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**
- **SweetSep™ AEX200 anion-exchange column**
- **High-resolution separation of 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® Carbohydrate 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 the SweetSep™ AEX200 column with 5 µm particle size. The combination of isocratic elution with a step gradient resulted in high-resolution 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 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 must be labelled with a 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 the ALEXYS carbohydrate analyzer with SweetSep™ AEX200 column in combination with a step gradient allows for fast high-resolution separation of the relevant mono- and disaccharides. To demonstrate the performance of the method several processed meat and fish samples were analyzed.

Figure 1: ALEXYS carbohydrate analyzer consisting of the ET 210 eluent tray, P 6.1L analytical pump, AS 6.1L autosampler, CT 2.1 column thermostat and DECADE Elite electrochemical detector.

Method

The HPAEC-PAD analysis was performed using the ALEXYS carbohydrate analyzer as shown in figure 1. The HPAEC-PAD system consists of an ET 210 eluent tray for Helium blanketing, P 6.1L quaternary LPG pump, AS 6.1L autosampler, CT 2.1 column thermostat and the DECADE Elite electrochemical detector. The SenCell with Au working electrode and HyREF (Pd/H_2) reference electrode was used for sensitive detection of the sugars. The system was operated under the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 7.3.2.

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 carbohydrate analyzer
Detector	DECADE Elite electrochemical detector
Columns	SweetSep™AEX200, 4 x 200 mm column, 5 µm SweetSep™AEX200, 4 x 50 mm precolumn, 5 µm Borate ion trap, 4 x 50 mm ID, 10 µm (All columns Antec Scientific)
Mobile phase (MP)	A: 15 mM NaOH B: 100 mM NaOH C: 100 mM NaOH + 100 mM NaOAc (resistivity > 18 MOhm.cm and TOC<10ppb) Eluents blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
Back pressure	about 220 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 - 15	15 mM NaOH	Elution & detection
15 - 23	100 mM NaOH	
23 - 28	100 mM NaOH, 100mM NaOAc	Column clean-up and regeneration
28 – 48	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 5 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 column clean-up and regeneration step is executed at t =23 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 20 minutes at the starting conditions, resulting in a total run time of 48 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, TOC < 5 ppb), which was sparged with nitrogen 5.0 (purity 99.999%) 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 nitrogen 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

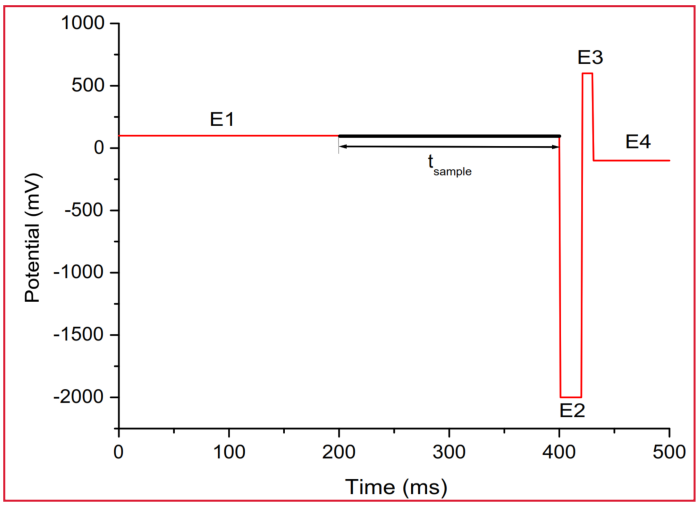

Figure 2: 4-step PAD potential waveform for the detection of carbohydrates.

consistent long-term peak area response and (2) minimal 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 shelf 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 0.1 - 7.5 mg/L were prepared by dilution of the stock standards with DI water.

Samples: Three commercially available meat and fish samples were purchased from a supermarket in the Netherlands. The following products were purchased and analyzed:

- Iglo Fish Cuisin Bretagne style
- Chicken cordon blue
- Porksteak spicy BBQ

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 and fish products: the two meat and one fish sample were prepared and analyzed using the procedure below.

