast and user-friendly analysis solution for the quantification of the TGOS content in food products following the AOAC 2001.02. An optimized method is presented using a high-resolution HPAEC column with 4 µm particle size. The column enables fast isocratic separation of all analytes of interest within 15 minutes, combined with excellent resolution for lactose and relevant isomers (Rs > 2.5). Furthermore, a shorter run time (40 min) in combination with a lower flow rate led to 50% less mobile phase consumption. As an alternative, the TGOS contents could be quantified by analysing both the galactose as well as the glucose release after hydrolysis. In that case product information from the manufacturer about the k-factor or degree of polymerization is not required.

Fructans are often used as additives in infant formula and pediatric/adult nutrition

Fructans are naturally occurring polysaccharides found in many fruits and vegetables. They are predominantly composed of fructose monomers with a chain length of 10 -60. Fructans with a shorter chain length are known as fructooligosaccharides (FOS). Fructans help to maintain a healthy and balanced gut microflora.

Application Note

Food & Beverage

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Monosaccharides
Disaccharides
Lactose
Galactooligosaccharides
Fructooligosaccharides
Fructans
Other oligo- and polysaccharides

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

Fructans in infant formula

- **ALEXYS Carbohydrate Analyzer**
- **AOAC 2016.14**
- **High-resolution isocratic HPAEC-PAD separation**
- **Fructose & glucose**

Summary

Fructans are naturally occurring carbohydrates found in many fruits, vegetables, and legume. They are polymers predominantly composed of fructose monomers with a length of 10 -60; Fructans with a shorter chain length are known as fructooligosaccharides (FOS). Fructans pass the stomach and small intestine unchanged and are therefore and source of water-soluble dietary fiber with a prebiotic function; They help maintain a healthy and balanced gut microflora by selectively stimulating the growth of one or a limited number of beneficial bacteria in the colon [1]. However, the intake of fructans can also have adverse effects, as the breakdown of fructooligosaccharides by bacterial fermentation could lead to symptoms similar to those of irritable bowel syndrome [2].

The AOAC method 2016.4 was recently developed for the quantification of fructans in infant formula and adult/pediatric nutritional formula [3]. This method is based on enzymatic hydrolysis of the fructans and subsequent analysis of the reaction products (fructose & glucose) with High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). The use of the DECADE Elite electrochemical detector and SenCell in combination with a high-resolution IC column with 4 µm particle size resulted in an excellent sensitivity and dynamic range. Therefore, this method allows for direct analysis of the fructan constituents without the need for post-column addition of NaOH as described in the original AOAC method. Four infant formula samples are analyzed as an example to demonstrate the performance of this method using the ALEXYS Carbohydrates Analyzer with DECADE Elite detector.

Introduction

Fructans are naturally occurring carbohydrates found in many fruits, vegetables, and legumes. They are classified into two groups, inulin-type and levan-type, which are composed of fructosyl units linked together via β(2–1) and β(2–6) glycosidic bonds, respectively, with an optional glucose residue linked via α(1–2) glycosidic bond at the reducing end [1]. The β(2–1), and β(2–6) glycosidic bonds prevent fructans from being digested like a typical carbohydrate; they reach the gut intact and are therefore available as a nutrient for the beneficial bacteria in the local microflora [2].

FOS as a functional food ingredient has been gaining significant interest due to its desirable organoleptic and prebiotic properties. Fructans can be as a food additive in processed products, for example it can be used as a low-caloric sweetener or a texture-improving ingredient of low-fat foods [4]. In infant formula and adult nutritionals FOS and other oligosaccharides, such as galactooligosaccharides, are often added for their prebiotic effect.

A method for the determination of fructans in infant formula and adult/pediatric nutritional formula was recently adopted as an AOAC final action method [3]. In AOAC method 2016.14 the total fructan content is determined indirectly based on the quantification of the monosaccharides fructose and glucose released after enzymatic treatment. The sample preparation consists of three steps:

(1) In the first step all carbohydrates containing fructose and glucose that are not fructans (i.e. sucrose and α-glucooligosaccharides) are completely hydrolyzed.

(2) Subsequently, the remaining oligosaccharides, including the fructans, are separated from the monosaccharides using solid phase extraction.

(3) In the last step the isolated fructans are hydrolyzed with a mix of fructanase into glucose and fructose.

The released monosaccharides glucose and fructose are then analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

HPAEC-PAD is the method of choice for the analysis of monosaccharides as it combines high selectivity with high sensitivity and a large dynamic range. The AOAC method 2016.14 utilizes post-column addition of NaOH to further extend the linear detection range. However, the use of post-column addition requires additional hardware and chemicals. In this application note it is demonstrated that, with the use of the ALEXYS carbohydrates analyzer, accurate and sensitive quantification of fructans is also possible without the use of post-column addition.

Figure 1: ALEXYS Carbohydrates Analyzer consisting of the ET210 eluent tray, AS6.1L autosampler, P6.1L isocratic pump and DECADE Elite electrochemical detector.

Method

The adapted AOAC 2016.14 method for total fructan analysis was evaluated using the ALEXYS Carbohydrates Analyzer as shown in figure 1. The HPAEC-PAD system consists of a P6.1L pump with integrated Solvent Switch Valve (SSV) capable of running step gradients, AS6.1L autosampler, ET210 eluent tray for Helium blanketing, and the DECADE Elite electrochemical detector. The SenCell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the sugars. The system was controlled by Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 7.2.10.

Requirements

The main method performance requirements for the determination of fructans in infant formula, as defined the AOAC Working Group for Fructans, are summarized in table 3 [5]. In this application note a small set of samples was analyzed to demonstrate that this method meets the key requirements.

Table 1

Adapted AOAC 2016.14 method

HPLC system	ALEXYS Carbohydrates Analyzer - isocratic
Detector	DECADE Elite electrochemical detector
Columns	CarboPac™ PA210-Fast-4µm IC column, 30 x 4.0 mm ID CarboPac™ PA210-Fast-4µm IC column, 150 x 4.0 mm ID BorateTrap™ Inline Trap Column, 50 x 4.0 mm ID, 20 µm All columns: Thermo Scientific™ Dionex™
Mobile phase (MP)	A: 9 mM NaOH B: 100 mM NaOH, 100 mM NaOAc Eluents prepared & blanketed with Helium 5.0
Flow rate	0.8 mL/min
Back pressure	about 290 bar
Injection	5 µL partial loop
Temperature	35 °C for separation and detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF RE, 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 0.2— 0.4 µA
ADF	0.05 Hz
Range	10 µA/V

Table 2

Step-gradient program

Time (min)	*Mobile phase*	*Description*
0 – 8	9 mM NaOH	Elution & detection
8 –13	100 mM NaOH, 100 mM NaOAc	Column clean-up and regeneration
13 - 33	9 mM NaOH	Equilibration, starting conditions

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.

In the AOAC method 2016.14 two sets of chromatographic conditions are described for an anion-exchange column with a 10 µm and 6.5 µm particle size, respectively. In this method (table 1) the same type of IC column was used but with a smaller particle size of 4 µm. The use of this specific column enables high-resolution separation with a short analysis time. An inline trap was used to trap borate ions to reduce tailing of the fructose peak. The temperature for both separation and detection was

Table 3

Method performance requirements

Parameter	*Value*
Analytical range (g/100g)	0.03 - 5.0
LOQ (g/100g)	≤ 0.03
Repeatability (RSD, %)	< 6
Reproducibility (RSD, %)	< 12
Recovery (%)	90 - 110

35°C, therefore no column thermostat was required and the column was placed inside the oven compartment of the DECADE Elite electrochemical detector. All analytes of interest (glucose, fructose and the internal standard N,N'diacetyl-chitobiose) are separated under isocratic elution (table 2). A strong column clean-up and regeneration step is executed at t =13 min to elute strongly retaining components and to remove carbonate ion (CO_3^{2-}) build up on the column. After the cleaning step the column is equilibrated for 15 minutes at the starting conditions, resulting in a total run time of 33 minutes.

Detection

For the sensitive detection of the saccharides the SenCell™ electrochemical flow cell was used in Pulsed Amperometric Detection (PAD) mode. This flow cell [5] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). The temperature for detection was set to 35°C. The cell current was typical about 0.2—0.4 µA using these PAD settings under the specified conditions.

Preparation of reagents, standards and samples

Buffers: Sodium acetate buffer (100 mM, pH 4.5) was prepared by diluting 2.9 mL glacial acetic in 450 mL of water, adjusting the PH to 4.5 with a 2M NaOH solution, and diluting it to a final volume 500 mL with water. Sodium maleate buffer (100 mM, pH 6.5) was prepared by dissolving 5.8 g maleic acid in 450 mL of water, adjusting the PH to 6.5 with a 2M NaOH solution, and diluting it to a final volume 500 mL with water. Both buffers were sterilized by filtration over a 0.2 µm Polyethersulfone (PES) syringe filter (25 mm Ø FFL/MLS) and kept at 4°C until use.

Enzyme solution: Sucrase/b-amylase/pullulanase/maltase solution and fructanase solution from the Fructan Assay Kit (K-FRUC, Megazyme International Ireland Ltd) were prepared according to the instructions of the manufacturer. The Sucrase/b-amylase/pullulanase/maltase solution was prepared by

Table 4

Sugar concentration in sugar standards

	Sugar standard concentration [µg/mL]		*Injection solution concentration [µg/mL]*	
Standard	*Fructose*	*Glucose*	*Fructose*	*Glucose*
Level 1	20	2	1.4	0.14
Level 2	200	50	14	3.5
Level 3	400	100	28	7
Level 4	600	150	42	10.5
Level 5	800	200	56	14
Level 6	1000	250	70	17.5

dissolving the freeze-dried enzymes in 22 mL sodium maleate buffer. The fructanase solution was prepared by dissolving the freeze-dried powdered enzymes in 22 mL sodium acetate buffer. Both enzyme solutions were aliquoted and stored at -20°C until use.

SPE reagents: The prewash solution for the SPE columns (80% ACN, 0.1% TFA) was prepared by diluting 80 mL acetonitrile and 100 µL TFA to a total volume of 100 ml with DI water. The wash solution (1 M NaCl) was prepared by dissolving 5.8 g NaCl in water and diluting it to an end volume of 100 mL with DI water. The SPE elution solution (25% ACN, 0.1% TFA) was prepared by diluting 25 mL of acetonitrile and 50 µL of TFA to 100 mL with DI water.

Standards: 10 g/L fructose stock solution, 5 g/L glucose stock solution, and 600 mg/L N,N'diacetylchitobiose (Megazyme International Ireland Ltd) internal standard solution were prepared in DI water and stored at -20°C until further use. Working standards were prepared by dilution of the stock solution with DI water according to the dilution scheme in AOAC method 2016.14. To prepare the injection solution for the calibration curve the sugar standards were diluted in exactly the same way as the samples during the enzymatic treatment and SPE extraction. In short, 140 µL of sugar standard was added to 70 µL internal standard, 140 µL DI water, 1050 µl SPE elution solution, 600 µl sodium acetate buffer and mixed well. As a result, the actual sugar concentration of the injection solution is lower than the calibration standards it represents, the actual concentration are shown in table 4.

Sample preparation:

Two infant formula products were purchased from a local supermarket for this study. One store-brand infant formula without any fructo-oligosaccharides (Infant formula A) and one name-brand infant formula containing 0.8 mg FOS per 100 mL reconstituted product (Infant formula B). To simulate a low-level FOS product, infant formula A was enriched with Orafti®Synergy1 Inulin-FOS powder. Additionally, the FOS concentration of Infant formula B was increased with the Inulin-FOS powder to simulate a high-level FOS product.

In total four samples were prepared according to the AOAC method 2016.14. An extensive description of the sample preparation can be found in the method [3]. The optional Carrez clarification was not performed. A flow-chart of the sample preparation is shown in figure 2.

- Infant formula A
- Infant formula A - enriched with fructans
- Infant formula B
- Infant formula B - enriched with fructans

Reconstitution and dilution: The infant formula powder was reconstituted in DI water according to instructions on the package and well homogenized. 9 grams of reconstituted product was added to 30 mL of water and the pH of the diluted product was confirmed to be between 5 – 9. The solution was heated using a water bath at 80°C with constant agitation for 20 minutes and cooled down to room temperature. The solution was diluted with DI water to and end volume of 50 mL and subsequently further diluted based on the expected fructan content according to the dilution scheme described in the AOAC method.

Hydrolysis of sucrose and α-glucans: 420 µL of N, N'-diacetylchitobiose internal standard solution was added to 840 µL of the diluted sample. 840 µL of buffered Sucrase/b-amylase/pullulanase/maltase solution was added and the solution was incubated at 40°C for 90 min.

Removal of monosaccharides: Graphitized carbon SPE columns (Supelclean™ ENVI-Carb™ SPE Tube 0.5g/6mL, Sigma Aldrich St. Louis, USA) were used for the removal of monosaccharides. These SPE columns have a higher bed weight than the SPE column in the original AOAC method, therefore all the working volumes were increases five-fold. The SPE columns were flushed 3x with 2 mL prewash solution followed by 3x 2 mL water. Subsequently, 2 mL of enzyme treated solution was loaded onto the SPE column followed by a washing step of 2x 5 mL of the NaCl wash solution and 4x 5 mL water. Finally, the trapped fructans were eluted using 4x 2 mL SPE elution solution. The eluate fractions were collected in one test tube and thoroughly mixed.

Hydrolysis of fructans: 200 µl of the sodium acetate buffer and 100 µl fructanase enzyme mixture was added to 700 µL of the eluate and incubated for 40 minutes at 40°C. Blank samples were prepared by mixing 300 µL of the sodium acetate buffer with 700 µL of the eluate and incubating for 40 minutes at 40°C. All samples were filtered over a 0.2 µm Polyethersulfone (PES) syringe filter (25 mm Ø FFL/MLS) prior to injection.

Calculations

The amount of glucose released from the constituted product (C_G) can be calculated by multiplying the measured glucose concentration (C_{GB}) with the dilution factor (D) and the dilution factor during extraction (total extraction volume V_A divided by sample mass m_A) and multiplied with 0.0001 (factor to convert analyte concentration in solution (in mg/mL) to analyte concentration in sample (in g/100 g)), as follows:

$$C_G = C_{GB} \times D \times \left(\frac{V_A}{m_A}\right) \times 0.0001$$

The amount of fructose released from the constituted product (C_F) can be calculated in a similar way, using the following formula:

$$C_F = C_{FB} \times D \times \left(\frac{V_A}{m_A}\right) \times 0.0001$$

The total fructan content (TF) can be calculated by adding the amount of released fructose (C_F), multiplied by 0.9 to correct for the water uptake during hydrolysis, to the amount of released glucose (C_G):

$$TF = (C_F \times 0.9) + C_G$$

The formula can be adapted to include a correction based on a blank measurement. In that case, first the concentration found in the blank (C_0) is subtracted from the concentration in the treated sample (C_B), as follows:

$$C_G = (C_{GB} - C_{G0}) \times D \times \left(\frac{V_A}{m_A}\right) \times 0.0001$$

And:

$$C_F = (C_{FB} - C_{F0}) \times D \times \left(\frac{V_A}{m_A}\right) \times 0.0001$$

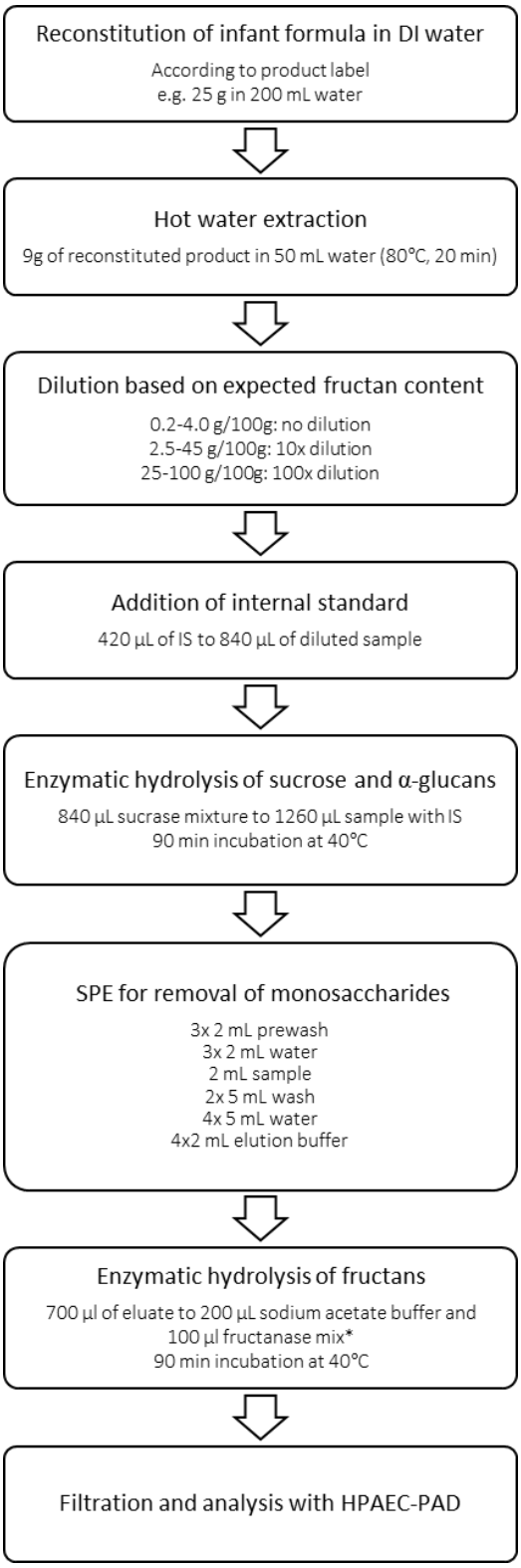

Figure 2: Flow chart of the sample preparation. The hydrolysis of sucrose/α-glucans and SPE were performed with 5x larger volumes than the original OAC method to accommodate for the higher bed weights of the SPE-columns. *) for the blank measurement the 100 µL of fructanase mix is replaced by 100 µL of sodium acetate buffer.

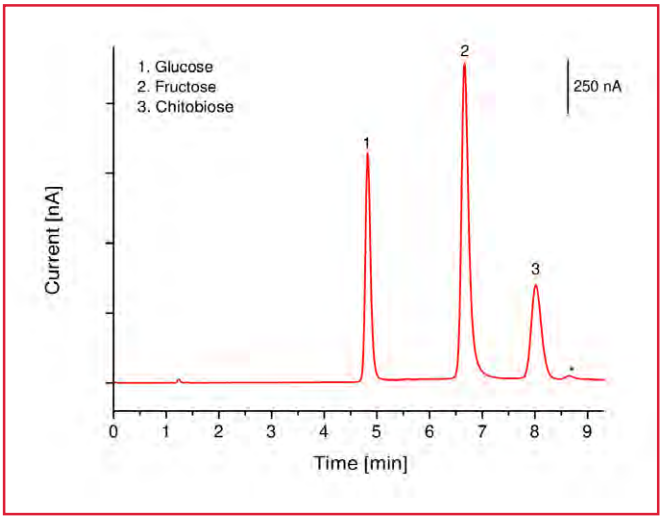

Figure 3: Chromatogram obtained with a 5 µL injection of the level 4 calibration standard containing glucose (1), fructose (2) and N,N'diacteylchitobiose (3). The peak originating from the organic solvents in the injections solution is marked with an asterisk.

Results

In figure 3 an example of a typical chromatogram of a calibration standard is shown. The injection solution consist of the sugar standard containing 600 µg/mL fructose and 150 µg/mL glucose diluted with internal standard solution, the SPE elution solution and the sodium acetate buffer. The actual sugar concentration in the injection solution are 42 µg/mL fructose, 10.5 µg/mL glucose, and 83.9 µg/mL chitobiose. All analytes of interest elute within 9 minutes. The total analysis time for each sample is 33 min, which includes a 5 min post-run step gradient for column clean-up and 15 min re-equilibration time. The peak efficiencies found for the sugars are in the range of 45 000 - 65 000 theoretical plates/meter. Glucose and chitobiose have a tailing of around 1.2. Fructose has a slightly higher tailing factor of 1.4.

The injected organic solvents from the SPE elution buffer cause a small disturbance in the baseline, as marked with an asterisk

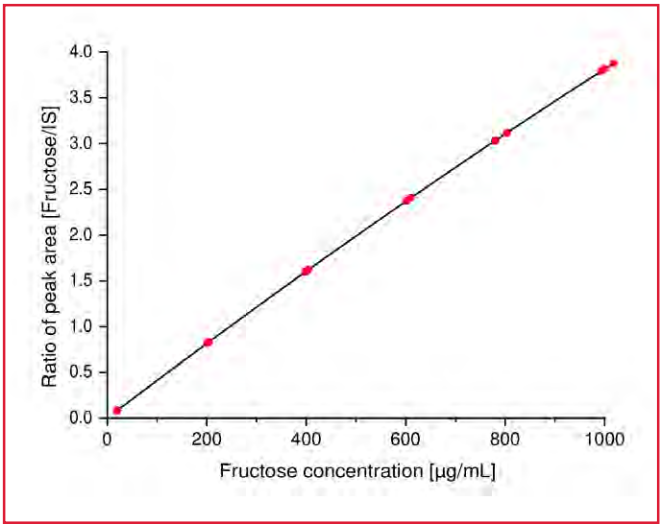

Fig. 4: Calibration curve of fructose using chitobiose as an internal std.

