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

Sugars in meat & fish

- **ALEXYS Carbohydrates Analyzer**
- **Fast and sensitive HPAEC-PAD analysis**
- **SenCell™ with Au working electrode**
- **Mono- and disaccharides**
- **Processed meat & fish products**

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

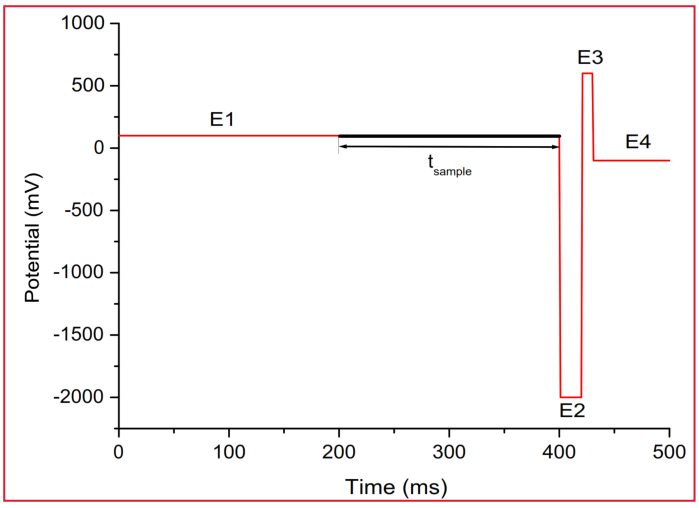

Figure 2: 4-step PAD potential waveform for the detection of carbohydrates.

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.

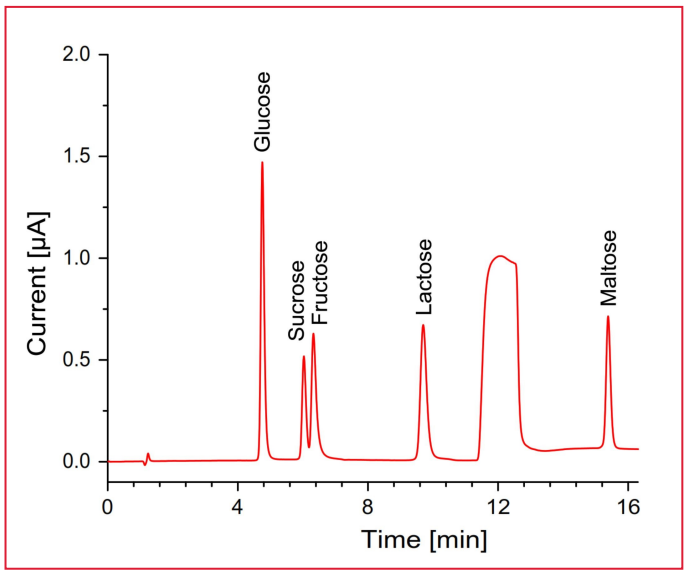

Figure 3: An example chromatogram obtained with a 10 µL injection of a 7.5 µg/L standard mix of glucose, sucrose, fructose, lactose and maltose in DI water.

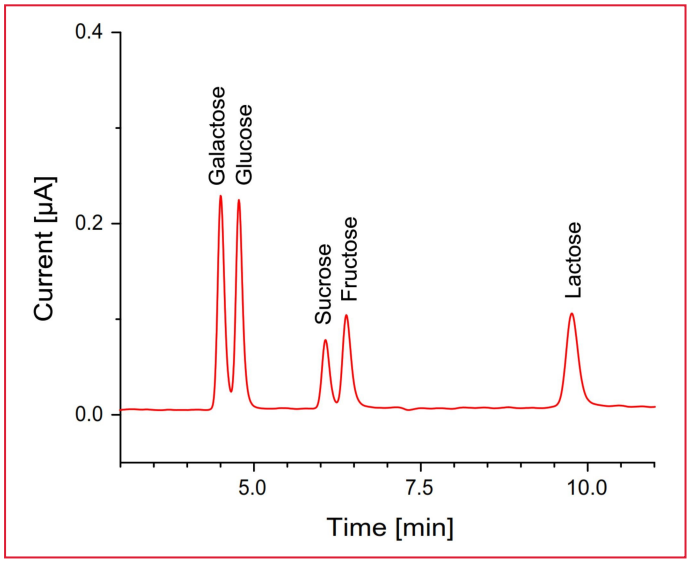

Figure 4: Zoom of the chromatogram (isocratic elution at 15 mM NaOH) obtained with a 10 µL injection of a 1 µg/L standard mix of sugars in DI water including galactose.