Procedure:

1. The sample was chopped in small pieces and homogenized using a homogenizer (Fisher Scientific PowerGen 125 homogenizer).
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 water bath (60°C) and stirred 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. 6 aliquots of 1 mL were pipetted into Eppendorf vials and then centrifuged for 10 minutes at 6000 rpm.
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 2.5 µg/L galactose, glucose, fructose sucrose, lactose, and maltose DI water. All saccharides except maltose elute within 15 minutes during the isocratic elution step with 15 mM NaOH. After 15 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 3 minutes allowing sensitive detection of Maltose. All compounds of interest eluted within 24 minutes, and the total run cycle time is 48 minutes due to

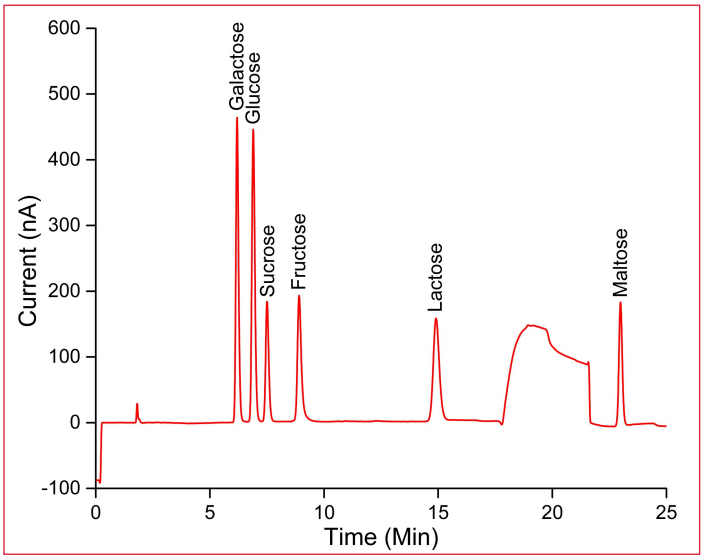

Figure 3: An example chromatogram obtained with a 10 µL injection of a 2.5 µg/L standard mix of galactose, glucose, sucrose, fructose, lactose and maltose in DI water.

the wash and equilibration step. The peak efficiencies found for the sugars ranged from 50.000 to 68.000 theoretical plates per meter with the exception of Maltose, which has a peak efficiency of 400.000 theoretical plates per meter. All peak tailing factors ranged from 1.0 to 1.3.

It is evident from figure 3 that despite their structural similarity of galactose and glucose (stereoisomers), they are baseline separated on the AEX200 column. Under the isocratic separation conditions (15 mM NaOH) the resolution of all sugars is above 1.5, allowing for accurate quantification of all carbohydrates if required.

Linearity

The linearity was investigated with standards dissolved in DI water in the concentration range of 0.1 - 7.5 mg/L . The obtained calibration curves are shown in figure 3. Real samples are diluted 50 times during sample preparation, so this calibration range corresponding to a sugar contents of 0.05 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 6 sugars.

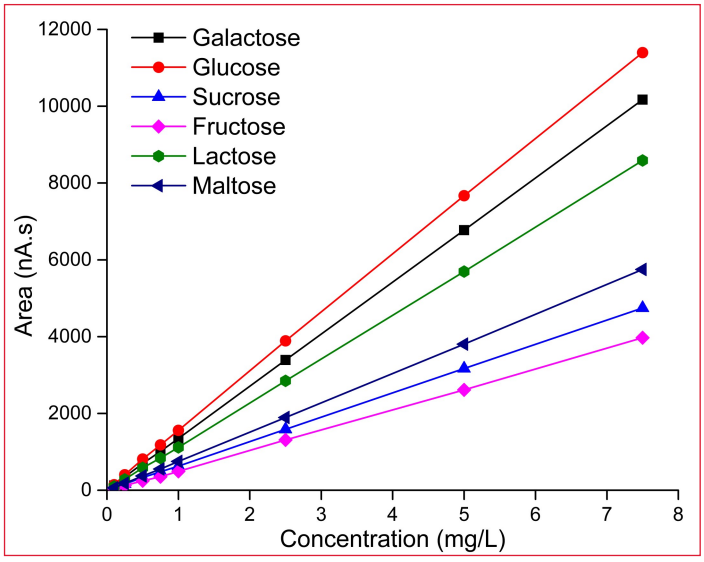

Figure 5: Calibration curves of galactose, glucose, fructose, sucrose, lactose and maltose in the concentration range of 0.1- 7.5 mg/L.