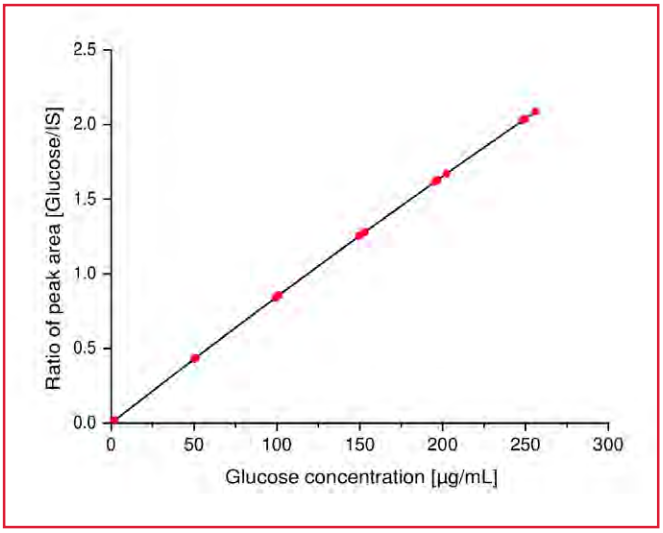

Fig. 5: Calibration curve of glucose using chitobiose as an internal std.

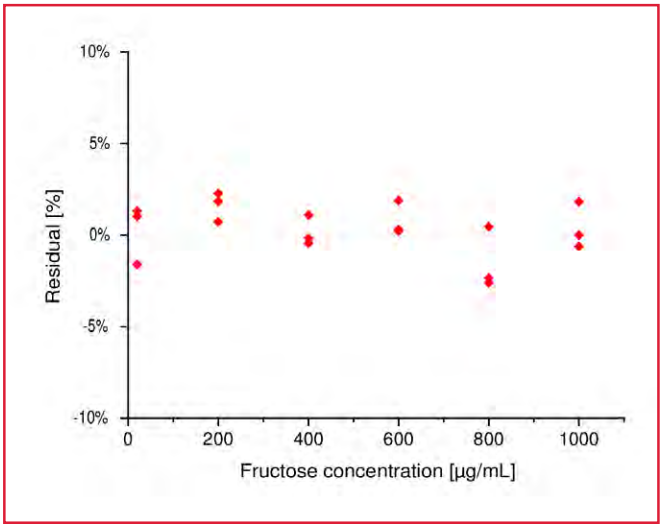

Fig. 6: Relative residual errors of the fructose calibration curve.

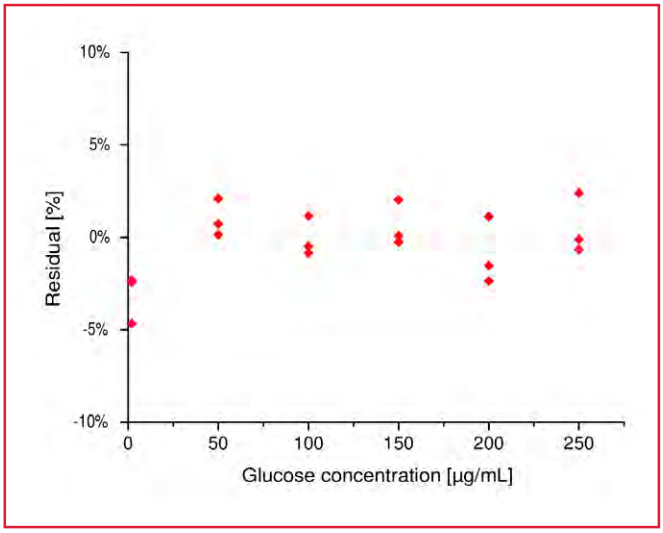

Fig. 7: Relative residual errors of the glucose calibration curve.

in figure 3. Initially, this small peak coeluted with the internal standard when using a separation temperature of 20 or 30°C as described in the AOAC. Therefore, the temperature setting was optimized to improve separation. At 35°C the peak was well separated from chitobiose and didn't interfere with the quantification of the saccharides.

Calibration

Electrochemical detection of saccharides has been shown to have a large linear dynamic range, however at high analyte concentrations, calibration curves of amperometric detectors may deviate from linearity, therefore a quadratic fit was used in AOAC method 2016.14. Three series of calibration standards were prepared and the quadratic fit of the curve was investigated by determination of the residual error for every calibration level. The standard curve of fructose and glucose using chitobiose as an internal standard are shown in figure 4 and 5, respectively.

The relative residual errors for the calibration curves for fructose and glucose are shown in the figure 6 and 7, respectively. The predicted concentration and the actual concentration of the standards correspond well with most of the residual errors below ± 3%. The generally accepted criteria for a good calibration model is that the lack-of-fit for the standards should be less than 5%, with the exception of the lowest standard, which may be higher, below < 10% [7]. The relative residual errors are small over the whole concentration range and fall well within the acceptance criteria. It is evident that a good quadratic fit can be obtained using this method without post-column addition of sodium hydroxide.

Repeatability

The relative standard deviation (RSD) of the retention time and peak area were determined for 6 repetitive injections with the level 1 (1.4 µg/mL fructose and 0.14 µg/mL glucose) and level 2 (14 µg/mL fructose and 3.5 µg/mL glucose) calibration standards (table 5). The RSD's for peak area and retention time for all sugars were below 2% and 0.15%, respectively. These data are well below the minimal required repeatability (< 6%, table 3) and demonstrate that with this method reproducible analysis of all the analytes of interest can be achieved.

Table 5

Repeatability of 5 µL injections of a level 2 and level 1 calibration standard (n=6)

	RSD's (%) L2 standard		*RSD's (%) L1 standard*	
Compound	t_R	*Area*	t_R	*Area*
Glucose	0.14	0.26	0.06	1.61
Fructose	0.10	0.48	0.08	0.72
Chitobiose	0.11	0.20	0.07	0.17

Table 6

Signal to noise ratio

Sample	*S/N Glucose*	*S/N Fructose*
Spiked infant formula A	170	701
L1 calibration standard	82	308

Limit of quantification

An infant formula A sample without Fructans was spiked with the LOQ level of fructan (0.03g /100g, see table 3) and the signal-to-nose (S/N) ratios were determined. The noise was calculated based on a 5-minute section of the baseline of a blank injection (ASTM noise, average peak-to-peak baseline noise of 10 segments of 0.5 min). The resulting S/N's are shown in table 6 and the chromatogram of the injection with sample with a low fructan concentration is shown as the red trace in figure 9. All signal to noise ratios are well above the limit of quantification (ten times the ASTM noise, i.e. a S/N of 10).

The excellent sensitivity is also evident from the S/N ratios of the lowest level calibration standard, as shown in table 6. The S/N's of the individual sugars are a least a factor 8 higher than minimally required for quantification (LOQ, S/N of 10). The sum of glucose and fructose in the level 1 calibration standard corresponds roughly to the equivalent of 0.011 g /100 g fructan content. The exact equivalent depends on the ratio fructose/glucose in the fructans, but these data already demonstrate that fructan contents down to 0.01 g per 100 g product can be reliably quantified.

Sample analysis

The four samples (2 consumer products and 2 enriched consumer products) were prepared and analyzed using the presented method. The obtained chromatograms are shown in figure 8 to 11. The samples and the sample blank are shown in an overlay in red and grey, respectively. For infant formula A (figure 8), the infant formula without any fructans, the sample and the sample blank correspond well. No fructose or glucose was detected after the second hydrolysis step with the fructanase mixture, which confirms no fructans were present in this product as stated on the label.

As infant formula A was confirmed to be free of fructans, it could be spiked with 0.03 g fructan per 100 g product, the mini-

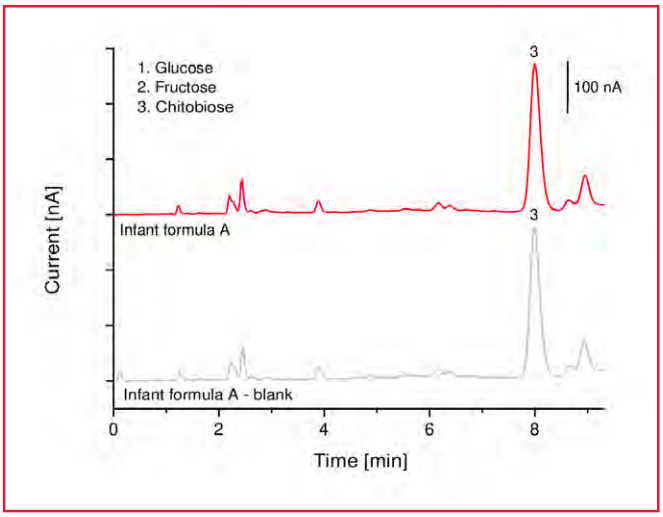

Figure 8: Overlay of the chromatograms obtained with the non hydrolyzed solutions (blank) of infant formula A (grey) and infant formula A (red).

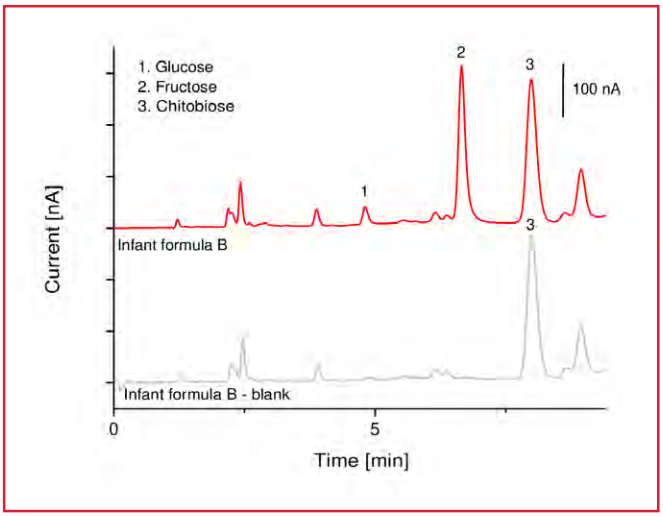

Figure 10: Overlay of the chromatograms obtained with the non hydrolyzed test solutions (blank) of infant formula B (grey) and infant formula B (red).

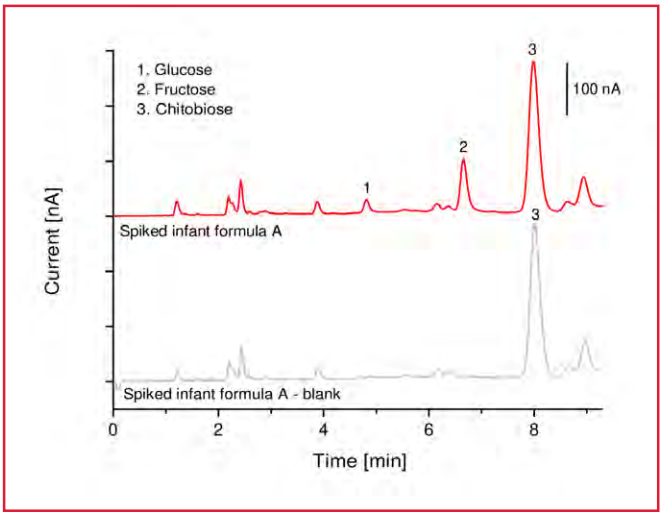

Figure 9: Overlay of the chromatograms obtained with the non hydrolyzed solutions (blank) of the spiked infant formula A (grey) and spiked infant formula A (red).

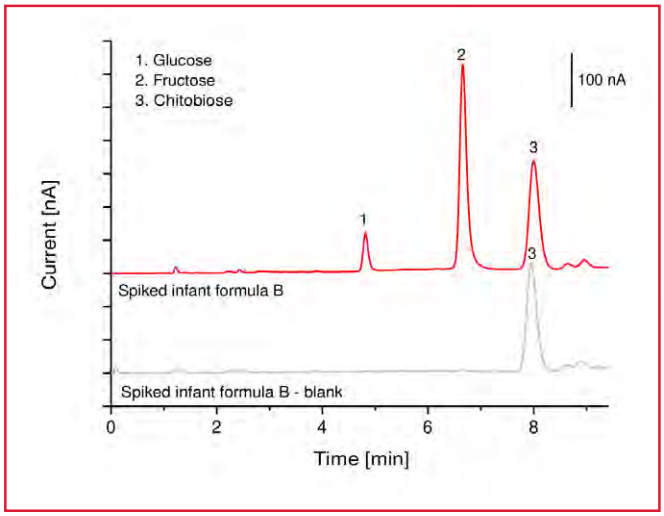

Figure 11: Overlay of the chromatograms obtained with the non hydrolyzed solutions (blank) of the spiked infant formula B (grey) and spiked infant formula B (red).

mal required limit of quantification for AOAC method 2016.14 [7]. After hydrolysis of the spiked sample, fructose and glucose are formed, as shown in figure 9. The S/N of glucose and fructose are 170 or higher (see also table 6), indicating that this method is suitable for the analysis of infant formula with low concentration levels of fructans.

The chromatogram of infant formula B, which contained fructans, is shown in figure 10. Fructose and sucrose are observed after hydrolysis with fructanase, which confirms that fructans are present in this sample. Infant formula B was also spiked with additional fructans to simulate a product with a higher fructan content. The spiked sample is shown in figure 11. All peaks other than glucose and fructose are greatly reduced in response, since this sample was additionally diluted based on its expected fructan content, as described in the AOAC method.

For all samples the total fructan (TF) content was calculated and the recovery for the spiked samples was determined. The recovery was calculated by subtracting the total fructan content from the infant formula (TF_{sample}) from the total fructan content from the spiked infant formula ($TF_{spiked\ sample}$), and subsequently dividing it by the total fructan added (TF_{spiked}), as described in the following formula:

$$\text{Recovery (\%)} = 100\% \times \frac{\text{TF}_{\text{spiked sample}} - \text{TF}_{\text{sample}}}{\text{TF}_{\text{spiked}}}$$

The results of the determination of the total fructan content and recovery are summarized in table 7. The found values correspond well with the expected fructan content. The fructan content found in infant formula B is in accordance with the content specification on the product label. Infant formula A was confirmed to be free of fructans. The recoveries, 97.7% and 104% for the low-level and high-level spiked infant formula respectively, fall well within the required range (90% - 110%).

For new type of products it should be verified that any interferences of the sample matrices, if present, have a neglectable impact on the quantification. Therefore the measurements were also performed with a blank correction included as described in the calculation section. These results are summarized in table 8.

Table 7

Total fructan content

Sample	g/100 g reconstituted product	Recovery
Infant formula A	0.00	-
spiked infant formula A	0.03	104%
Infant formula B	0.09	-
spiked infant formula B	1.70	97.7%

The results are near identical, only the recovery of the spiked infant formula A is slightly lower. The blank correction had no significant impact on the quantified (absolute) amounts of fructan in the sample. From this data it is evident that no blank measurement and subsequent correction is required for these specific infant formula products.

Table 8

Total fructan content after blank correction

Sample	g/100 g reconstituted product	Recovery
Infant formula A	0.00	-
spiked infant formula A	0.03	103%
Infant formula B	0.09	-
spiked infant formula B	1.70	97.7%

Conclusion

The ALEXYS Carbohydrate Analyzer provides a fast and sensitive analysis solution for the determination of fructans in infant formula and adult nutritionals following the AOAC 2016.14. An optimized method is presented which meets all the key performance requirements for the analysis of fructans as defined by the AOAC Working Group for Fructans. The use of a DECADE Elite and SenCell in combination with a high-resolution IC column enables sensitive and accurate quantification of fructans over a wide concentration range, without the need for post-column addition of sodium hydroxide.

References

1. Niness, Kathy R. Inulin and oligofructose: what are they?. The Journal of nutrition 129.7 (1999), 1402S-1406S.
2. Olesen, Merete, and Eivind Gudmand-Høyer. Efficacy, safety, and tolerability of fructooligosaccharides in the treatment of irritable bowel syndrome. *The American journal of clinical nutrition* 72.6 (2000), 1570-1575.
3. Spichtig, Véronique, et al. Determination of Fructans in Infant Formula and Adult/Pediatric Nutritional Formula by Anion-Exchange Chromatography with Pulsed Amperometric Detection after Enzymatic Treatment: Collaborative Study, Final Action 2016.14. *Journal of AOAC International* 103.5 (2020), 1301-1317.
4. Shoaib, Muhammad, et al. Inulin: Properties, health benefits and food applications. *Carbohydrate polymers* 147 (2016), 444-454
5. AOAC SMPR 2014.002: Fructans in Infant Formula and Adult/Pediatric Nutritional Formula, Journal of AOAC International 98.4 (2015), 1038–1039.
6. Louw, H.J. Brouwer, N. Reinhoud, Electrochemical flow cell, (2016) US patent 9310330
7. Brunt, Kommer, et al. Dual-Laboratory Validation of a Method for the Determination of Fructans in Infant Formula and Adult Nutritionals: First Action 2016.14. Journal of AOAC International 100.3 (2017), 753-767.

Ordering information

System	
180.0055W	ALEXYS Carbohydrates Analyzer - isocratic
116.4321	SenCell 2 mm Au HyREF
Software*	
195.0035	Clarity CDS single instr. incl LC, AS module

*) The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

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Q10, ubiquinols

Characterization of Inulin-type fructan mixtures

- **ALEXYS Carbohydrate Analyzer**
- **High-resolution HPAEC-PAD separation**
- **Profiling of Inulin and fructo-oligosaccharide**
- **Dietary fiber, prebiotic and food additive**
- **Quantitation of short-chain FOS**

Fructans are polymers consisting of fructose found widely distributed in nature as plant storage carbohydrates. Inulin-type fructans (ITF) are present in many plant species including wheat, onion, bananas, garlic and chicory. Estimates suggest a person consuming an average European diet ingests 3-11 g inulin-type fructans daily [1]. Fructans are a form of dietary fiber as they are not digested in the upper gastrointestinal trac. They can act as an energy source for the gut microbiota and have the potential to promote the growth of specific beneficial bacteria. Furthermore, ITF is increasingly being used as an food additive, for example as a low-caloric sweetener or a texture-improving ingredient of low-fat foods [2].

Fructans exist as heterogeneous blends of polymers, with degrees of polymerization ranging from 2 to 60 subunits. The properties of inulin-type fructan as a functional food ingredient depend mainly on the average degree of polymerization (DP). The average DP can vary, depending on source, harvest time, and processing conditions [3]. In this application note a method is presented for the analysis of the chain-length distribution of Inulin-type fructans. The use of High-performance anion-exchange chromatography (HPAEC), coupled with pulsed amperometric detection (PAD), enables the complete separation and sensitive detection of individual oligosaccharides. To demonstrate the specificity and sensitivity of this method, the oligo- and polysaccharide distribution of several inulin samples was characterized by HPAEC-PAD using the ALEXYS® Carbohydrates Analyzer

Introduction

Fructans are polymers consisting of fructose building blocks and serve as an energy storage carbohydrate in many plants. Inulin-type fructans (ITF) are present in over 36,000 plant species and are widely used in the food industry because of its nutritional and functional properties. Estimates suggest a person consuming an average European diet ingests 3-11 g inulin-type fructans daily [1]. Inulin-type fructans are linear polymers where the fructose residues are bound to one another by β-2,1 linkage with an optional terminal glucose unit. Hence, all inulin-type fructans can be described with the generic chemical structure GF_n (with G as optional glucose, F as fructose, and n indicating the number of fructose moieties).

Inulin is typically extracted as native inulin from a food source such as chicory root. Native inulin is a heterogenous blend of oligo- and polysaccharides with a degree of polymerization (DP) ranging from 2 to 60. Some of the fructans have a glucose unit at reducing end (GF_n type fructans), while others do not include a glucose residue at all (F_n type fructans). The functional properties of inulin mainly depend on the degree of polymerization. To achieve its desired properties, native inulin can be processed into more purified food ingredients, such as long-chain inulin (DP 10-60) or the shorter fructo-oligosaccharides (FOS, DP 2-7). Short-chain FOS (scFOS, DP 2-4) can be obtained by the enzymatic elongation of a sucrose with fructose moieties [2].

The β-2,1 linkages prevent inulin from being digested like a typical carbohydrate. The fructans are not broken down by human digestive enzymes and are therefore available as a nutrient for beneficial bacteria. The shorter fructans are fermented first by the colonic microflora, the longer chain inulin is fermented twice slower than FOS, and therefore stimulate the metabolic activity of the improved flora in more distal parts of the colon [4]. As the fructans resist enzymatic digestion and reach the gut intact, they are also considered dietary fiber from a food-labeling perspective.

Inulin-type fructans are also increasingly being used as food additive. Several commercial grades of inulin are available that have a neutral, clean flavor and used to improve the mouthfeel, stability, and acceptability of healthier food products. For example, short-chain inulin is soluble and sweet with minimal caloric value and can be used to partially replace sucrose in low-sugar products. Long-chain inulin is less soluble and more viscous and can be used to simulate the presence of fat in low-fat products [5].