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

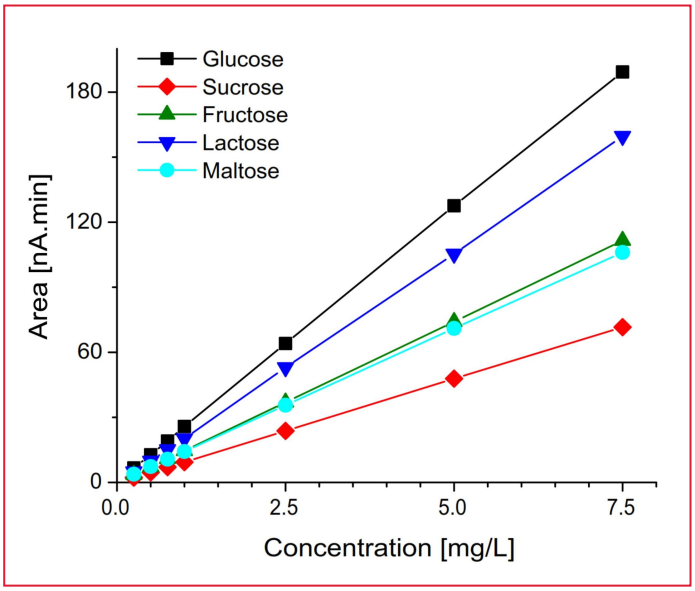

Figure 5: Calibration curve of glucose, fructose, sucrose, lactose and maltose in the concentration range of 0.25 - 7.5 mg/L

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 analyzer.

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

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.

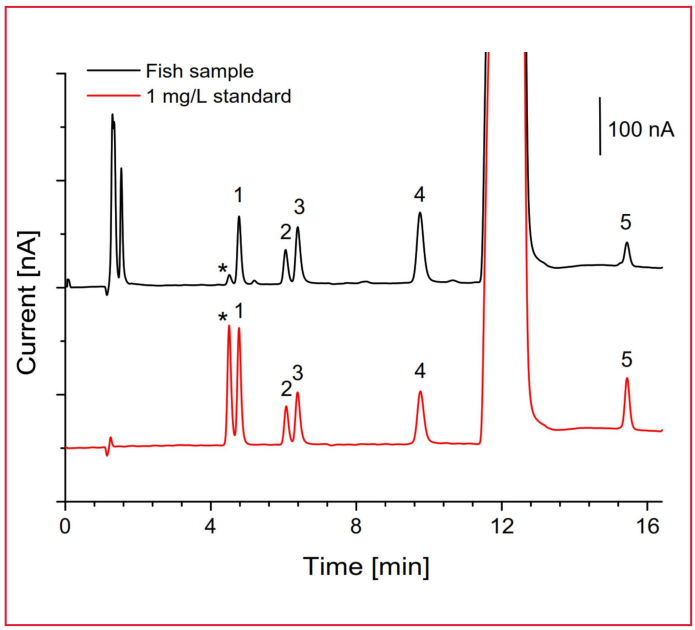

Figure 6: Chromatogram of the fish sample (black) and a 1 mg/L standard (red, -35% offset). Labeled peaks: * Galactose, 1. Glucose, 2. Sucrose, 3. Fructose, 4. Lactose, 5. Maltose.

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.

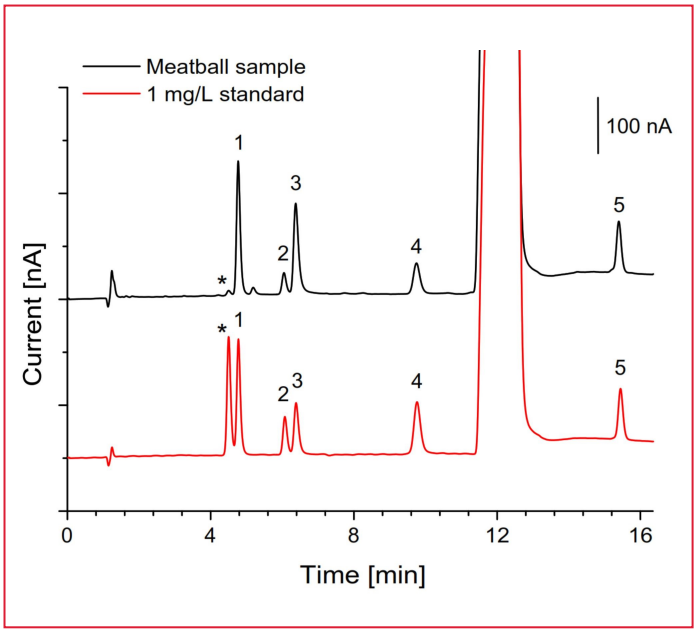

Figure 7: Chromatograms of the meatball sample (black) and a 1 mg/L standard (red, -35% offset). Labeled peaks: * Galactose, 1. Glucose, 2. Sucrose, 3. Fructose, 4. Lactose, 5. Maltose.

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.

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.

References

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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.

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