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 0.00 - 0.19% for all analytes. The RSD for peak areas for all sugars was < 1.1% for the 0.1 mg/L standard and < 0.6% 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*
Galactose	0.06	0.38	0.08	0.63
Glucose	0.06	0.44	0.07	0.66
Sucrose	0.00	0.38	0.08	0.74
Fructose	0.07	0.59	0.19	1.07
Lactose	0.04	0.16	0.14	0.73
Maltose	0.10	0.40	0.13	0.96

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. The calculated LODs ranging from 2 to 22 ng/mL demonstrate the excellent sensitivity of the method.

Table 4

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

	Limit of detection		*Limit of Quantification*	
Compound	*nM*	*ng/mL*	*nM*	*ng/mL*
Galactose	14	2	45	8
Glucose	13	2	42	8
Sucrose	12	4	41	14
Fructose	34	6	112	20
Lactose	16	6	54	19
Maltose	64	22	212	73

Sample analysis

Two meat and one fish products with an unknown concentration of galactose, glucose, sucrose, fructose, lactose and maltose were analyzed with the presented method. An overlay of the chromatograms of the chicken sample (black) and a 0.5 mg/L standard + galactose (red) is shown in figure 6. Glucose, fructose, lactose and maltose were present in the chicken sample. The large baseline disturbance between 18 - 21 minutes is due to the onset of the step gradient (15 —> 100 mM NaOH), as explained on page 4. The overlay of the chromatograms of the pork sample (black) and 0.5 mg/L standard (red) is shown in figure 7. In this sample glucose, sucrose, fructose and maltose were present. Figure 8 shows the overlay of the chromatograms of the fish sample (black) and 0.5 mg/L standard (red). This sample consist of galactose, glucose, sucrose, fructose, lactose and maltose.

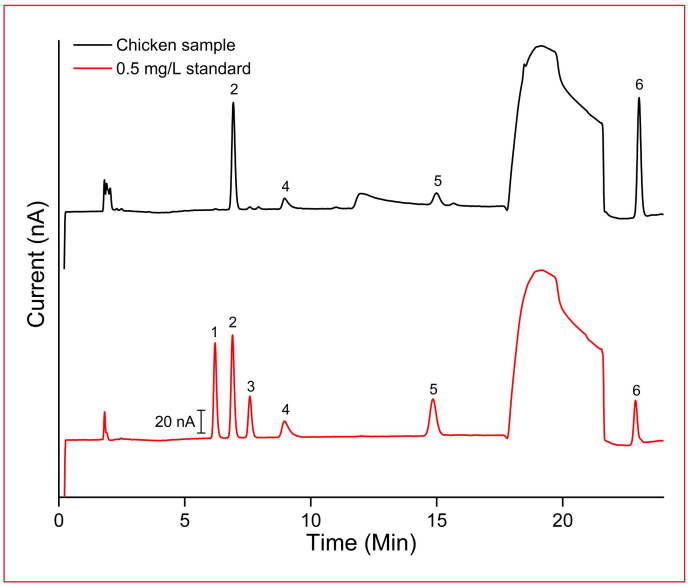

Figure 6: Chromatogram of the chicken sample (black) and a 0.5 mg/L standard (red). Labeled peaks: 1. Galactose, 2. Glucose, 3. Sucrose, 4. Fructose, 5. Lactose, 6. Maltose.