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.

High-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric (PAD) detection has already been demonstrated as a powerful tool to profile chain-length distribution of FOS and inulin [6]. Evaluation of the chain length distribution of Inulin-type fructans is important as it affects the nutritional and functional properties. In this application note a method to profile Inulin-type fructans is described using HPAEC-PAD. Several grades of Inulin-type fructans with varying chain length distributions were analyzed to demonstrate the performance of the method.

Additionally, quantification of Inulin-type fructans as dietary fiber is also required for food-labeling purposes. However, the lack of commercial standards hampers the direct quantification of fructans with a DP over 5. A method based on the enzymatic hydrolysis followed by analysis HPAEC-PAD suitable for the quantification of all lengths of fructans is described in application note 220_022: Determination of Fructans in infant formula and adult nutritionals. The quantification of scFOS is possible with the same method for profiling as commercial standards are available for these short oligos. The direct quantification of the relevant free mono– and disaccharides and scFOS is demonstrated with the quantification of scFOS in a prebiotic food supplement

Table 1

Conditions

HPLC system	ALEXYS Carbohydrates Analyzer - gradient
Detector	DECADE Elite electrochemical detector
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA210 guard column, 30 x 4.0 mm ID Thermo Scientific™ Dionex™ CarboPac™ PA210 analytical column, 150 x 4.0 mm ID
Mobile phase (MP)	A: 100 mM NaOH B: 100 mM NaOH, 500 mM NaOAc Eluents prepared & blanketed with Helium 5.0
Flow rate	0.8 mL/min
Back pressure	about 310 bar
Injection	10 µL
Temperature	25 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF RE, 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 0.2— 0.4 µA
ADF	0.05 Hz
Range	10 µA/V

Table 2

Gradient program for Inulin ($DP_{max} \geq 9$)

Time (min)	*Mobile phase*	*Description*
0	100 mM NaOH, 25 mM NaOAc	Elution & detection
60	100 mM NaOH, 450 mM NaOAc	
60 - 75	100 mM NaOH, 25 mM NaOAc	Equilibration, starting conditions

Table 3

Gradient program for FOS ($DP_{max} \leq 8$)

Time (min)	*Mobile phase*	*Description*
0	100 mM NaOH, 25 mM NaOAc	Elution & detection
12	100 mM NaOH, 110 mM NaOAc	
12 - 22	100 mm NaOH, 450 mM NaOAc	Column clean-up and regeneration
22 - 37	100 mM NaOH, 25 mM NaOAc	Equilibration, starting conditions

Method

The method for profiling of Inulin-type fructans was evaluated using the ALEXYS Carbohydrates Analyzer as shown in figure 1. The HPAEC-PAD system consists of a P6.1L pump quaternary LPG pump AS6.1L autosampler, ET 210 eluent tray for Helium blanketing, CT 2.1 column oven and the DECADE Elite electrochemical detector. The SenCell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the carbohydrates. The system was operated under the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 7.2.10.

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. An high resolution anion-exchange column with small particle size (4 µm) was chosen for the separation of the fructans. This column is specifically recommended for the analysis of small oligosaccharides up to pentasaccharides, but perform also well in the separation of homologous series of larger carbohydrates such as Inulin-type fructans, as shown in this application note.

The CT 2.1 column thermostat was used to perform the separation at 25°C. For the analysis of inulin with a degree of polymerization up to 65 the gradient shown in table 2 was used. All polysaccharide are eluted in within 60 minutes with a 100—450 mM sodium acetate gradient in 100 mM NaOH. Subsequently, the column is equilibrated for 15 minutes at the starting conditions, resulting in a total run time of 75 minutes

For the analysis of shorter oligos with a max DP of 8 a shorter gradient program was used (table 3). The gradient is exactly the same for the first 12 minutes. After elution of the oligosaccharides with a DP ≤ 8 a strong column clean-up and regeneration step is executed at t =12 min to elute strongly retaining components and to remove carbonate ions (CO_3^{2-}) build up on the column. After the clean-up step the column is equilibrated for 15 minutes at the starting conditions, resulting in a total run time of 37 minutes.

Detection

For the pulsed amperometric detection of oligosaccharides the Antec SenCell electrochemical flow cell is used. This flow cell [6] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/ H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform was applied as described in table 1. The temperature for detection was set to 35°C. The cell current was typical about 0.2—0.4 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 [7], resulting in less flow cell maintenance and system down time.

Sample preparation

Four products containing inulin-type fructans were obtained for this study. Inulin powder from chicory was purchased from 2 suppliers: Carbosynth (Compton, United Kingdom) and Sigma -Aldrich (Saint Louis, USA). Two commercial consumer products were obtained from local stores: Jarrow Formulas Inulin-FOS food supplement containing Orafti® Synergy1 and Swanson® Probiotics Lactobacillus Rhamnosus with FOS dietary supplement capsules containing probiotics and NUTRAFLORA® scFOS.

One gram of powder or the content of one capsule (0.4 g) was dissolved in 50 mL of water, filtered over a 0.2 µm Polyethersulfone (PES) syringe filter (25 mm Ø FFL/MLS) and diluted to its final concentration with water. Samples were kept at 4°C and injected within 24 hours.

Characterization of Inulin

The 3 samples containing inulin were characterized according to the method described in table 1 and 2. An overlay of the chromatograms is shown in figure 2. Note that these chain-length distribution can be primarily interpreted qualitatively; the response factor decreases linearly with the chain length [8], hence the qualitative distribution is biased towards the lower mass fructans and does therefore not represent the exact quantitative distribution.

The chromatogram of a 10 µL injection of 200 ppm Inulin from Carbosynth is shown as the red trace in figure 2. The inulin consist predominantly of GF_n type fructans ranging from DP 3 (GF_2) to approximately DP 66 (GF_{65}). Additionally, this sample contains a substantial amount of free sugars (glucose, fructose and sucrose). Based on the chain-length distribution of this mixture and the presence of free sugars this was characterized as native inulin, which is the crude hot-water extract of inulin. This sample demonstrates the excellent separation of the presented method. The GF_n and F_n type fructans are baseline separated until GF_7. Because the GF_n type series and the F_n type series show slightly different retention behavior, they inevitably overlap, resulting in the coelution of components starting with GF_8 and F_8.

The chromatogram of a 10 µL injection of 200 ppm inulin from Sigma-Aldrich is shown as the grey trace. The chain length of this mixture varies from DP 8 (GF_9) to approximately DP70 (GF_{70}) and is free of any mono- and disaccharides. This mix of inulin has been processed to remove all oligomers resulting in high-molecular weight inulin, and is also known as long-chain inulin.

The two inulin powders from both manufacturers were labeled as “Inulin from chicory” without any additional specification. Further characterization revealed that inulin powders consisted of different grades of Inulin. The terms Inulin, FOS and terms of proprietary mixes of the two are often used inconsistently throughout industry and academia [9]. In the most generic sense inulin is a term that covers all fructans of the inulin-type, however, it can also be used to specifically describe the hot-water extract from an Inulin-containing material.

The Inulin-FOS food supplement, shown as the black trace in figure 2, was confirmed to be inulin enriched with FOS. This blend of inulin-type fructans consists of two distinct chain-length distributions. The first distribution consist of F_n and GF_n type oligomers up to a DP of 7. The second distribution consists of solely GF_n type fructans ranging from DP 9 to DP 65.

Characterization of FOS

The fructo-oligosaccharide samples were analysed according to method described in table 1 and 3. The chromatograms of the FOS samples are shown in figure 3. The gradient used for the FOS samples has exactly the same slope as the gradient used for inulin samples, hence the retention times of the oligomers match retention times from the low-mass components of the inulin samples exactly.

A chromatogram of a 10 µL injection of a 10 ppm sugar standard containing glucose (G), fructose (F), sucrose (GF), kestose (GF_2), nystose (GF_3), and fructosyl nystose (GF_4) is shown in figure 3 in grey.

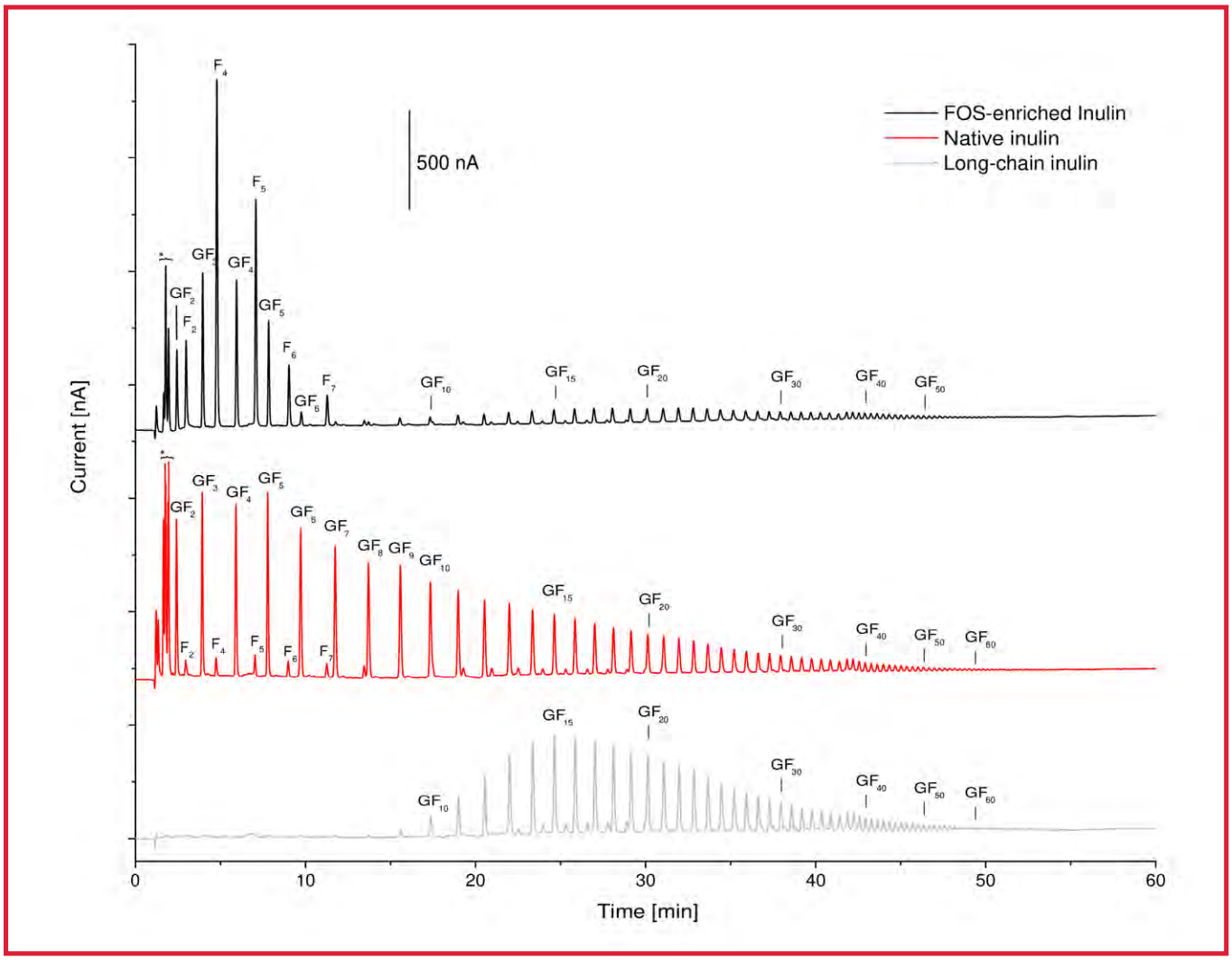

Figure 2: Overlay of 10 µL injections of 200 PPM of inulin-type fructan. The Inulin-FOS mixture is shown in black and was confirmed a mix of FOS and long-chain inulin. The Inulin for chicory from Carbosynth is shown in red and was characterized as native inulin. The Inulin for chicory from Sigma-aldrich is shown in grey and was characterized as purified long-chain inulin. A part of the fructans are labeled with their respective generic chemical structure GF_n (with G as glucose, F as fructose, and n indicating dp). *The free sugars (glucose, fructose and sucrose) are indicated with an asterisk.

The short-chain FOS is shown as the black trace in figure 3. scFOS is synthesized enzymatically by the extension of sucrose, therefore it consists exclusively of GF_n type fructans with DP3 to 5 (GF_{2-4}). Additionally, it contains some free sugar in the form of sucrose (GF).

The red trace represents the FOS content of the Inulin-FOS mixture. This FOS consist mostly of substantial more F_n type than GF_n type fructans, which is typical for FOS obtained by partial hydrolysis of inulin. The hydrolysis of an inulin chain with (mostly) one terminal glucose, results in multiple oligomers with a terminal reducing fructose and only one oligomer with a terminal glucose.

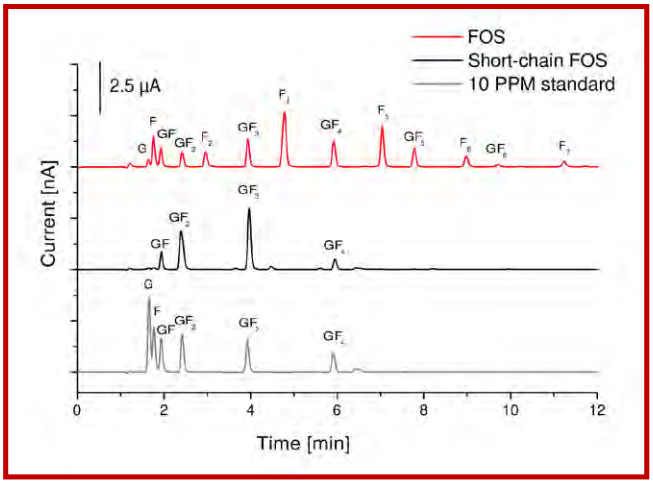

Figure 3: Overlay of 10 µL injections of 100 PPM Inulin-FOS mixture (red), 100 ppm scFOS (black), and standard solution (grey).

Quantitation of free sugars and scFOS

The quantification of most inulin-type fructans is limited by the lack of commercially available sugar standards. However, for scFOS and mono- and disaccharides all standards are available and therefore mixtures containing only scFOS can be quantified using the presented method.

The chromatogram of the 10 ppm calibration standard containing glucose (G), fructose (F), sucrose (GF), kestose (GF_2), nystose (GF_3), and fructosyl nystose (GF_4) is shown in figure 3 in grey. The free sugars (glucose, fructose and sucrose) are not completely baseline separated (R_{G-F} = 1.0, R_{F-GF} = 1.4), but can still be reliably quantified as shown by the repeatability and linearity data. All oligos are baseline separated (R > 1.5) and show no significant tailing with symmetry factors around 1.2.

Linearity and LOQ

The linearity was investigated with standards dissolved in DI water in the concentration range of 0.25 - 25 mg/L . The obtained calibration curves are shown in figure 4. The linearity is excellent in this concentration range with correlation coefficients for peak area better than 0.9994 for all 6 sugars.

The LOQ was calculated as the analyte response corresponding to 10x the ASTM noise determined on a 5-minute section of the baseline (average peak-to-peak baseline noise of 10 segments of 0.5 min). The ASTM noise was 1.1 nA and the calculated concentration LOQ's of the free sugars were 33, 53, and 71 µg/L for glucose, fructose and sucrose, respectively.

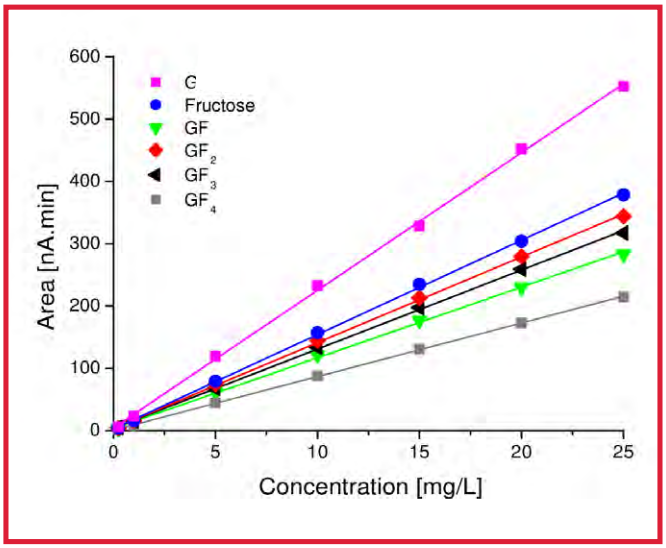

Figure 4: Calibration curve of the glucose (G), fructose (F), sucrose (GF), kestose (GF_2), nystose (GF_3), and fructosyl nystose (GF_4) in the concentration range of 0.25 - 25 mg/L.

Table 4

Repeatability of 10 µL injections of a 0.25 and 1 mg/L sugar standard mix in DI water (n=5)

	RSD's (%) 1 mg/L		*RSD's (%) 0.25mg/L*	
Compound	t_R	*Area*	t_R	*Area*
G	0.23	0.22	0.18	0.46
F	0.21	0.44	0.24	0.46
GF	0.24	0.14	0.23	0.87
GF_2	0.17	0.72	0.19	1.18
GF_3	0.19	0.52	0.23	1.38
GF_4	0.21	0.71	0.16	2.36

The concentration LOQ's for the scFOS were 63, 73, and 131 µg/L for GF_2, GF_3, and GF_4, respectively. During the sample preparation 1 g is dissolved in 50 mL of water, hence the highest LOQ of 131 µg/L for GF_4 corresponds to 0.65 mg/100 g product, which is a factor 150 below the required LOQ for labelling purposes (0.1g/100g).

Repeatability

The relative standard deviation (RSD) of the retention time and peak area were determined for 5 repetitive injections with a 1 mg/L and 0.25 mg/L sugar standard in water. Retention times were stable, with RSD values around 0.20% for all oligos and free sugars independent of concentration. The RSD values for peak area are shown in table 5. The RSD's for peak area were < 2.5% for the 0.25 mg/L standard and typically < 0.75% for the 1 mg/L standard. The data demonstrate that with this method reproducible analysis of all the components, including the free sugars, can be achieved.

Quantification of free sugars

The free sugar content for all four samples was quantified using the presented method. The obtained values and the specified maximum free sugar content for the fructan powders are summarized in table 5. The mono– and disaccharide content in the inulin samples are all in accordance with the specifications. The prebiotic supplement capsules containing scFOS did contain other additional ingredients, therefore no maximum free sugar content could be specified.

Table 5

Free sugars content

Fructan type	*Glucose [g/100g]*	*Fructose [g/100g]*	*Sucrose [g/100g]*	*Total [g/100g]*	*Specified [g/100g]*
scFOS	-	0.2	3.0	3.2	-
Inulin-FOS	0.4	3.3	2.8	6.5	≤ 8
Native inulin	0.9	2.5	4.1	7.6	≤ 10
Long-chain Inulin	-	-	-	-	≤ 0.1

Quantification of short-chain FOS

The short-chain FOS content of the prebiotic supplement as shown in figure 3 was quantified and compared to the specified chain-length distribution. The found values are summarized in table 6. The relative amount of the separate short-chain oligomers fall within the specified range and the total amount meets the required minimal amount of 95 mg per prebiotic supplement capsule.

Table 6

scFOS quantification

Compound	*Measured [mg/capsule]*	*Specified content [mg/capsule]*
GF2	36.5	30 - 42
GF3	50.0	45 - 57
GF4	13.2	5 - 15
Total scFOS	99.7	>95

Conclusion

The ALEXYS Carbohydrates Analyzer, in combination with the SenCell flow cell offers an dedicated and optimized analysis solution for the characterization of mixtures of inulin-type fructans, quantification of short-chain FOS, and determination of free sugar content. The chain-length distribution of complex Inulin mixtures can be determined within 75 minutes, quantification of short-chain FOS and free sugars can be performed in just 37 minutes. The results obtained from various samples, ranging from short-chain FOS to long-chain inulin, show that this method is well suitable for the characterization of heterogenous blends of inulin-type fructans.

References

[1] Niness, Kathy R. Inulin and oligofructose: what are they?. The Journal of nutrition 129.7 (1999), 1402S-1406S.

[2] Shoaib, Muhammad, et al. Inulin: Properties, health benefits and food applications. *Carbohydrate polymers* 147 (2016), 444-454.

[3] Kriukova, Yana, et al. Chain length distribution of inulin from dahlia tubers as influenced by the extraction method. *International journal of food properties* 20.sup3 (2017), S3112-S3122.

[4] Moerman, Frank T., Marinella B. Van Leeuwen, and Jan A. Delcour. Enrichment of higher molecular weight fractions in inulin. *Journal of Agricultural and Food Chemistry* 52.12 (2004), 3780-3783.

[5] Tárrega, A., J. D. Torres, and E. Costell. Influence of the chain -length distribution of inulin on the rheology and microstructure of prebiotic dairy desserts. *Journal of Food Engineering* 104.3 (2011), 356-363.

[6] Rossi, Maddalena, et al. Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. *Applied and environmental microbiology* 71.10 (2005), 6150-6158.

[7] Rocklin, Roy D., Alan P. Clarke, and Michael Weitzhandler. Improved long-term reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform. *Analytical chemistry* 70.8 (1998), 1496-1501.

[8] Timmermans, J. W., et al. Quantitative analysis of the molecular weight distribution of inulin by means of anion exchange HPLC with pulsed amperometric detection. *Journal of Carbohydrate Chemistry* 13.6 (1994), 881-888.

[9] Kelly, Greg. Inulin-type prebiotics--a review: part 1. *Alternative Medicine Review* 13.4 (2008).

Ordering information

System	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT 2.1 Column thermostat
Software*	
195.0035	Clarity CDS single instr. incl LC, AS module

*) The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

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Summary

Dairy products play a vital role in a healthy and balanced diet providing essential vitamins and minerals like calcium. Lactose-intolerance is a wide-spread condition, which prevents a large number of people of consuming dairy products as a part of their daily diet. It is estimated that an average of 65% of the global population is suffering from lactose-intolerance [1]. The global market for 'lactose-free' dairy products is rapidly growing and the criteria for 'lactose-free' labelled products are becoming stricter. In the EU for instance the threshold limit for lactose has been lowered to 10 mg per 100 gr product in the last years in a number of EU member states [2]. To check the lactose contents in these products there is a need for fast, sensitive and selective analytical methods.

In this application note a 'green' method is presented for fast and sensitive analysis of lactose, lactose isomers and lactulose using the DECADE Elite electrochemical detector and SenCell. The method is based on separation by High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). The use of a narrow-bore HPAEC column with 4 µm particle size, allowed the separation of all compounds of interest in less than 8 min in combination with a four-fold reduction of mobile phase usage. A variety of dairy product were analyzed to demonstrate the versatility of the method. Besides the quantification of low concentrations of Lactose and derivatives also the major sugars in dairy products (Galactose, Glucose, Sucrose and Fructose) can be analyzed using the presented method.

Introduction

Lactose is the main carbohydrate found in milk and dairy products (cow milk contains approximately 5% Lactose). It is a disaccharide composed of the monosaccharides D-glucose and D-galactose, joined in a ß-1,4-glycosidic linkage. In humans lactose is metabolized into glucose and galactose in the intestines by the enzyme lactase. Lactose intolerance is a condition caused by the inability to digest lactose due to a lactase deficiency. The most common symptoms of lactase deficiency are intestinal discomforts such as cramps, diarrhea, bloating, and gas. The reduction of lactase activity in humans starts already at infancy and might develop into a lactose intolerance during adulthood [3]. It is estimated that an average of more than 65% of the global population is suffering from lactose-intolerance. Rates of lactose intolerance vary between regions, from less than 10% in Northern Europe to as high as 95% in parts of Asia and Africa.

The global demand for 'lactose-free' dairy and other food products is rapidly growing and a large amount of commercial 'lactose-free' product are available nowadays. The majority of these products are produced by enzymatic hydrolysis using lactase containing yeast (*Kluyveromyces* strains), resulting in a reduction of lactose concentration up to < 0.01%. Currently there is no legislation in the US and EU with respect to the lactose concentration limits in 'lactose-free' labelled products, except for infant formulae [4]. However, in many EU member states a lactose threshold level of 10 mg per 100 g of product is adopted for 'lactose-free' labelled dairy [2]. To check if products meet these requirements there is a need for fast, sensitive and selective analytical methods to quantify lactose.

Besides Lactose, dairy products can also contain lactose isomers, such as allolactose, epilactose and Lactulose. These isomers can be formed enzymatically [5] or by heat treatment such as pasteurization [6]. Lactulose is a recognized laxative and food additive for digestive comfort. Furthermore, both epilactose and lactulose are considered prebiotic lactose isomers. The presence of these isomers, with their small structural differences, hampers the quantification of such low levels of lactose. Good chromatographic separation is necessary to avoid coelution and thus overestimation of the lactose contents in dairy samples. A wide range of different methods are available to measure lactose in food products based on different techniques (enzymatic assays, Mid Infrared, gravimetry, differential pH, polarimetry and HPLC), most of them are lacking sufficient selectivity and sensitivity [7].

High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) is the method of choice. It combines superior selectivity with sensitive detection. Due to the presence of hydroxyl groups which can be oxidized, carbohydrates can be detected without derivatization using pulsed amperometric detection with pico- and femtomol sensitivity [8-10]. Several HPAEC-PAD methods for quantification of lactose are reported in literature based on conventional Anion-exchange columns with 6.5 - 10 µm particle sizes [11,12].

In this application note a 'green' method is presented based on a new type of HPAEC column with 4 µm particle size for the fast and sensitive analysis of lactose and its isomers.

Method

The LC-EC conditions are listed in table 1. The analysis was performed using HPLC with a quaternary low-pressure gradient pump, autosampler and thermostatted column compartment. For detection a DECADE Elite electrochemical detector with SenCell flow cell (see figure 1) was used. The DECADE Elite was controlled via a PC using the Antec Dialogue Elite software.

Figure 1: Left: SenCell with Au working electrode and Pd/H_2 (HyREF) reference electrode. Right: DECADE Elite electrochemical detector.

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. An anion-exchange column was chosen for the separation of lactose and isomers (Table 1) . This column type with small particle size (4 µm) and small internal diameter (2 mm) combines high-resolution separation with low

Table 1

Conditions	
LC system	Quaternary HPLC system
Detector	Antec DECADE Elite electrochemical detector
Columns	CarboPac™ PA210-Fast-4µm IC column, 30 x 2.0 mm ID, 4 µm + 150 x 2.0 mm ID, 4 µm BorateTrap™ Inline Trap Column, 50 x 4.0 mm ID, 20 µm All columns: Thermo Scientific™ Dionex™
Mobile phase (MP)	MP A: deionized (DI) water (resistivity > 18 MOhm.cm and TOC<10ppb) MP B: 200 mM KOH Eluents blanketed with Helium 5.0
Flow rate	0.2 mL/min
Back pressure	About 290 bar (during isocratic elution)
Injection	2.5 µL
Temperature	30 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE 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 0.2— 0.4 µA
ADF	0.5 Hz
Range	500 nA/V or 5 µA/V

Table 2

Step-gradient program			
Time (min)	*Mobile phase*	*B (%)*	*Description*
0 - 10	20 mM KOH	10	Isocratic elution and detection
10 - 15	100 mM KOH	50	Column clean-up and regeneration
15 - 30	20 mM KOH	10	Equilibration, starting conditions

consumption of mobile phase. A trap column was installed in the solvent line between pump and autosampler to suppress tailing of the fructose and lactulose peaks due to complexation of these particular sugars with borate.

The temperature for separation was set at 30 °C . The analysis is based on a step-gradient, see Table 2. At a concentration of 20 mM KOH, 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 100 mM KOH is therefore necessary to remove the bound carbonate ions and late eluting compounds like oligo-saccharides present in dairy samples. This regeneration step assures reproducible retention behavior for each run. The total cycle time for each run is 30 minutes.

For some samples it might be necessary to increase the time of the washing (and/or equilibration) step a bit to avoid late eluting interference in subsequent runs.

Note that all samples shown as example in this application note are analyzed with a wash step of 5 min (table 2). Under this conditions only the chocolate paste sample showed some late eluting interferences in the next run. Therefore, after analysis of this particular sample, a blank run was executed for clean-up.

To minimize the introduction of carbonate ions in the mobile phase the eluents were carefully prepared manually using a carbonate-free 50% w/w KOH solution (commercially available). The diluent was DI 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 KOH 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 (0.5 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 Lactose and isomers the Antec SenCell electrochemical flow cell is used. This novel flow cell [14] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/ H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform was applied as shown in figure 2. The

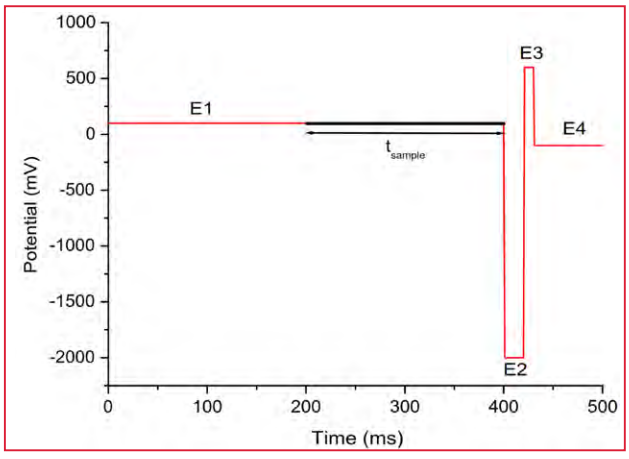

Figure 2: 4-step PAD potential waveform for the detection monosaccharides and other carbohydrates.

temperature for detection was set to 35°C. The cell current was typical about 0.2—0.4 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 [15], resulting in less flow cell maintenance and system down time.

Preparation of standards, reagents and samples

Standards: 10 mM stock standards of the individual sugars were prepared in 95/5 (v/v%) water/acetonitrile. To prevent fast degradation 5% Acetonitrile was added to suppress bacterial and fungal growth. Stock standards under these conditions are stable for more than a month in the fridge at 4° C. Working standards in the concentration range of 100 nM—100 µM were prepared by dilution of the stock standards with DI water.

Carrez reagents: a Carrez clarification procedure is used for deproteination of the dairy samples. By removing all proteins also the enzymatic activity will be quenched, eliminating any unwanted conversion of the sugars inside the sample during the analysis process. The Carrez I solution was prepared by dissolving 15.0 g potassiumhexacyanoferrate(II) trihydrate in 100 mL of DI water in a volumetric flask. The Carrez II solution was prepared by dissolving 30.0 g zinc sulfate heptahydrate in 100 mL of water in a volumetric flask. Both Carrez solutions were filtered over a 0.2 µm syringe filter prior to use.

Sample preparation: The following lactose-free products were prepared and analyzed using the method described below:

- Semi-skimmed milk
- Cream cheese
- Low-fat yoghurt

Procedure:

1. 0.5 gram of dairy sample was weighted in a 50 mL volumetric flask and 10 mL DI water added.
2. Subsequently, 100 µL Carrez I and 100 µL Carrez II reagent was added (the solution was shortly vortexed after each addition of reagent). Followed by addition of DI water up to a total volume of 50 mL.
3. The solution was allowed to stand for 30 minutes and vortexed again to obtain a homogeneously turbid solution.
4. A few milliliters of the turbid sample solution was centrifuged 15 min in 2 mL Eppendorf vials at 6000 RPM.
5. The supernatant was collected in a plastic 5 mL syringe and filtered over a 0.20 µm PE (Polyethersulfone) syringe filter.
6. 2.5 µL of the filtered supernatant was injected into the LC system and analyzed.

For the following dairy products a slightly modified procedure was used in step 1:

- Matured cheese
- Chocolate paste
- Chocolate-covered rice cake

1. 0.5 gram of (crushed) sample was weighted in a 50 mL disposable centrifuge tube (with cap) and 10 mL DI water added. The tubes with sample solutions were vortexed and transferred in a water bath of 65°C and heated for 20 minutes. During the heating process the tubes were taken out of the bath a few time and vortexed shortly to assure optimal dissolution and mixing.

Subsequently, steps 2 - 6 from the procedure described for yoghurt, cheese and milk samples were followed.

Results

In Figure 3 an overlay is shown of two chromatograms obtained with a 2.5 µL injection of a 10 µM standard mix containing the following sugars: fucose, arabinose, galactose, glucose, sucrose, fructose, allolactose, lactose, lactulose and epilactose. In the standard mix shown in the top chromatogram (red curve) also raffinose was present. Raffinose might be found in whole grain and cacao products. A concentration of 10 µM corresponds to 3.4 mg/L (ppm) of lactose and lactose isomers. All compounds of interest eluted within 8 minutes, and the total run cycle time is 30 minutes due to the wash and equilibration step. The analysis time is at least two times shorter than reported with conventional HPAEC columns [11,12], resulting in a significant improvement in sample throughput. The retention time of Lactose was approximately 5.6 minutes. Not all sugars are completely baseline separated (resolution < 1.5), but under these conditions reliable quantification of lactose is still possible of the two raffinose containing samples shown as example in this application note. The elution order of Raffinose , between lactose and lactulose, deviates from the method reported in reference [13] in which a column was used with the same stationary phase.

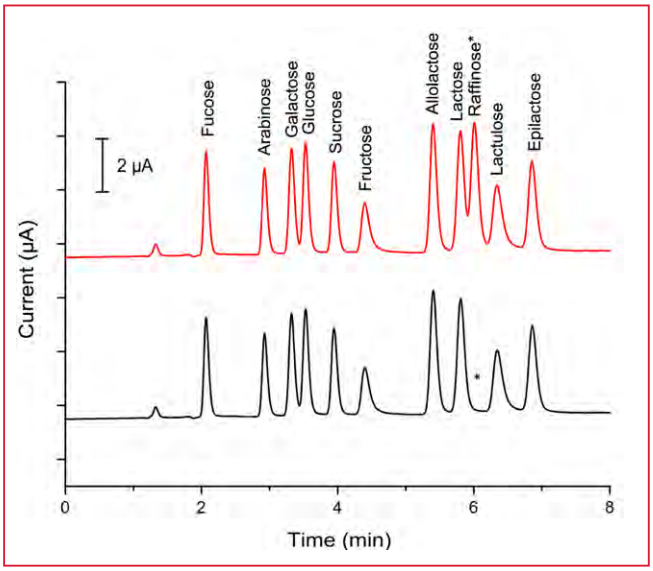

Figure 3: Two chromatogram of a 2.5 µL injection 10 µM sugar standard mix. (1) Fucose, (2) Arabinose, (3) Galactose, (4) Glucose, (5) Sucrose, (6) Fructose, (7) Allolactose, (8) Lactose, (9) Raffinose (10) Lactulose and (11) Epilactose. *) Raffinose only present in the mix of the top chromatogram (in red).

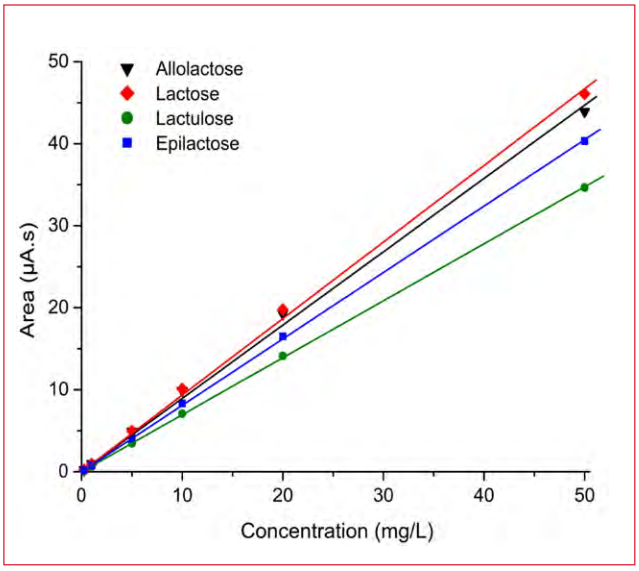

Figure 4: Calibration curve of Allolactose, Lactose, Lactulose and Epilactose in the concentration range of 0.25 - 50 mg/L.

The peak efficiencies found for the sugars ranged from 20.000 to 85.000 theoretical plates/meter (fucose and epilactose, respectively). Fructose and lactulose showed slight tailing with a tailing factor between 1.7 - 1.8.

Linearity

The linearity was investigated in the concentration range of 100 nM - 100 µmol/L for all 11 sugars shown in figure 3. This corresponds with a concentration range of 34 µg/L - 34 mg/L for lactose and its isomers. In this concentration range the linearity is excellent and correlation coefficients for peak area were better than 0.999 for all sugars.

The calibration curves for lactose and the lactose isomers, used for the actual quantification of samples, are shown in figure 4 The linear correlation coefficient was 0.9999 or better for all 4 sugars. The calibration curve (0.25— 50 mg/L) lays within the expected concentration range of lactose in the samples. During sample preparation the dairy product is 100 x diluted. Therefore, a concentration of 10 mg lactose in 100 gram dairy product (upper limit for lactose in 'lactose-free' products) corresponds to a concentration of 1 mg/L (ppm) lactose in the samples actually injected into the LC system.

Repeatability

The repeatability of the method and system was evaluated by repetitive injections with a 0.1, 1 and 10 µM sugar standard (mix of 10 sugars), corresponding with a concentration of 0.034, 0.34 and 3.4 mg/L for lactose and the isomers, respectively.

Table 3

Repeatability of 2.5 µL injections of a 10, 1 and 0.1 µM sugar standard mix in water (n=10)

	RSD's (%) 10 µmol/L		*RSD's (%) 1 µmol/L*		*RSD's (%)* 0.1 µmol/L*	
Compound	t_R	*Area*	t_R	*Area*	t_R	*Area*
Fucose	0.04	0.59	0.07	1.27	0.08	1.70
Arabinose	0.06	0.32	0.08	1.53	0.09	1.70
Galactose	0.06	0.23	0.08	0.30	0.12	1.52
Glucose	0.07	0.21	0.10	0.37	0.12	1.63
Sucrose	0.10	0.22	0.10	0.57	0.14	0.86
Fructose	0.08	0.20	0.11	1.12	0.17	2.22
Allolactose	0.11	0.22	0.12	0.36	0.16	1.04
Lactose	0.11	0.21	0.12	0.53	0.15	1.60
Lactulose	0.11	0.22	0.13	0.84	0.12	1.67
Epilactose	0.12	0.19	0.15	0.68	0.17	1.69

*) n=5 for the repeatability test with the 0.1 µmol/L standard.

The relative standard deviations (RSD) for retention time and peak area for the different concentrations of sugar standards are listed in table 3. The good repeatability of the method is evident from the low RSD values obtained. RSD's for retention time were < 0.2%. For peak area the RSD's were < 1% for all sugars in the 10 µmol/L standard and < 2% for most of the sugars in the 100 nmol/L standard. These data demonstrate that with this method reproducible analysis of lactose and related sugars can be achieved.

LOD and LOQ

The Limit of Detection (LOD) for all sugars are shown in table 4 in mg/L (ppm) and molar concentrations. The LOD's 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 noise was calculated based on a 5 minute section of the baseline close to the peaks of interest. The average responses of 5 replicate injections obtained with a 100 nmol/L standard mix in the 500 nA/V range were used to calculate the LOD's for all sugars.

Table 4

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

	Limit of detection (LOD)		*Limit of Quantification (LOQ)*
Compound	*mg/L (ppm)*	*nmol/L*	*mg/L (ppm)*
Fucose	0.001	9	0.005
Arabinose	0.002	10	0.005
Galactose	0.001	8	0.005
Glucose	0.001	7	0.004
Sucrose	0.003	9	0.010
Fructose	0.003	19	0.012
Allolactose	0.002	7	0.008
Lactose	0.002	7	0.008
Lactulose	0.005	14	0.016
Epilactose	0.003	10	0.011

The excellent sensitivity of the method is evident from table 4. Detection limits around 10 nmol/L are attainable for most of the sugars. The calculated LOQ (10x S/N) for lactose is approximately 0.010 mg/L, which is a factor 100 below the upper limit of the lactose concentration expected in samples of 'lactose-free' labelled products.

Sample analysis

In total 6 commercially available 'Lactose-free' labelled products were purchased from supermarkets in the Netherlands and analyzed using the presented method, see table 5.

Table 5

'Lactose-free' labelled products

Product	*Lactose content on product label*
Semi-skimmed milk UHT*	Lactose < 10 mg / 100 mL
Cream cheese	Lactose < 10 mg / 100 g
Low-fat yoghurt	Lactose < 10 mg / 100 g
Matured cheese	Lactose < 10 mg / 100 g
Chocolate paste	Lactose < 100 mg / 100 g
Chocolate-covered rice cake	Lactose < 100 mg / 100 g

*) Ultra Hight Temperature sterilization.

The series of products include dairy, chocolate paste and chocolate-covered cookies. The lactose contents specified on the product labels ranged from < 10 mg to < 100 mg Lactose per 100 gr or mL of product. All samples were prepared following the sample preparation procedure described in the method section. The contents of lactose and lactose isomers in the samples was determined in two different ways, using:

- Calibration curve based on standards (0.25 - 50 mg/L)
- Standard addition method

The quantification with the standard addition method was based on a single point calibration by spiking the sample in the first dilution step during sample preparation with a known amount of a standard containing allolactose, lactose, lactulose and epilactose. The spike concentration was 3.3 µM (1.1 mg/L) for all 4 components in the final sample.

By using standard addition the method accuracy could be assessed, by calculating the sample recovery based on the responses of the analytes in the sample, spiked sample and 3.3 µM standard.

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

The chromatograms of the samples are shown in figure 5 - 10. Every figure contains an overlay of chromatograms of the sample (red), spiked sample (black) and the 3.3 µM standard (grey).