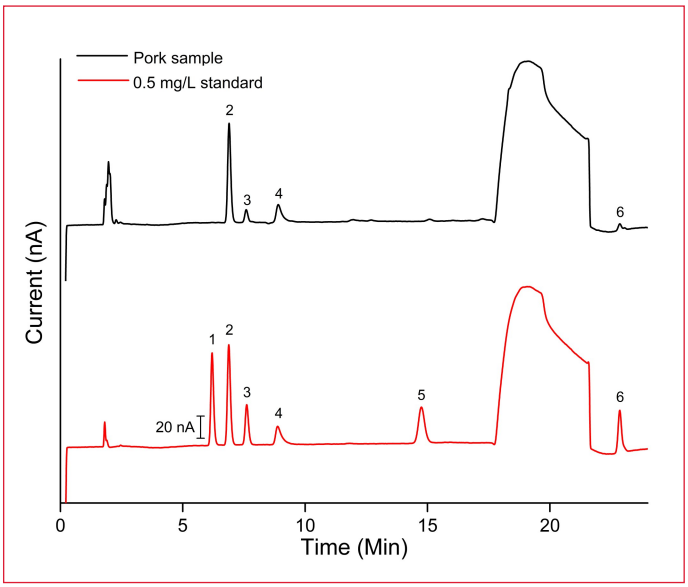

Figure 7: Chromatograms of the pork sample (black) and a 0.5 mg/L standard (red). Labeled peaks: 1. Galactose, 2. Glucose, 3. Sucrose, 4. Fructose, 5. Lactose, 6. Maltose.

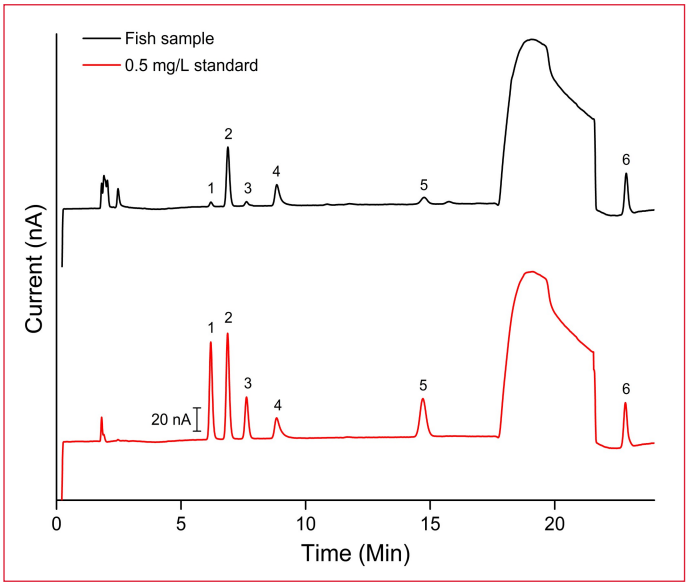

Figure 8: Chromatogram of the fish sample (black) and a 0.5 mg/L standard (red). Labeled peaks: 1. Galactose, 2. Glucose, 3. Sucrose, 4. Fructose, 5. Lactose, 6. Maltose.

The sugar content of the chicken, pork and 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 0.1—7.5 mg/L.

Table 5

Sugar content samples [mg/100g product]

Compound	*Chicken*	*Pork*	*Fish*
Galactose	-	-	11
Glucose	272	258	144
Sucrose	-	78	23
Fructose	165	225	234
Lactose	80	-	40
Maltose	674	26	225
Total content	1191	586	678

Table 6

Sugar content on nutrition label products [g/100g product]

	Chicken	*Pork*	*Fish*
Sugar content	0.7	0.4	0.9

In table 6 the added sugar contents on the nutrition labels of the products are summarized. For the fish sample the content quantified with HPAEC-PAD was about 22% lower than the amount stated on the product label. A higher amount of added sugar was found in both the pork and chicken sample compared to the amount listed on the product label. This could have several reasons, the sugar contents listed on the nutrition label is an average value, and the difference might be attributed to variations in batch-to-batch production, or due to the heterogeneous composition of the processed meat & fish samples. All analyzed products either were marinated or covered with a sugar containing crust or sauce. Only a small amount of the products was sampled for analysis in this case, and the sugar composition & contents may vary throughout the product.

References

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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 SweetSep™ AEX200 column with 5 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 24 minutes. The total runtime including column clean-up and equilibration is 48 min.

Ordering information

Detector only	
176.0035B	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
Recommended ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Column	
260.0010	SweetSep™ AEX200, 4 x 200 mm column, 5 µm
260.0015	SweetSep™ AEX200, 4 x 50 mm precolumn, 5 µm
260.0030	Borate ion trap, 4 × 50 mm column, 10 µm
260.0100#	Pre-column filter PEEK, 0.5 µm
Software*	
195.0035	Clarity CDS single instr. incl LC, AS module

*) In case samples might contain particulate matter it is advised to use a pre-column filter.

#) Antec ECD drivers are available for Chromeleon CDS, OpenLAB CDS and Empower CDS. The ALEXYS Carbohydrates Analyzer (full system) 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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