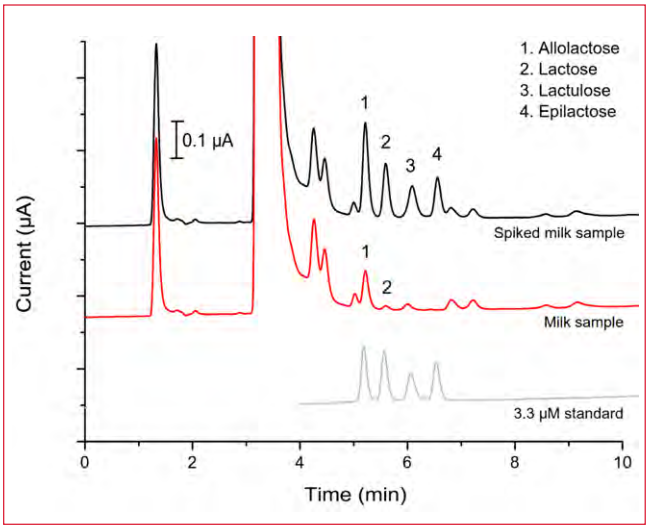

Figure 5: Chromatogram of the milk sample (red), spiked milk sample (black) and 3.3 µM standard of lactose and isomers (grey).

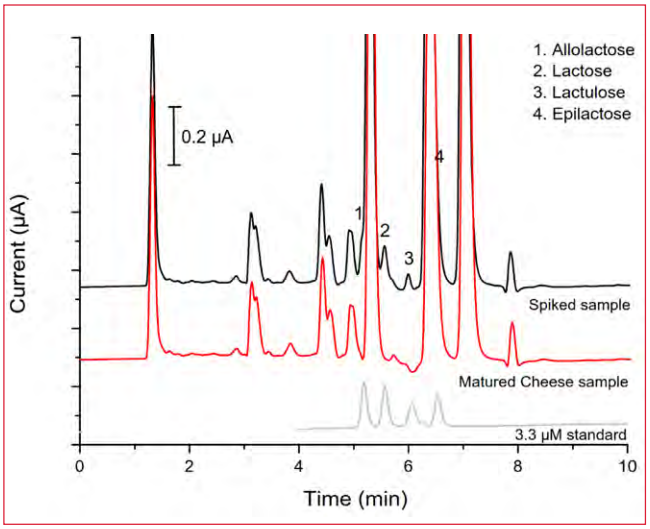

Figure 8: Chromatogram of the matured cheese sample (red), spiked matured cheese sample (black) and 3.3 µM standard (grey).

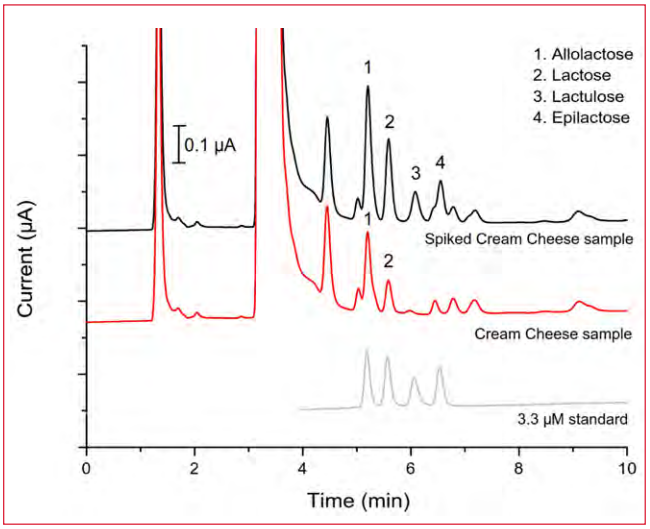

Figure 6: Chromatogram of the cream cheese sample (red), spiked cream cheese sample (black) and 3.3 µM standard (grey).

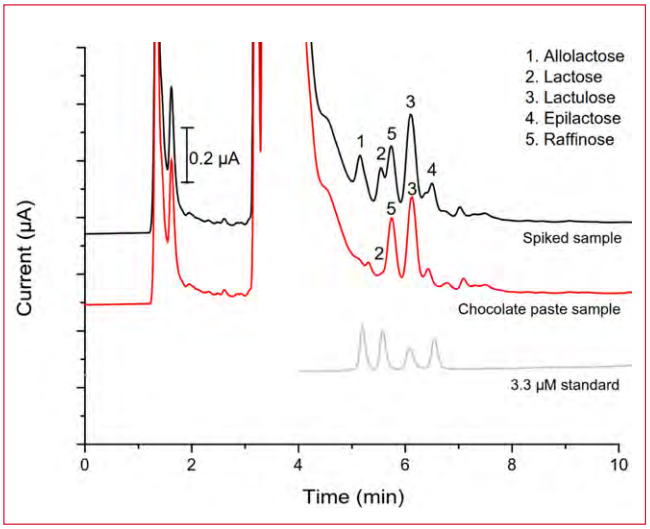

Figure 9: Chromatogram of the chocolate paste sample (red), spiked chocolate paste sample (black) and 3.3 µM standard (grey).

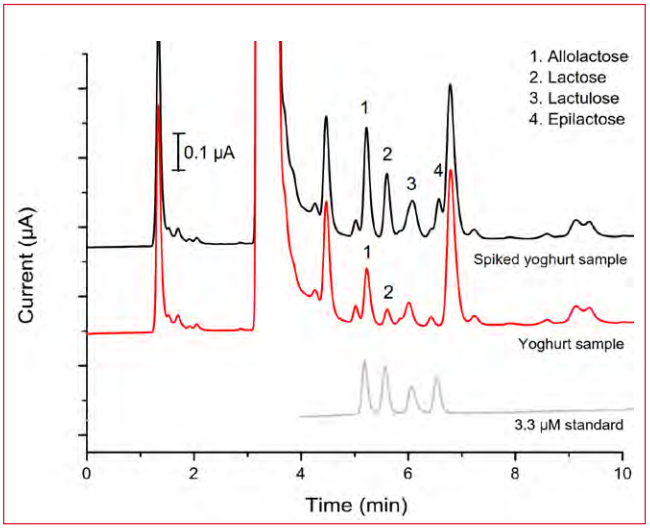

Figure 7: Chromatogram of the yoghurt sample (red), spiked yoghurt sample (black) and 3.3 µM standard (grey).

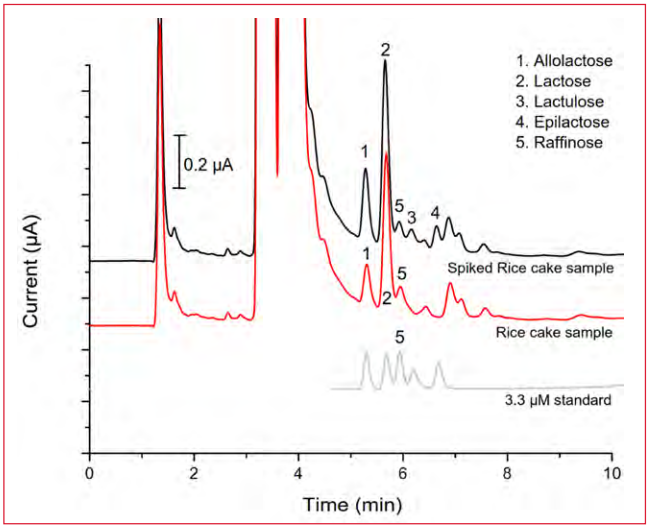

Figure 10: Chromatogram of the chocolate-covered rice cake sample (red), spiked rice cake sample (black) and 3.3 µM standard (grey).

The chromatograms in red, shown in figure 5-10, represent the unspiked samples. The lactose-related compounds present in the specific samples are marked with a number above the peaks, corresponding with the legend in the top right corner of the plot.

In none of the analyzed samples epilactose was found and lactulose was present in the chocolate paste sample only (figure 9). The concentration of lactulose in the sample was calculated based on standard addition and corresponds to 51 mg lactulose per 100 gram product (recovery 91.3%). The yoghurt sample shows an unidentified interference which coelutes closely to lactulose. The matured cheese (figure 8) did not contain a detectable amount of lactose or any of the other lactose isomers. Cheese is a fermented product and during fermentation lactose is converted into lactic acid.

In both the chocolate paste and chocolate-covered rice cake sample raffinose was present. Despite the presence of Raffinose it was possible to quantify the amount of lactose in the samples with sufficient accuracy. For lactose and allolactose , which are present in the majority of the 'lactose-free' products, the amounts found in the analyzed samples are listed in table 6.

Table 6

Lactose & allolactose, contents and sample recovery

Product	*Lactose*		*Allolactose*	
	mg per 100 g product	*Recovery (%)*	*mg per 100 g product*	*Recovery (%)*
Semi-skimmed milk UHT	0.7	95.6	7.5	96.8
Cream cheese	6.7	90.5	17.6	94.4
Low-fat yoghurt	3.3	91.7	12.2	95.9
Matured cheese	-	94.6	-	-*
Chocolate paste	0.6	85.9	-	98.0
Chocolate-covered rice cake	60.5	87.1	13.9	95.2

*) recovery could not be calculated due to coeluting interference.

The concentrations shown in table 6 were calculated using the the standard addition method (single point calibration). There was a good correlation between the values in table 6 and the the concentrations calculated based on the calibration curve. The sample recovery (see table 6) found for lactose, ranged between 86% - 96%.

The lactose contents in all the 'lactose-free' products analyzed with the presented HPAEC-PAD method, fall within the specified limit of < 10 mg/100 g (dairy products) or < 100 mg/100 g (Chocolate paste, Chocolate-covered rice cake) listed on the product labels.

Analysis of glucose, galactose and other sugars

Although, the main focus in this application note is quantification of lactose and isomers, the method is also suitable to assess the contents of the other sugars present in dairy products (see figure 3). For accurate quantification of high abundant sugars it might be necessary to dilute the sample. As an example the milk sample shown in figure 5 was diluted 10x more to to get the response of Galactose en Glucose in range for quantification. The resulting chromatogram is shown in figure 11.

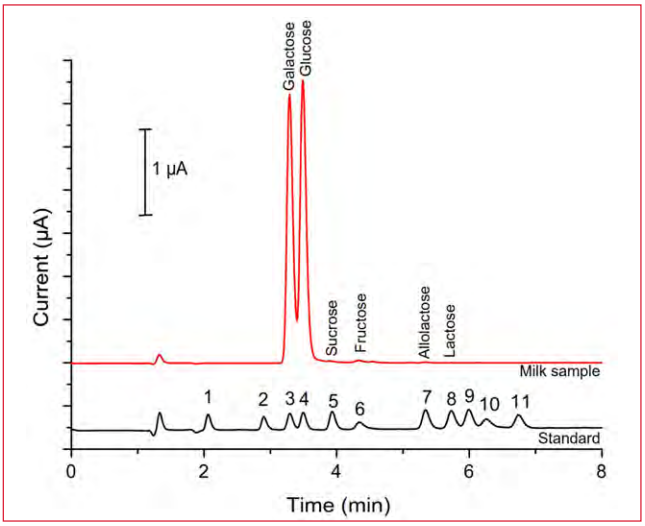

Figure 11: Chromatogram of a 10 x dilution of the worked-up milk shown in figure 5 (red) and a standard sugar mix for reference (black). See figure 3 for the legend of the sugars in the standard.

Table 7

Contents of sugars in milk sample

Sugar	*Contents (mg/100 g)*
Galactose	1451
Glucose	1685
Sucrose	4.6
Fructose	22.9
Allolactose	7.5
Lactose	0.7
Total	3172

The sugars detected in the milk sample are tagged in the chromatogram. The calculated concentrations of the sugars in the sample are listed in table 7. It is evident that galactose and glucose, which are formed due to hydrolysis of lactose are the main sugar constituents in the 'lactose-free' milk product. The total amount of sugar found (3.2 g/100 g) is in agreement with the product label (an average sugar contents of 3.0 g/100 g).

References

1. T.M Bayless, et al. , Lactase Non-persistence and Lactose Intolerance, Curr Gastroenterol Rep, 19 (2017) , 23.
2. ESFA panel, Scientific Opinion on lactose thresholds in lactose intolerance and galactosaemia, EFSA Journal , 8(9) (2010), 1777 https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2010.177
3. National Institute of Health, Lactose-intolerance, https://ghr.nlm.nih.gov/condition/lactose-intolerance
4. EU directive 2006/141/EC on infant formulae, L401/1
5. E. Rentschler et al., Enzymatic production of lactulose and epilactose in milk, J Dairy Sci., 98(2015), 6767
6. A. Olano et al., Kinetics of lactulose, galactose and epilactose formation during heat-treatment of milk, Food Chemistry, 34, (1989), 239
7. L.M.L. Nollet, F.Toldra, Handbook of Dairy Foods Analysis, CRC press, Boca Raton, 2010, chapter 6 & page 397
8. D.C. Johnson, D. Dobberpuhl, R. Roberts, P. Vandeberg, Review: Pulsed amperometric detection of carbohydrates, amines and sulfur species in ion chromatography- the current state of research, J. Chromatogr., 640 (1993), 79 -96
9. D.C. Johnson, W.R. LaCourse, LC with pulsed ECD at gold and platinum, Anal. Chem., 62 (1990), 589A – 597
10. W.R. LaCourse, Pulsed Electrochemical Detection in High Performance Liquid Chromatography, John Wiley & Sons, New York, 1ed, 1997
11. W.B. van Scheppingen et al., Selective and sensitive determination of lactose in low-lactose dairy products with HPAEC-PAD, J. Chromatogr. B, 1060 (2017), 395
12. L. Monti et al., Lactose, galactose and glucose determination in naturally “lactose free” hard cheese: HPAEC-PAD method validation, Food Chemistry, 220 (2017), 18
13. M. Aggrawal, J. Rohrer, Fast and sensitive determination of Lactose in Lactose-free products using HPAE-PAD, Thermo Fisher Scientific Application note 72632 (2018)
14. H. Louw, H.J. Brouwer, N. Reinhoud, Electrochemical flowcell, (2016) US patent 9310330
15. 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

Conclusion

The DECADE Elite detector, in combination with the SenCell flow cell and a narrow-bore ‘fast-4µm’ HPAEC column, offers a fast and sensitive analysis solution for the quantification of lactose and isomers in commercial Lactose-free products. The presented HPAEC-PAD method allows fast separation (within 8 min) of Lactose and isomers. Besides the quantification of low concentrations of lactose also the major sugars in dairy products (Galactose, Glucose, Sucrose and Fructose) can be analyzed using the presented method. A four-fold reduction of mobile phase usage was achieved by using a 2 mm ID column instead of a standard bore version (4 mm ID).

Figure 12: Recommended instrument configuration for this application: the ALEXYS Carbohydrate Analyzer
The system consists of a P6.1L pump with integrated Solvent Switch Valve (SSV) capable of running step gradients, an AS6.1L autosampler, an ET 210 Eluent tray for helium blanketing, a CT 2.1 column oven with broad temperature range, and the DECADE Elite electrochemical detector. The ALEXYS Carbohydrate Analyzer can be operated under DataApex™ Clarity™ CDS (version 8.3 and up) or Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software (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
Recommended ALEXYS analyzer	
180.0055W	ALEXYS Carbohydrate Analyzer
116.4321	SenCell 2 mm Au HyREF
Software*	
195.0035	Clarity CDS single instr. incl LC, AS module

*) 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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Analysis of lactose and isomers in dairy and meat products

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- **Fast and sensitive HPAEC-PAD analysis**
- **Lactose, allolactose, epilactose and lactulose**
- **Dairy, meat, and meat analogues**
- **SenCell™ with Au working electrode**

Summary

Dairy products play a vital role in a healthy and balanced diet providing essential vitamins and minerals like calcium. Lactose-intolerance is a wide-spread condition, which prevents a large number of people of consuming dairy products as a part of their daily diet [1]. The global demand for low-lactose food products is rapidly growing and a large number of commercial 'lactose-free' product are available nowadays. While commonly found in dairy products, lactose can also be present in processed food products such as deli meats and meat analogues [2]. High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) is a fast, sensitive and selective analytical method to quantify the lactose contents in such products [3].

This application note is complementary to the previously published application note 220_009 - Analysis of Lactose and Isomers in 'Lactose-free' Labelled Products [3]. In application note 220_009 a fast, reliable, and sensitive method is presented for the analysis of lactose and isomers using the DECADE Elite electrochemical detector and SenCell in combination with a narrow-bore (2mm ID) anion-exchange column with 4 µm particle size. In this publication the same method is evaluated on the new Antec ALEXYS Carbohydrates Analyzer in combination with a standard-bore (4mm ID) anion-exchange column. The ALEXYS Carbohydrates Analyzer is a dedicated LC system solution for the quantification of lactose in 'lactose-free' products. A variety of commercial food samples including dairy, meat and meat analogues were analysis to demonstrate the performance and versatility of the system.

Introduction

Lactose is a disaccharide composed of the monosaccharides D-glucose and D-galactose, joined in a ß-1,4-glycosidic linkage. It is the main carbohydrate found in milk and most dairy products. The concentration of this disaccharide depends on the origin of the milk, and ranges from 7.0 mg/100mL in human breast milk to 4.2 mg/100 ml in goat milk [4]. To digest and absorb lactose from the digestive system it is hydrolyzed into glucose and galactose in the intestines by the enzyme lactase.

While lactose is the most important energy source during the first year of human life, most humans cease to produce the enzyme lactase after the weaning phase and as a result become lactose intolerant [4]. As the lactose is not digested due to a deficiency of lactase, it is fermented by intestinal bacterial flora, resulting in the production of gas leading to diarrhea, flatulence, and/or abdominal pain. Most people with lactose intolerance can still tolerate some amount of lactose, and the digestive symptoms can be reduced by limiting the intake of lactose to a level that can be tolerated.

Lactose is not only present in dairy products but can also be found as a food additive in processed food products such as meat or meat analogues. For example, lactose can be used to add texture or bind water in processed meats, such as sausages and cold cuts [2]. In order to know if these products can be considered 'lactose free' (< 0.01% w/w), and safe to consume for lactose intolerant people [5], there is a need for fast and accurate analytical method to quantify lactose.

In this application note a LC solution is presented for fast and sensitive analysis of lactose, lactose isomers and lactulose using the new ALEXYS Carbohydrates analyzer based on the DECADE Elite electrochemical detector and SenCell. A variety of commercial food samples including dairy, meat and meat analogues were analysis using a standard-bore anion-exchange column to demonstrate the performance and versatility of the ALEXYS HPAEC-PAD system.

Method

The analysis was performed using the ALEXYS Carbohydrates Analyzer as shown in figure 1. The ALEXYS Carbohydrates Analyzer consists of a P6.1L pump with integrated Solvent Switch Valve (SSV) capable of running step gradients, AS6.1L autosampler, ET 210 eluent tray for Helium blanketing, CT 2.1 column oven and the DECADE Elite electrochemical detector. The SenCell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the sugars. The system was operated under the Thermo Scientific™

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.

Chromeleon™ Chromatography Data System (CDS) software version 7.2.10.

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. An anion-exchange column was chosen for the separation of lactose and isomers (Table 1).

This column type with small particle size (4 µm) results in high-resolution separation, allowing short analysis time. No trap column was required as no tailing of the fructose and lactulose peaks was present. The temperature for separation was set at 30 °C using the CT 2.1 column thermostat. The analysis is based on a step-gradient. Both, potassium hydroxide (KOH) or sodium hydroxide (NaOH) can be used to prepare the mobile phase for separation and the column clean-up step.

During isocratic elution, 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 with a strong eluent as rinsing solution is therefore necessary to remove the

Table 1

Conditions

HPLC system	ALEXYS Carbohydrate Analyzer
Detector	Antec DECADE Elite electrochemical detector
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast -4µm guard column, 30 x 4.0 mm ID, 4 µm Thermo Scientific™ Dionex™ CarboPac™ PA210-Fast -4µm analytical column, 150 x 4.0 mm ID, 4 µm
Mobile phase (MP)	A: see gradient program B: see gradient program Eluents prepared & blanketed with Helium 5.0
Flow rate	0.8 mL/min
Back pressure	about 300 bar
Injection	10 µL
Temperature	30 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF RE, 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 0.2— 0.4 µA
ADF	0.05 Hz
Range	10 µA/V

Table 2

Step-gradient program dairy samples

Time (min)	*Mobile phase*	*Description*
0 - 9	14 mM KOH*	Isocratic elution and detection
9 - 14	100 mM KOH	Column clean-up and regeneration
14 - 30	14 mM KOH	Equilibration, starting conditions

*) Both KOH or NaOH can be used as eluent.

bound carbonate ions and late eluting compounds, see table 2. This regeneration step assures reproducible retention behaviour for each run. The total run time is 30 minutes.

To minimize the introduction of carbonate ions in the mobile phase the eluents were carefully prepared manually using a carbonate-free 50% w/w KOH or NaOH solution and electrochemical grade sodium acetate salt (all commercially available). The diluent was DI water (resistivity >18 MΩ.cm), which was sparged with Helium 5.0 using the sparging function of the ET 210 eluent tray (figure 2). The eluent tray was also used to pressurize the head space above the mobile phase with Helium gas (0.2—0.4 bar He overpressure) during the analysis. Blanketing of mobile phase with an inert gas minimizes the introduction of CO_2 in the mobile phase and the subsequent formation of CO_3^{2-} ions, ensuring reproducible analysis.

Detection

For the pulsed amperometric detection of lactose and isomers

Figure 2: ET 210 eluent tray with PPCO bottles. The eluent tray enables blanketing & sparging of LC mobile phases with an inert gas atmosphere in an user-friendly and easy way.

the Antec SenCell electrochemical flow cell is used. This flow cell [6] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/ H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform was applied as shown in figure 3. The temperature for detection was set to 35°C. The cell current was typical about 0.2—0.4 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 [7], resulting in less flow cell maintenance and system down time.

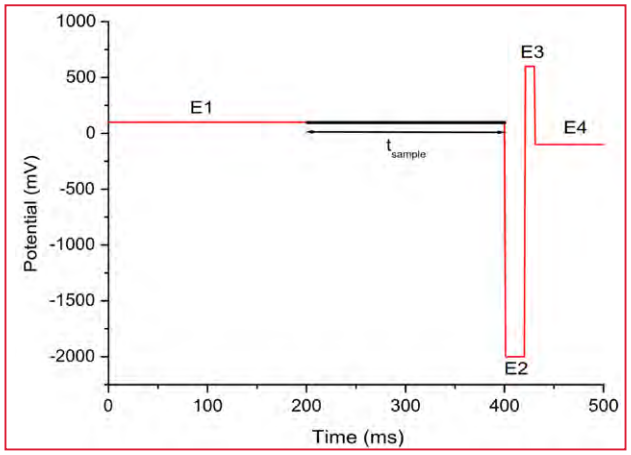

Figure 3: 4-step PAD potential waveform for the detection of carbohydrates.

Preparation of standards, reagents and samples

Standards: 50 mM stock standards of the individual sugars were prepared in 95/5 (v/v%) water/acetonitrile to increase the storage life. Stock standards under these conditions are stable for approximately 1 month in the fridge at 4°C. Working standards in the concentration range of 100 nM—100 µM were prepared by dilution of the stock standards with DI water.

Carrez reagents: a Carrez clarification procedure is used for deproteination of the dairy samples. By removing all proteins also the enzymatic activity will be quenched, eliminating any unwanted conversion of the sugars inside the sample during the analysis process. The Carrez I solution was prepared by dissolving 15.0 g potassium hexacyanoferrate(II) trihydrate in 100 mL of DI water in a volumetric flask. The Carrez II solution was prepared by dissolving 30.0 g zinc sulfate heptahydrate in 100 mL of water in a volumetric flask. Both Carrez solutions were filtered over qualitative filter paper prior to use.

Sample preparation dairy products:

The following lactose-free products were prepared and analyzed using the method described below:

- Semi-skimmed milk
- Whipping cream
- Cream cheese
- Yoghurt

Procedure:

1. 0.5 gram of dairy sample was weighted in a 50 mL volumetric flask and 10 mL DI water added.
2. Subsequently, 100 µL Carrez I and 100 µL Carrez II reagent was added (the solution was shortly vortexed after each addition of reagent). Followed by addition of DI water up to a total volume of 50 mL.
3. The solution was allowed to stand for 30 minutes and vortexed again to obtain a homogeneously turbid solution.
4. The turbid sample solution was centrifuged 15 min in 50 mL conical tubes at 2500 xG. For the lactose-free whipping cream, no clear supernatant could be obtained by centrifugation. Therefore the solution was allowed to stand for another 30 min, and clear supernatant was aspirated from the bottom of the tube.
5. The supernatant was collected in a plastic 5 mL syringe and filtered over a 0.20 µm PES (Polyethersulfone) syringe filter.
6. 10 µL of the filtered supernatant was injected into the LC system and analyzed.

Results

In figure 4 a typical chromatogram of a 10-µL injection of the 10 µM standard mix is shown. Not all carbohydrates are completely baseline separated (resolution > 1.5). For example raffinose elutes closely to lactose and lactulose. Raffinose might be found in whole grain and cacao products. The peak efficiencies found for the sugars ranged from 27.000 to 65.000 theoretical plates per meter with peak tailing factors < 1.5.

Separation

The elution order of the 11 carbohydrates, as shown in figure

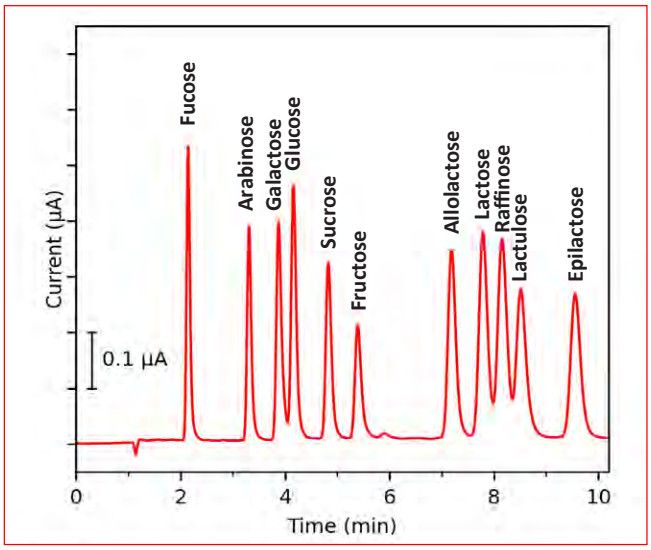

Figure 4: Chromatogram of a 10 µL injection of a 10 µM standard mix in water (eluent 14 mM KOH). The mix is composed of the following sugars: fucose, arabinose, galactose, glucose, sucrose, fructose, allolactose, lactose, raffinose, lactulose and epilactose.

4, is the same as previously shown in the application note [3]. Elution with a mobile phase of 14 mM hydroxide results in the best separation for this set of carbohydrates. However, if component from the sample matrix interferes with the analysis, the concentration of OH^- can be adjusted to prevent coelution and improve the separation. The retention times of the carbohydrates in the standard mix as function of the concentration hydroxide ranging from 13 to 20 mM are shown in figure 5.

As shown in figure 5, the retention times of the different carbohydrates are not affected equally by a change in hydroxide concentration (pH) which can result in a swap in elution order. For example, the retention time of the

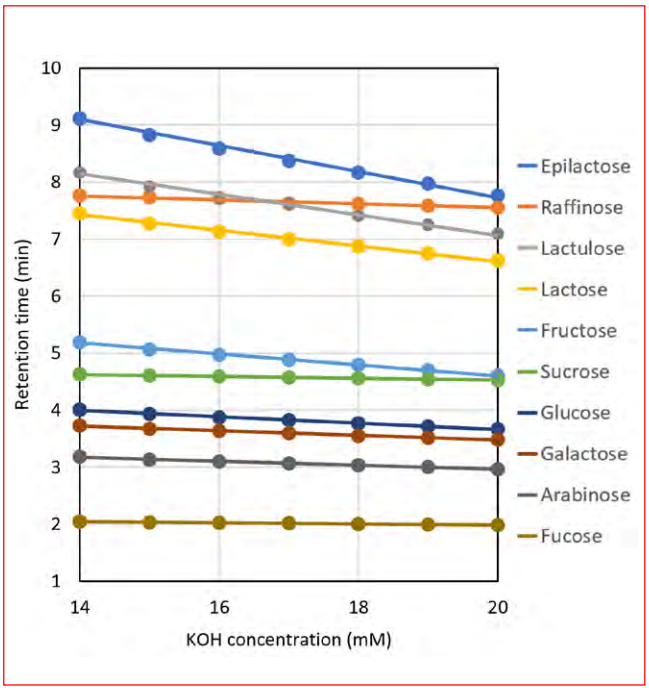

Figure 5: Retention times of all carbohydrates (except allolactose) in the standard mix as function of the hydroxide concentration.

disaccharide sucrose is less affected by an increasing concentration of hydroxide than the closely eluting monosaccharide fructose. The same holds for the trisaccharide raffinose and the closely eluting disaccharides lactose and epi-lactose. By selecting the right concentration of hydroxide, coelution of the sugars of interest and interferences can be prevented and the separation optimized.

Two chromatograms of 500 nM standard mix eluted with a mobile phase containing 14 mM NaOH (black) or 14 mM KOH (red) are shown in figure 6. It is evident that the counter ion of the hydroxide has no influence on the separation.

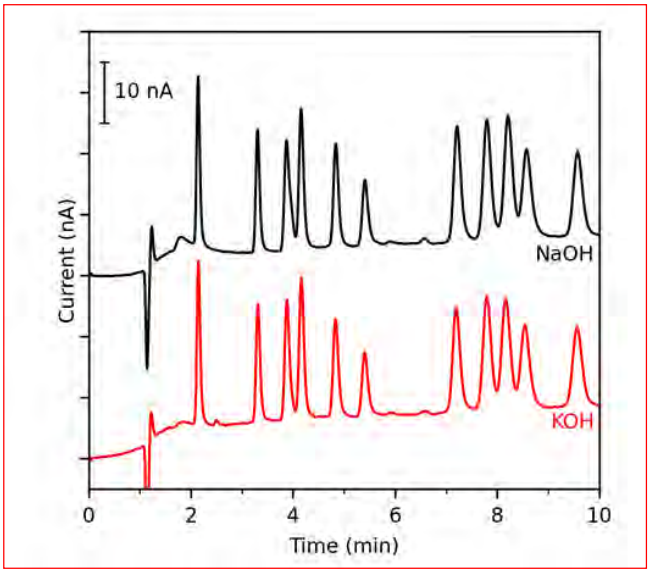

Figure 6: Chromatograms of a 10 µL injections of the 500 nM standard mix eluted with 14 mM KOH (red) and 14 mM NaOH (black).

Repeatability

The relative standard deviation (RSD) of the retention time and peak area were determined for 10 repetitive injections with a 0.1, 1 and 10 µM standard, corresponding with 0.034, 0.34 and 3.4 mg/L for lactose and the isomers, respectively. Retention times were stable, with RSD values below 0.20% for all 11 carbohydrates independent of concentration. The RSD values for peak area are shown in table 5. The RSD's for peak area were < 3% for the 0.1 µM standard and typically < 0.5% for the 1 and 10 µM standard. The only exception is raffinose, which has slightly higher RSD since this component is not baseline separated under these LC conditions. The data demonstrate that with this method reproducible analysis of all the components can be achieved.

Table 5

Repeatability of 10 µL injections of a 10, 1 and 0.1 µM sugar standard mix in water (n=10)

Compound	*RSD area (%) 0.1 µM*	*RSD area (%) 1 µM*	*RSD area (%) 10 µM*
Fucose	1.07	0.22	0.08
Arabinose	1.28	0.31	0.09
Galactose	1.11	0.40	0.16
Glucose	1.31	0.20	0.15
Sucrose	2.37	0.25	0.13
Fructose	2.97	0.50	0.30
Allolactose	2.15	0.50	0.10
Lactose	2.07	0.49	0.43
Raffinose	3.28	0.43	0.67
Lactulose	2.91	0.53	0.42
Epilactose	2.59	0.40	0.36

LOD/LOQ and linearity

The Limit of Detection (LOD) and limit of quantification (LOQ) for all sugars are shown in table 6. The LOD's and LOQ's were calculated as the analyte response corresponding to 3x and 10x the ASTM noise (average peak-to-peak baseline noise of 10 segments of 0.5 min), respectively. The noise was calculated based on a 5-minute section of the baseline close to the peaks of interest. The average responses of 5 replicate injections obtained with the 0.1 µM standard mix were used to calculate the LOD's for all sugars.

Table 6

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

	Limit of detection		*Limit of Quantification*	
Compound	*nM*	*µg/L*	*nM*	*µg/L*
Fucose	7.7	1.3	25.8	4.2
Arabinose	10.5	1.6	34.9	5.2
Galactose	9.6	1.7	32.0	5.8
Glucose	8.9	1.6	29.7	5.3
Sucrose	12.7	4.4	42.4	14.5
Fructose	19.8	3.6	65.9	11.9
Allolactose	11.9	4.1	39.7	13.6
Lactose	11.1	3.8	37.0	12.7
Raffinose	11.2	5.7	37.4	18.9
Lactulose	14.6	5.0	48.6	16.6
Epilactose	15.6	5.3	52.1	17.8

The LOQ of lactose of 12.7 µg/L demonstrates the excellent sensitivity of the method. During sample preparation the dairy product is 100 x diluted. Therefore, a concentration of 10 mg lactose in 100 gram product corresponds to a concentration of 1000 µg/L (ppm) lactose in the sample vials. Therefore. the reported LOQ is factor 70 below the upper limit of the lactose concentration expected in 'lactose-free' products.
The linearity was investigated for the concentration range of 0.1 to 100 µmol/L. The calibration curves for lactose and the lactose isomers, used for the actual quantification of samples, are shown in figure 7. The linear correlation coefficient was 0.9997 or better for all 4 sugars.

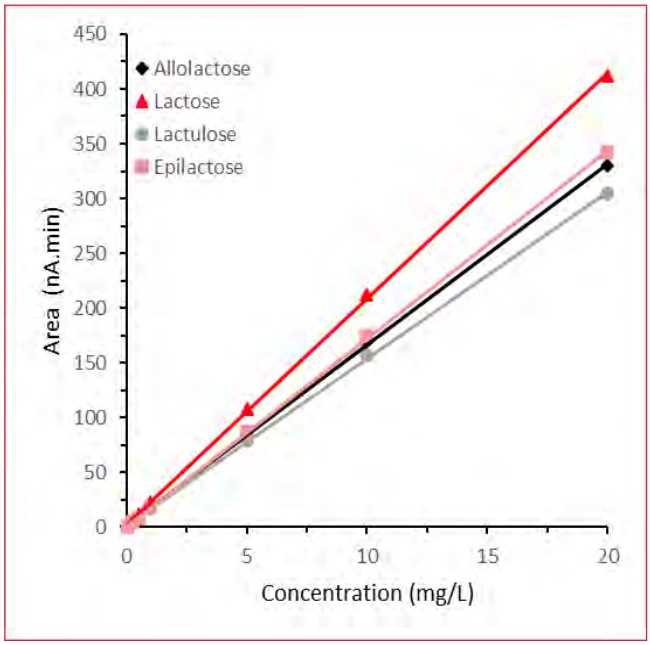

Figure 7: Calibration curve of Allolactose, Lactose, Lactulose and Epilactose in the concentration range of 0.05 - 20 mg/L.

Dairy sample analysis

In total 4 commercially available 'Lactose-free' labelled products were purchased from supermarkets in the Netherlands and analyzed using the presented method.

Table 7

'Lactose-free' labelled dairy products

Product	*Lactose content according to label*
Semi-skimmed milk	< 0.01 g/100 mL
Cream cheese	< 0.01 g/100 g
Yoghurt	< 0.01 g/100 mL
Whipping cream	< 0.1 g/ 100 g

The lactose contents specified on the product labels ranged from < 10 mg to < 100 mg Lactose per 100 g or mL of product. All samples were prepared as described in the method section.

The contents of lactose and lactose isomers in the samples was determined in two different ways, using:

- Calibration curve based on standards (0.05 - 20 mg/L)
- Standard addition method

The quantification with the standard addition method was based on a single point calibration by spiking the sample in the first dilution step during sample preparation with a known amount of a standard containing allolactose, lactose, lactulose and epilactose. The spike concentration was 1 ppm (1 mg/L) for all 4 components in the final sample.

By using standard addition, the method accuracy could be assessed, by calculating the sample recovery based on the responses of the analytes in the sample, spiked sample and 1 ppm standard.

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

In figure 8 the chromatograms of the samples are shown. The sample is shown in red, the spiked in black and the 1 ppm standard containing allolactose (1), lactose (2), lactulose (3) and epilactose (4) is shown in grey. In every 'lactose-free' product tested allolactose and lactose was found, the values are listed in table 8 . Notably, in the semi-skimmed milk lactose was

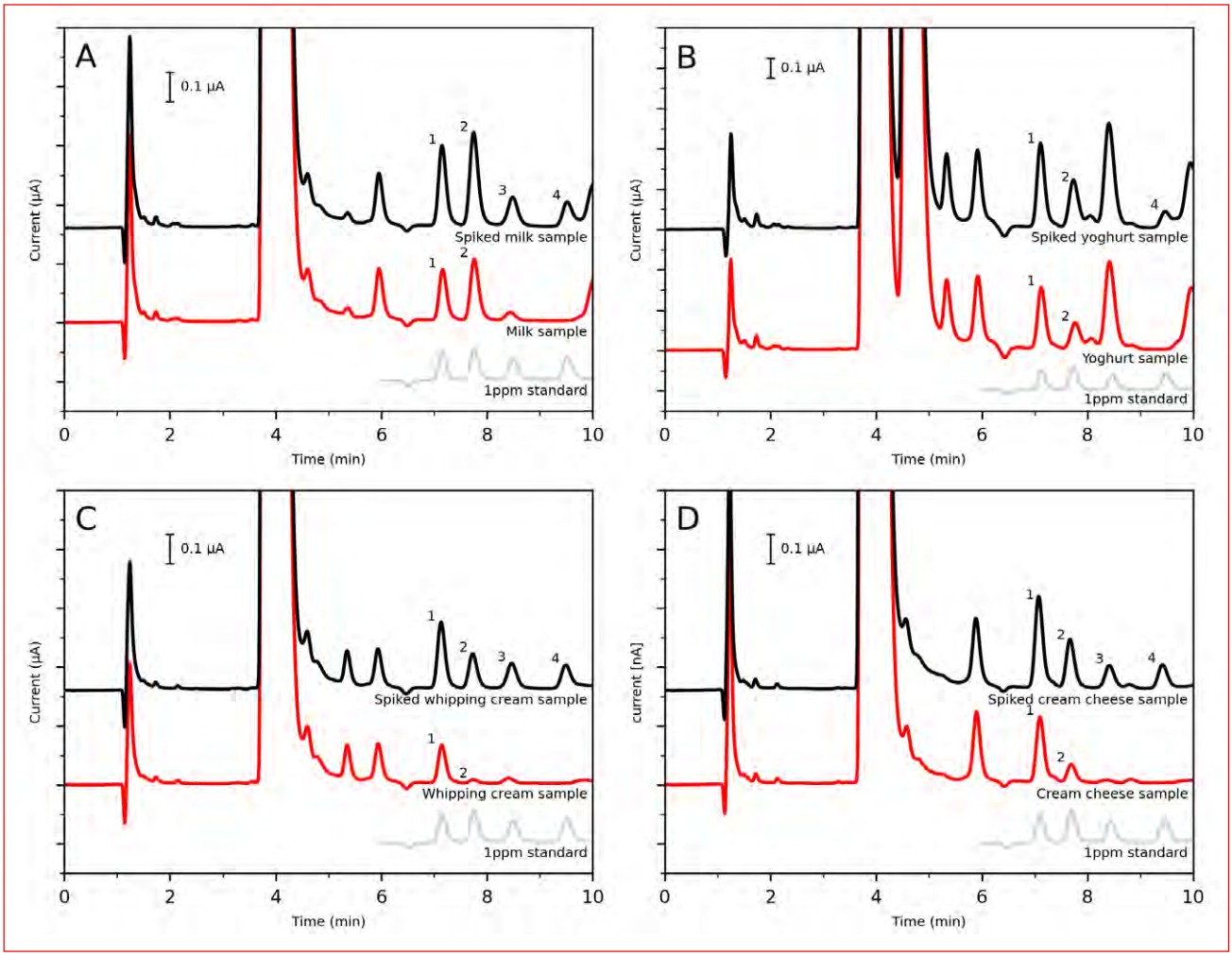

Figure 8: Chromatograms of the dairy samples. A: Milk sample (red), spiked milk sample (black), and 1 ppm standard (grey). B: Yoghurt sample (red), spiked yoghurt sample (black), and 1 ppm standard (grey). C: Whipping cream sample (red), spiked whipping cream sample (black), and 1 ppm standard (grey). D: Cream cheese sample (red), spiked cream cheese sample (black), and 1 ppm standard (grey). Labeled peaks: 1. Allolactose, 2. Lactose, 3. Lactulose and 4. Epilactose.

present at a concentration of 19 mg/100 mL, which is more than specified on the product label (10 mg/100 mL).

In all samples a significant amount of allolactose was present, ranging from 14 to 30 mg/100 mL. This underlines the need for selectivity to differentiate between lactose and allolactose to accurately quantify the amount of lactose. None of the samples contained a detectable amount of lactulose or epilactose. However, it has to be noted that under the selected mobile phase conditions (14 mM NaOH) unknown peaks are eluting closely at the retention times of lactulose, making quantification difficult. By using a higher concentration of KOH (> 20 mM) as eluent it is possible to separate lactulose from the interferences for better identification, see reference [8].

The concentrations in this table 8 were calculated using the standard addition method (single point calibration). There was a good correlation between the values in table 8 and the concentrations calculated based on the calibration curve. The sample recovery found for lactose, ranged between 90% - 97%.

Table 8

Lactose & allolactose, contents and sample recovery

Product	*Lactose*		*Allolactose*	
	mg/ 100 g product	*Recovery*	*mg/ 100 g product*	*Recovery*
Semi-skimmed milk	19	95%	17	99%
Cream cheese	5.8	90%	25	92%
Yoghurt	9.7	97%	30	102%
Whipping cream	5.8	93%	14	92%

Meat and meat analogue samples

For the analysis of lactose in meat and meat analogues three commercial meat products were analyzed, see table 9.

Table 9

Meat and meat analogues

Sample	*Product description*
A	Vegan meat analogue based on pea-protein
B	Breaded chicken with processed-cheese filling
C	Dry-cured ham

It should be noted that the analyzed meat samples are products which are not specifically labelled as 'lactose-free' products.

The samples were prepared using the sample preparation procedure described below:

1. The sample was homogenized using a turrax.
2. 1 gram of sample was weighted in a 100 mL volumetric flask and 50 mL DI water added.
3. the sugars were extracted by placing the flask in hot shaking water bath (60°C) for 20 minutes
4. Subsequently, 100 µL Carrez I and 100 µL Carrez II reagent was added. Followed by addition of DI water up to a total volume of 100 mL.
5. The solution was allowed to stand for 30 minutes and vortexed again to obtain a homogeneous turbid suspension.
6. The turbid sample solution was centrifuged 10 min at 4000 xG
7. The supernatant was collected, diluted 40 times, and filtered over a 0.20 µm Polyethersulfone (PES) syringe filter.
8. 10 µL of the filtered supernatant was injected into the LC system and analyzed.

See table 1 and 10 for the LC conditions and step-gradient program used for the analysis of the meat samples. Whereas for the dairy samples 100 mM hydroxide was sufficient to regenerate the column, the meat samples required a stronger column clean-up solution. A solution consisting of 100 mM hydroxide and 100 mM acetate was sufficient to remove all late-eluting compounds. Column regeneration was also more effective with a column clean-up solution containing NaOAc, leading to increased retention, hence a longer isocratic elution time of 12 minutes was used for the meat samples.

Table 10

Step-gradient program meat samples

Time (min)	*Mobile phase*	*Description*
0 - 12	15 mM NaOH	Isocratic elution and detection
12 - 17	100 mM NaOH, 100 mM NaOAc	Column clean-up and regeneration
17 - 30	15 mM NaOH	Equilibration, starting conditions

The chromatograms of the samples and a 0.5 µM standard are shown in figure 9 in red and grey, respectively. With the gradient program used in this method raffinose (10) elutes after lactulose (9) and before epilactose (11). The sugar contents of the food products are summarized in table 11. The concentrations shown were calculated using a calibration curve based on standards ranging from 0.5 - 10 µmol/L.

Table 11

Carbohydrate content meat and meat analogues

Carbohydrate	*Sample A (mg/ 100 g product)*	*Sample B (mg/ 100 g product)*	*Sample C (mg/ 100 g product)*
Arabinose	8	-	7
Galactose	15	6	11
Glucose	543	367	39
Sucrose	964	17	-
Fructose	404	89	20
Lactose	-	61	-
Raffinose	56	-	-

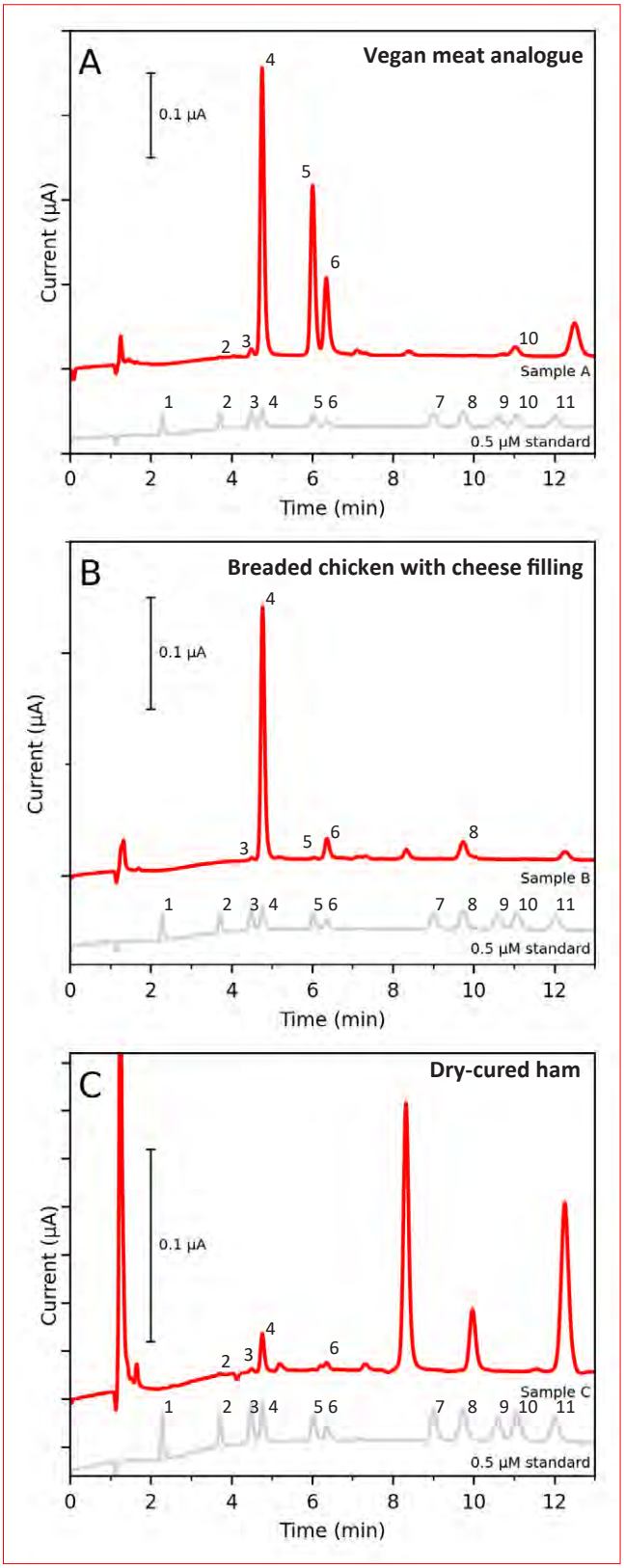

Figure 9: Chromatograms of samples A to C (red) and 0.5 µM standard (grey). Labeled peaks: 1. Fucose, 2. Arabinose, 3. Galactose, 4. Glucose, 5. Sucrose, 6. Fructose, 7. Allolactose, 8. Lactose, 9. Lactulose, 10. Raffinose , 11. Epilactose.

The highest amount of carbohydrates was found in sample A, the vegan meat analogue. Lactose was only present in sample B, the chicken-based product with processed-cheese filling. The lactose concentration in this product was 61 mg per 100 g product which evidently originates from the cheese filling. Sample C, the dry-cured ham, contained the lowest amount of carbohydrates of the three samples.

References

1. T.M Bayless, et al. , Lactase Non-persistence and Lactose Intolerance, Curr Gastroenterol Rep, 19 (2017), 23.

2. K. Kyriakopoulou et al., Functionality of ingredients and additives in plant-based meat analogues, Foods 10.3 (2021), 600.

3. Antec Scientific, Analysis of Lactose and Isomers in 'Lactose-free' Labelled Products, Application note, 220_009

4. N. Silanikove et al., The interrelationships between lactose intolerance and the modern dairy industry: global perspectives in evolutional and historical backgrounds." Nutrients 7.9 (2015): 7312-7331.

5. ESFA panel, Scientific Opinion on lactose thresholds in lactose intolerance and galactosaemia, EFSA Journal, 8(9) (2010), 1777

6. H. Louw, H.J. Brouwer, N. Reinhoud, Electrochemical flowcell, (2016) US patent 9310330

7. R.D. Rocklin, et al., Improved long-term reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform, Anal. Chem, 70 (1998), 1496 – 1501

8. M. Aggrawal, J. Rohrer, Fast and sensitive determination of Lactose in Lactose-free products using HPAE-PAD, Thermo Fisher Scientific Application note 72632 (2018)

Conclusion

The ALEXYS Carbohydrates Analyzer, in combination with the SenCell flow cell offers an dedicated and optimized analysis solution for the fast and sensitive quantification of lactose and isomers in dairy, meat and other products. All lactose isomers are eluting within 10 minutes using the selected LC conditions. The total run time including column clean-up and equilibration is 30 min. In addition to lactose, also other major sugars in processed food, such as galactose, glucose and sucrose can be quantified using the presented method. Optionally, an stronger column cleaning step with sodium acetate can be programmed, for sample matrices containing large concentrations of strongly retaining contaminants.

Ordering information

System	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quartenary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT 2.1 Column thermostat
Software*	
195.0035	Clarity CDS single instr. incl LC, AS module

*) 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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Sugars made by the photosynthesis are essential for the plant's metabolism

Plant roots release a wide range of carbon-containing compounds such as sugars, i.e., mono-, di- and trisaccharides. The measurement of changes in sugar levels in plants tissue and root exudates is important in terrestrial ecology.

Application Note

Environment

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Carbohydrates
Monosaccharides
Other oligo- and polysaccharides

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Bisphenol A
Catechins
Flavonoids
Antioxidants
Resveratrol
Epicatechin
Quercetin
Other (poly)phenols
Chloro- and nitrophenols

Herbicides
Glyphosate
Aminomethylphosphonic acid (AMPA)

Carbohydrate Analysis in Plant Ecology

- **Mono-, di-, and trisaccharides in plants**
- **Samples: leaf extracts and root exudates**
- **Pulsed Amperometric Detection (PAD)**
- **SenCell with Au working electrode**
- **Sensitive & selective analysis**

Summary

In this application note the analysis of mono-, di and trisaccharides in plants (leaves and root exudates) is demonstrated using the DECADE Elite electrochemical detector and SenCell, in combination with an Agilent 1260 Infinity Bio-Inert LC system. The method is based on separation by High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) using a 4-step potential waveform.

The method development and all data presented in this application note are courtesy of Mrs. Ciska E. Raaijmakers, Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands. NIOO-KNAW is a research institute focused on fundamental and strategic research in the field of animal -, plant and microbial ecology in terrestrial and freshwater environments. The scientists of the department of Terrestrial Ecology are experts in the ecology, physiology and chemistry of plants, and in soil ecology, entomology and behavioral biology. They investigate the interactions between plants, the animals and diseases that damage plants above and below the ground, and the natural enemies of these attackers [1].

Introduction

Plants, like all organisms, require energy for growth and this is achieved via photosynthesis. In photosynthesis light energy is converted into chemical energy in the form of sugars by the so-called chloroplasts (most abundant in leaf cells). These carbohydrates, or sugars, are essential for the plants metabolism (plants growth and quality) and also provide nutrition to natural enemies. Plants are the primary food source on earth for a wide range of above ground and below ground organisms. Plant roots release a wide range of carbon-containing compounds into its rhizosphere (an area of a few mm surrounding the root), the so-called root exudates. Among these components, sugars, amino acids and organic acids are released in the largest quantities. The level of sugars in plant tissue and the rhizosphere can be influenced by the plant response caused by shoot and /or root damage. Therefore, the measurement of changes in sugar levels in plants tissue and root exudates is important in terrestrial ecology [2].

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 plant extracts and root exudate samples.

Method

The HPAEC-PAD conditions that were used are listed in Table 1. The analysis was performed with an HPLC system with a quaternary low-pressure gradient pump, autosampler, a thermostatted column compartment, and the DECADE Elite electrochemical detector with SenCell flow cell (Table 1).

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. 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 method for the analysis of sugars in plants is based on isocratic separation using an anion exchange column and alkaline mobile phase (100 mM NaOH, pH 13) followed by pulsed amperometric detection on a gold (Au) working electrode (SenCell). For optimal separation the column

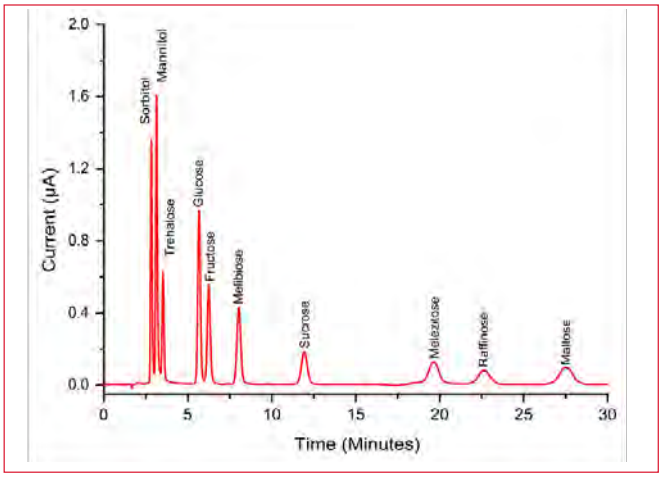

Figure 1: Chromatogram of a standard mix consisting of 10 ppm sorbitol, mannitol, trehalose, glucose, fructose, melibiose, sucrose, melezitose, raffinose and maltose in water (5 µL injection). Conditions as in Table 1.

temperature was set to 20 °C in the separate thermostatted oven compartment of the LC system (Agilent).

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

Table 1

Conditions	
LC system	HPLC system with quaternary LPG mixer (for automated column cleaning)
Detector	DECADE Elite electrochemical detector
Columns	BorateTrap™ Inline Trap Column, 50 x 4.0 mm ID, 20 µm (between LC pump and injector) CarboPac™ PA1 IC column, 50 x 2.0 mm ID, 10 µm + 250 x 2.0 mm ID, 10 µm All columns: Thermo Scientific™ Dionex™
Mobile phase	Isocratic elution with 100 mM NaOH (carbonate-free) in water. The mobile phase is continuously sparged with Helium 5.0
Flow rate	0.25 mL/mL
Temperature	20 °C for separation 30 °C for detection
$V_{injection}$	5 µL
Flow cell	SenCell™ with Au WE and HyREF™ (Pd/H_2) RE, AST setting 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	2 µA/V
ADF	0.1 Hz
I-cell	1 - 2 µA

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 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 bar overpressure) during the analysis to minimize the build-up of carbonate ions in the mobile phase, and to assure a reproducible analysis. For more details about mobile phase preparation and precautions see the application note Carbohydrates in Food Products[3].

To eliminate the presence of borate contaminants in the mobile phase 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, especially for sugar alcohols (reduced monosaccharides) like sorbitol and mannitol. If borate is present in the mobile phase, it binds to both the anion-exchange stationary phase and carbohydrate molecules. A carbohydrate-borate complex is eluted less efficiently than the carbohydrate molecule itself, resulting in peak tailing or co-elution of the alcohol sugars. For the example described in this application, the installation of the trap was crucial, otherwise no selection of the alcohol sugars was possible.

Detection

For the pulsed amperometric detection [4] of the mono-, di and trisaccharides, the Antec SenCell electrochemical flow cell is used, controlled by the DECADE Elite electrochemical detector. This flow cell [5] has a confined wall-jet design flow and consists of a Au working electrode (WE), HyREF (Pd/ H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform is applied (Figure 2). This particular waveform results in an excellent reproducibility and

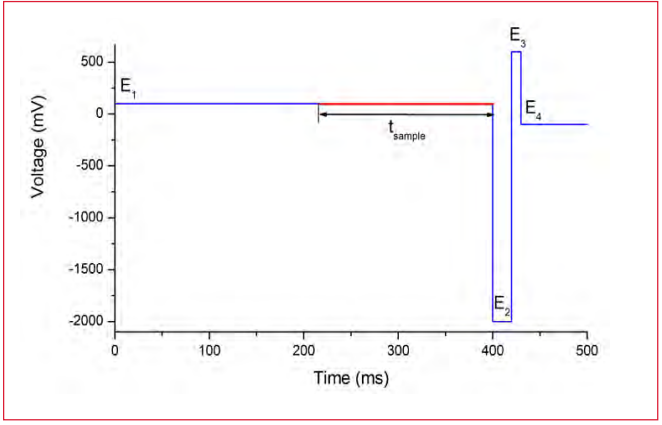

Figure 2: 4-step PAD potential waveform for the detection FDG and impurities.

minimal electrode wear [6]; i.e. resulting in less flow cell maintenance and system down time. The cell current was typical about 1 – 2 µA under the specified conditions. The temperature for detection was set to 30 °C.

Sample preparation

In the section below, the sample preparation procedures are described for [1] plant leaf samples and [2] root exudate samples.

[1] Plant samples:

After collection plant samples were kept frozen until freeze-drying. Sample material was freeze-dried before analysis. After drying, samples were stored in a desiccator or air free bags until extraction, to avoid moist contamination. Extraction of the freeze-dried plant samples was performed in the following way:

- Dry sample material was crumbled and mixed in a dish.
- An Eppendorf tube containing 2 metal balls was filled with as much as possible crumbled dry sample material and ground using a Retch grinding machine (2 min 30 b/s).
- 50.0 mg of ground plant material was put into a 2 mL Eppendorf tube with perforated cap.
- First extraction:
  - 1 mL 70/30 % (v/v) MeOH/Water was added to the tube and the solution vortexed.
  - The solution was boiled for 5 minutes and subsequently sonicated for 15 min in an ultrasonic bath.
  - After sonication the solution was centrifuged for 10 min at 10000 rpm
  - The supernatant was transferred to a clean 2 mL Eppendorf tube using a pipette.
- Second extraction:
  - Again 1 mL 70/30 % (v/v) MeOH/Water was added to the original Eppendorf tube containing the remaining plant leaf sediment, and sonicated for 15 min in an ultrasonic bath.
  - After sonication the solution was centrifuged for 10 min at 10000 rpm.
  - The supernatant of the second extraction was transferred to the Eppendorf tube containing the supernatant of the first extraction.
- The total volume of the combined supernatant in the Eppendorf tube was adjusted to 2 mL by adding 70/30 % (v/v) MeOH/water solution*.
- The obtained extracts were kept in the freezer (-20 °C) until analysis.

For HPLC analysis, the extracts were diluted 100x, by pipetting 10 µL into a HPLC vial and adding 990 µL MilliQ water, followed by capping and mixing. Subsequently, 5 µL of this solution was injected into the HPLC system for analysis.

*) adjustment to 2 mL of the combined supernatant was done gravimetrically using the following procedure: three clean 2 mL Eppendorf tubes were filled with 2 mL 70/30 % (v/v) MeOH/Water and weighted on an analytical balance. The average mass of the three tubes (at 0.001 g precise) was taken as reference and the weight of the tubes with combined supernatant were adjusted to that weight by adding 70/30 % (v/v) MeOH/Water.

[2] Root exudate samples:

Samples are obtained by flushing and filtering plant root cultures growing in glass-wool-water medium. 1 Liter of water extract used for flushing is subsequently freeze dried and concentrated to a volume of 1 mL, from which 5 µL is injected into the HPLC system for analysis.

Results

In Figure 1 a typical chromatogram is shown of a 5 µL injection of a 10 ppm standard mix of 10 saccharides in water. All compounds elute within 30 minutes.

Table 2

Linearity, repeatability and detection limit (LOD)

	Ret. Time (min)	*Corr. coeff. R (-) **	*RSD, Area (%) ***	*LOD (ppb) ****	*LOD (nM) ****
Sorbitol	2.82	0.9999	1.41	27	148
Mannitol	3.13	0.9997	0.79	13	71
Trehalose	3.53	0.9999	1.70	21	61
Glucose	5.68	0.9999	1.58	16	89
Fructose	6.25	0.9997	1.97	21	117
Melibiose	8.05	0.9999	0.44	22	64
Sucrose	11.95	0.9999	0.81	38	111
Melezitose	19.68	0.9988	0.73	36	71
Raffinose	22.68	0.9999	1.78	26	52
Maltose	27.56	0.9998	0.53	29	85

* The linearity was determined using a 5 point calibration curve based on a 0.5, 1, 2.5, 5 and 10 ppm standard.

** The RSD of the peak area was determined with the 1 ppm standard (n=10.)

*** The LOD's were calculated based on the response (area) of the lowest calibration standard of 0.5 ppm (n=10), where the LOD = 3.3 x standard deviation of the response / slope. See reference [7], ICH guideline .

Linearity, repeatability and detection limit (LOD)

The linear response of the saccharides was investigated in the concentration range of 0.5 – 10 ppm. The obtained correlation coefficients were better than 0.999 for peak area for all compounds of interest (see Table 2).

The relative standard deviation (RSD) for peak area was determined for 10 replicate injections of the 1 ppm standard mix of the saccharides dissolved in water. The RSD for peak area was < 2% for all sugars (see Table 2). The RSD's for the retention times of all components is typically ≤ 0.2%.

The Limit of Detection (LOD) for all saccharides are shown in Table 2 in ppb and molar concentrations. the LOD's were calculated based on the calibration curves (Area), as LOD = 3.3σ/S, where σ = the standard deviation obtained from the response (Area) of 10 repetitive injections of the 0.5 ppm standard, and S is the slope of the calibration curve [5]. The concentration detection limits for the sugars obtained were in the range of 50 – 150 nmol/L (10 – 40 ppb).

Plant samples

This section shows two examples of the analysis of mono-, di and trisaccharides in actual plant samples. Figure 3 shows a chromatogram of a 5 µL injection of a leaf sample of the *Brassica oleracea* (Brussels sprouts), and Figure 4 shows a chromatogram of a root exudate sample of a tomato plant. The main peaks observed in both samples are originating from sucrose, fructose and glucose.

The calculated carbohydrate contents (glucose, fructose, sucrose and maltose) for both samples are listed in Table 3. These concentrations correspond to saccharide levels typically found in such plant samples.

Table 3

Mono-, di- and trisaccharide content in two plant samples

Compound	*Concentration*	
	*(1) **Leaf** from Brassica oleracea (ng/mL) **	*(2) **Root exudate** from tomato plant (mg/L)***
Glucose	3.7	1.5
Fructose	3.2	2.8
Sucrose	3.1	1.5
Maltose	0.2	0.2

* concentration defined as ng sugar per mg freeze dried plant material.

** sugar concentration in mg/L (ppm) in the 1 Liter of collected water extract used for flushing of the roots.

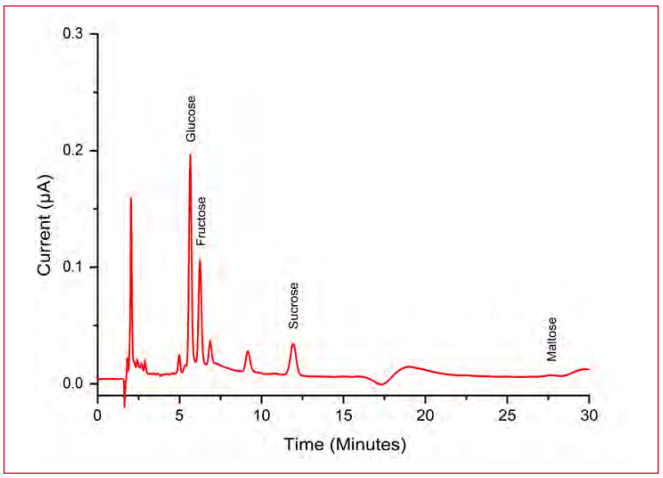

Figure 3: Chromatogram of a 5 µL injection of a *Brassica oleracea* (Brussels sprouts) leaf sample, obtained using the extraction method described in the sample preparation section of this note.

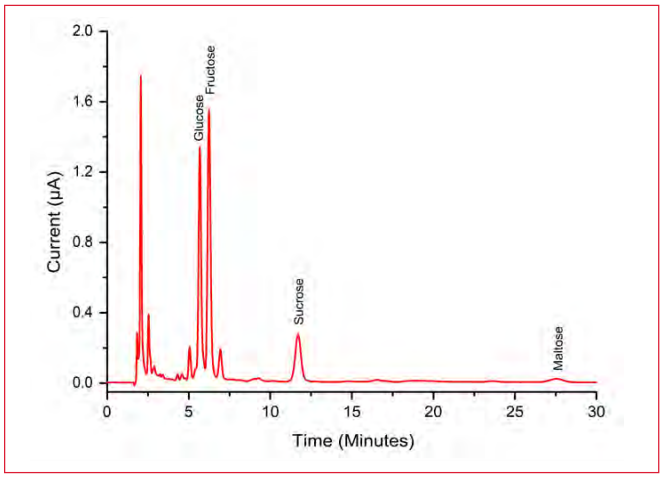

Figure 4: Chromatogram of a 5 µL injection of root exudate of a tomato plant sample obtained by flushing and filtering tomato root cultures growing in glass -wool-water medium.

References

1. Netherlands Institute of Ecology (NIOO-KNAW), https://nioo.knaw.nl/en/about-nioo
2. M. Van Geem, Y. A. Harvey, A.M. Cortesero, C.E. Raaijmakers, R. Gols, Interactions Between a Belowground Herbivore and Primary and Secondary Root Metabolites in Wild Cabbage, J. Chem. Ecol., 41 (2015) 41, 696–707 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4568014/pdf/10886_2015_Article_605.pdf 41:696–707. doi:10.1007/s10886-015-0605-7
3. Antec, Carbohydrates in Food Products, Application note, 220_002
4. W.R. Lacourse, Pulsed Electrochemical Detection In High Performance Liquid Chromatography, John Wiley & Sons, New York, 1ed,1997.
5. H. Louw, H.J. Brouwer, N. Reinhoud, Electrochemical flow cell, (2016) US patent 9310330
6. 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
7. ICH guideline, Validation of Analytical Procedures: Methodology, Q2(R1) (1994), 11 http://www.ich.org/fileadmin/ Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_ R1/Step4/Q2_R1 Guideline.pdf

Conclusion

The DECADE Elite detector in combination with the SenCell flow cell offers a user-friendly and sensitive detection solution for the analysis of saccharides in plant extracts or root exudates using HPAEC-PAD. Easy integration of the DECADE Elite into a third-party LC systems like the Agilent 1260 Infinity Bio-Inert LC system is provided by means of a software drivers for the DECADE Elite or dedicated analog output-to-ADC and remote cables.

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, 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 - for connection to 3rd party HPLC	
176.0035A	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
Recommended ALEXYS analyzer	
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

#) other 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.

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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.

Sugar Alcohols

Introduction

Sugar alcohols (also called polyols or alditols) are organic compounds, typically derived from sugars, containing one hydroxyl group (–OH) attached to each carbon atom. Sugar alcohols are used widely in the food industry as thickeners and artificial sweeteners [1]. Sugar alcohols are non-cariogenic or, in case of xylitol, even anti-cariogenic, have a low glycemic index and insulin index (useful in obesity and diabetes), are digested more slowly and also have osmotic properties showing water holding properties beneficial to the colon. Due to their laxative effects the approval is restricted and the products with more than 10% added polyols must bear the words "excessive consumption may produce laxative effects" [2].

High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) can be used as a tool for the compositional analysis of sugar alcohols. This note shows the proof of principle for the analysis of a mix of sugar alcohols using the ALEXYS Carbohydrate Analyzer.

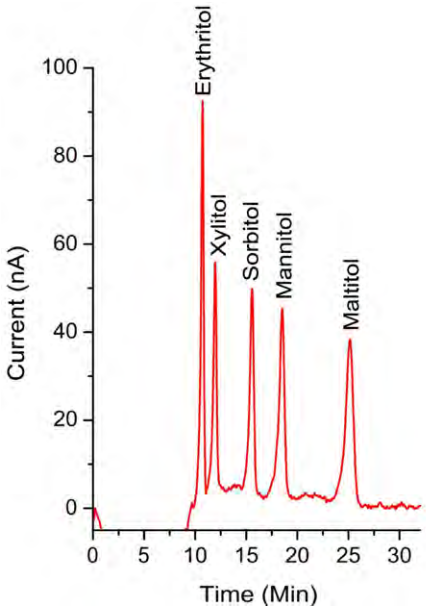

Fig 2. *10µL injection of a 10µM mix of sugar alcohols; (1) erythritol, (2) xylitol, (3) sorbitol, (4) mannitol and (5) maltitol in water.*

Fig. 1*. ALEXYS Carbohydrate Analyzer. The system is equipped with a Solvent Selection Valve (SSV) and capable of running step gradients for column clean-up. The system can be operated under DataApex™ Clarity™ CDS (version 8.3 and up) or Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software (version 7.2 SR 5 and up).*

Table 1. LC-EC conditions

HPLC	ALEXYS Carbohydrate Analyzer
Columns	CarboPac™ MA1, 250 x 4.0 mm ID + 50 x 4.0 mm ID BorateTrap™ Inline Trap Column, 50 x 4.0 mm ID, 20 µm All columns: Thermo Scientific™ Dionex™
Mobile phase	A) 500mM NaOH , B) 700mM NaOH mobile phases blanketed with Helium 5.0
Flow rate	0.4 mL/min
Back pressure	about 85 bar
Injection	10 µL (Partial loop fill)
Temperature	32 °C for separation & detection
Flow cell	SenCell with Au WE, HyREF, AST 2
E-cell	Waveform A
I-cell	about 0.3 µA
ADF	0.5 Hz
Range	1 µA/V

Method

The method for the analysis of sugar alcohols is based on isocratic separation (500 mM NaOH) with an anion exchange column followed by post-run column flush (700 mM NaOH) for improved repeatability. Mobile phase eluents were carefully prepared manually using a carbonate-free 50% w/w NaOH solution and sparged with Helium 5.0 prior to use. To eliminate the presence of borate contaminants in the mobile phase, a trap column was installed in the solvent line between pump and autosampler. For detection, a 4-step potential waveform was applied, resulting in an excellent reproducibility and minimal electrode wear [3]; i.e. resulting in less flow cell maintenance and system down time.

An example chromatogram of a 10 µM standard mix of 5 sugar alcohols is shown in figure 2. From figure 2 it is evident that all component peaks show some peak fronting. This behavior is also shown in the different application examples in the column manual [4] of the manufacturer and is considered an as expected performance. When a post-run column flush (700 mM NaOH) is used for improved repeatability, extra stabilization time is needed after the peaks are eluted. A longer equilibration time results in a more stable baseline at the beginning of the next run.

The relative standard deviations (RSD's) for retention time, peak area and height of 10 µL injections of a 10 µM standard mix of 5 sugar alcohols in water are shown in Table 2 to demonstrate the repeatability of the method.

Table 2. Repeatibility, 10 µM standard mix (n=6)

Compound	*Ret. time (RSD%)*	*Area (RSD%)*	*Height (RSD%)*
Erythritol	0.07	1.11	0.64
Xylitol	0.07	2.27	1.76
Sorbitol	0.08	2.77	1.58
Mannitol	0.09	2.98	2.15
Maltitol	0.08	3.87	1.93

Ordering information

ALEXYS analyzer	
180.0055W	ALEXYS Carbohydrate Analyzer
116.4321	SenCell 2 mm Au HyREF
Software*	
195.0035	Clarity CDS single instr., incl. LC, AS module

*) The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

References

1. Wikipedia, Sugar alcohol, https://en.wikipedia.org/wiki/Sugar_alcohol
2. Eurofins Scientific Media Center, https://www.eurofins.com/media-centre/newsletters/food-newsletter-nr45-july-2014/sugar-alcohols-content/
3. 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
4. CarboPac MA1 Column Product Manual, https://assets.thermofisher.com/TFS-Assets/CMD/manuals/Man-065546-CarboPac-MA1-Column-Man065546-EN.pdf

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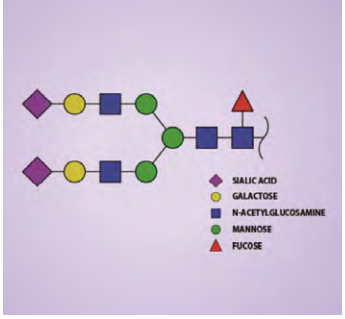

The finest LC-EC Applications for Food & Beverage analysis

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

Compositional Analysis of Glycans

- **Mono- and disaccharides in glycans/glycoproteins**
- **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.

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.

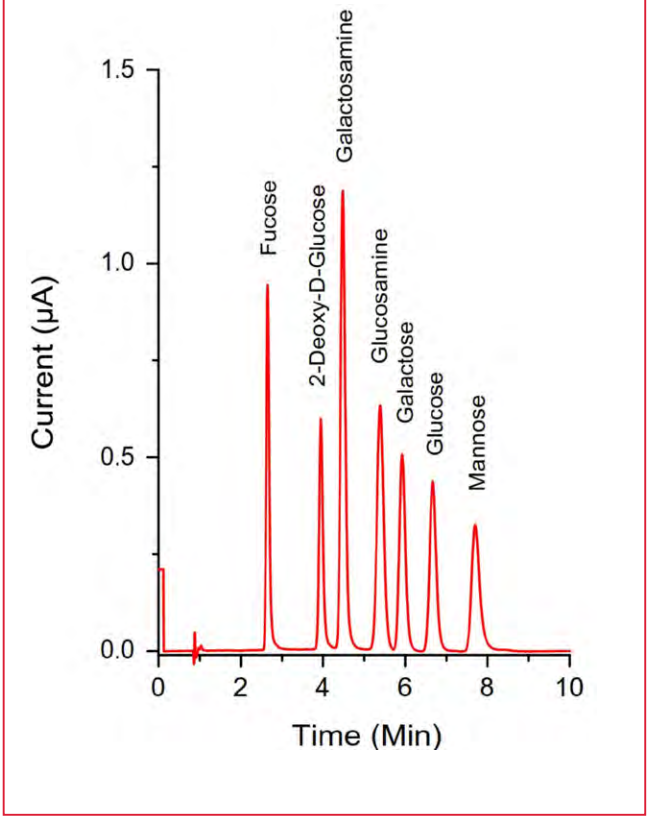

Figure 1: Chromatogram of a 10 µL injection of a 10 µM standard mix of 7 monosaccharides in water: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and

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
Back pressure	About 180 bar
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

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

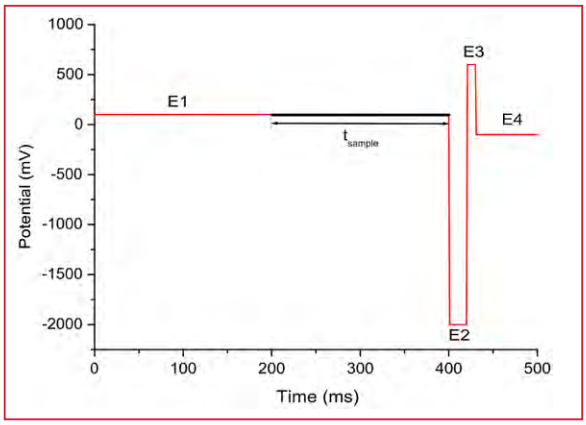

Figure 2: 4-step PAD potential waveform for the detection mono-saccharides and other carbohydrates.

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 ≥ 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. 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.

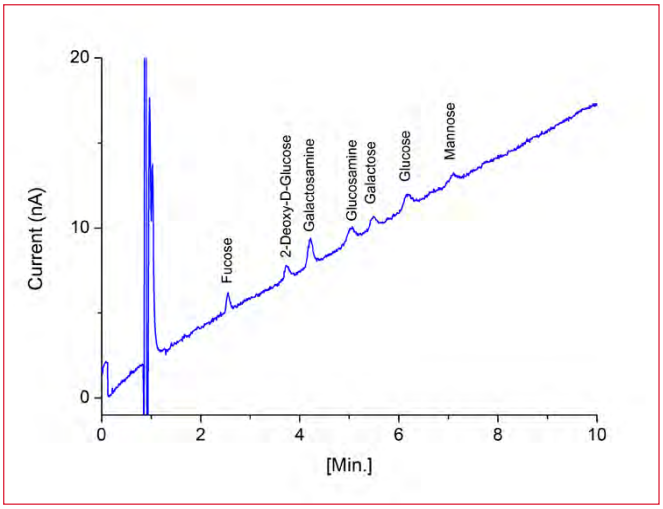

Figure 3: Chromatogram of a 10 µL injection of a 10 nM standard mix of 7 monosaccharides in water: (1) L-Fucose, (2) 2-Deoxy-D-Glucose, (3) Galactosamine, (4) Glucosamine, (5) Galactose, (6) Glucose and (7) Mannose.

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.

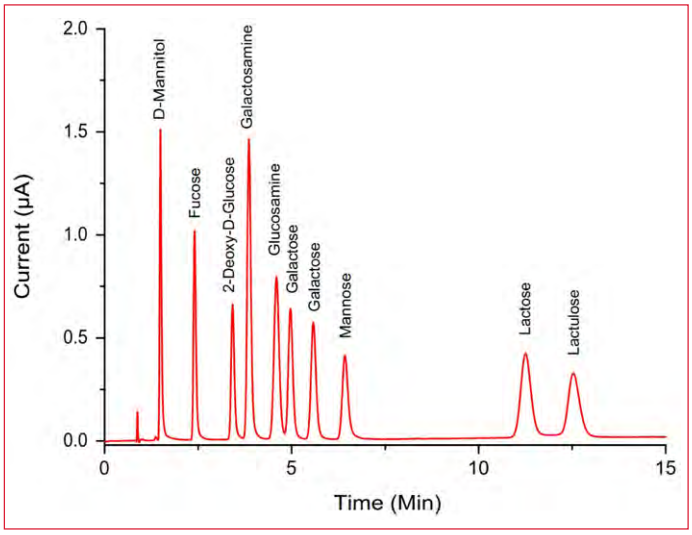

Figure 4: Chromatogram of a 10 µL injection of a 10 nM standard mix of 10 mono- and disaccharides in water: (1) Mannitol, (2) Fucose, (3) 2-Deoxy-DGlucose, (4) Arabinose, (5) Glucosamine, (6) Sucrose, (7) Xylose, (8) Fructose, (9) Lactose and (10) Lactulose.

References

1. K.F. Aoki-Kinoshita, An introduction to Bioinformatics for Glycomics, PLoS Comput Biol, vol 4 issue 5 (2008), 1– 6
2. D.C. Johnson, D. Dobberpuhl, R. Roberts, P. Vandeberg, Review: Pulsed amperometric detection of carbohydrates, amines and sulfur species in ion chromatography- the current state of research, J. Chromatogr., 640 (1993), 79 - 96
3. D.C. Johnson, W.R. LaCourse, LC with pulsed ECD at gold and platinum, Anal. Chem., 62 (1990), 589A – 597
4. W.R. LaCourse, Pulsed Electrochemical Detection in High Performance Liquid Chromatography, John Wiley & Sons, New York, 1ed, 1997

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
6. Antec, Carbohydrates in food, Application note, 220.002 rev. 04
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

#) Antec ECD drivers are available for Chromeleon CDS, OpenLAB CDS and OpenLAB Chemstation 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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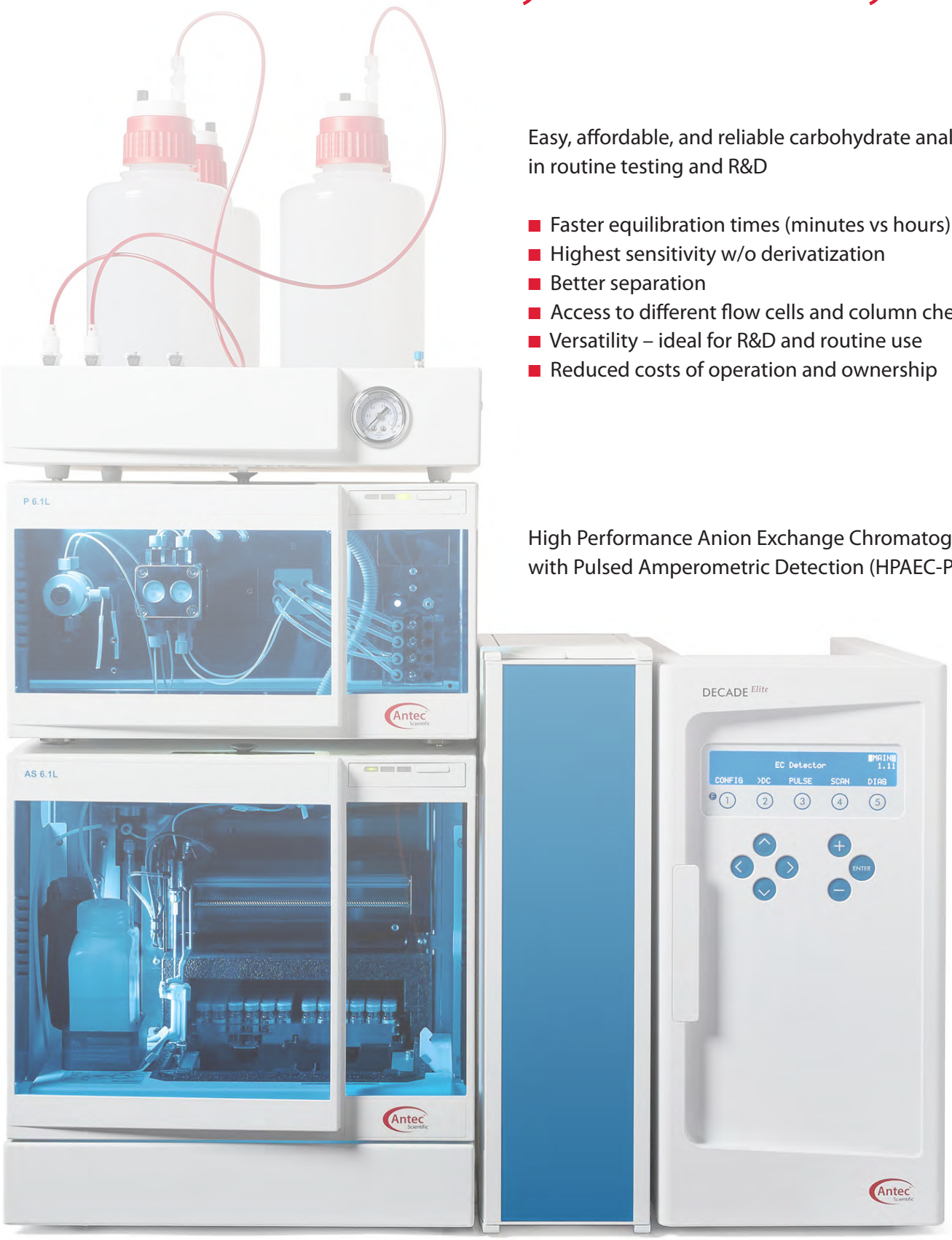
ALEXYS™ Carbohydrate Analyzer



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High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Approximately 65% of the global population is suffering from lactose-intolerance

Lactose is the main carbohydrate found in milk and dairy products but can also be found as a food additive in meat, e.g., sausages, salami, meat balls and fish products. Lactose is a disaccharide composed of the monosaccharides D-glucose and D-galactose, joined in a ß-1,4-glycosidic linkage.

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