Application Note

Carbohydrate Analysis

The finest HPAEC-PAD applications for carbohydrate analysis

Food and Beverage
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Sugars in meat & fish
Carbohydrates in food according to AOAC
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Carbohydrates in Dutch candy
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Oligo– and Polysaccharides in honey
Sugars in beer

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Fructans in infant formula
TGOS in food products
Profiling of FOS

Lactose Free Products
Lactose in dairy & meat
Lactose in lactose-free products

Artificial Sweeteners
Sugar alcohols
Sucralose

Glycoproteins
N-glycans
Monosaccharides
Sialic acids

Carbohydrates in urine

- **ALEXYS™ Carbohydrate Analyzer**
- **SweetSep™ AEX20 anion-exchange column**
- **Intestinal permeability probes**
- **Analysis of human urine sample**

Summary

Detection of carbohydrates in urine plays an important role in evaluating carbohydrates metabolism and overall health condition. As an example, high glucose level in urine may indicate diabetes, while the amount of several non-absorbable carbohydrates in urine can provide insights to the gastrointestinal function. For instance, lactulose-to-mannitol ratio is widely recognized as a reliable indicator of intestinal permeability [1—3]. Therefore, a method with high selectivity and sensitive detection of urinary carbohydrates is essential for assessing intestinal health.

Carbohydrates in urine can be separated and detected using high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). HPAEC-PAD is a powerful technique, offering highly selective separation and sensitive detection of carbohydrates, often with minimal sample treatment. In this application note, a HPAEC-PAD method is evaluated for the analysis of carbohydrates in urine using the ALEXYS™ Carbohydrate Analyzer and the new SweetSep™ AEX20 column. This column contains dual ion-exchange sites (quaternary amine and tertiary amine) enabling fast and efficient separation of mono– and disaccharides. To assess the applicability of the presented method, a human urine sample was analyzed.

Introduction

The consumption of ultra-processed foods (UPFs) is becoming increasingly common worldwide, replacing fresh or minimally processed foods in many diets. In some countries, UPFs contribute up to about 60% of the daily energy intake [4], and it raises concerns about its long-term health effects. One major concern is related to the high amount of additives in the UPFs, in particular sugars. The World Health Organization (WHO) strongly recommends reducing the intake of sugars to < 10% of total energy intake, and suggests even further reduction in sugar intake to below 5% of total energy intake [5]. However, this recommendation is in contrast with the increase in UPFs consumption in the diet.

Excessive sugar intake in the diet has been linked to various health conditions including diabetes and gastrointestinal disorders such as irritable bowel syndrome (IBS), or inflammatory bowel diseases (IBDs) such as Crohn's disease and colitis [6]. These conditions are often associated with impaired intestinal function, leading to poor absorption of specific sugars. For instance, xylose which is naturally present in many fruits and vegetables, is normally excreted in urine [6,7]. If urinary xylose levels in urine are lower than normal, it may indicate malabsorption and can be directly linked to several diseases. Several carbohydrates serve as a marker for intestinal diseases, such as lactulose, mannitol, rhamnose, 3-O-methylglucose, etc. [1-3, 8, 9]. The levels of these sugars in urine are commonly assessed as an indicator for intestinal health. Therefore, it is crucial to have a method with good selectivity and sensitivity for accurate analysis of these individual sugars in urine.

In this application note, a method is presented for separation and detection of 10 sugars which are commonly linked to a health problems [1-3, 6-9]. The 10 sugars are mannitol, 3-O-methylglucose, rhamnose, galactose, glucose, sucrose, xylose, ribose, lactose, and lactulose. High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is the analysis technique of choice, providing excellent separation in combination with highly sensitive detection of the sugars of interest. Typically carbohydrates can be detected using HPAEC-PAD up to pico– and femtomole sensitivity [10]. In this study the new SweetSep™ AEX20 column is used for the separation of these sugars. To evaluate the applicability of the method, a human urine sample was analyzed.

Figure 1. ALEXYS Carbohydrate Analyzer.

Method

The analysis of carbohydrates in urine was performed using the ALEXYS™ Carbohydrate Analyzer as shown in Figure 1, This dedicated HPAEC-PAD system consists of the ET210 eluent tray (for storage and N_2 blanketing of mobile phases during the analysis), a P6.1L quaternary LPG pump, AS6.1L autosampler, CT2.1 column thermostat, and the DECADE Elite electrochemical detector. The SenCell™ with Au working electrode and HyREF (Pd/H_2) reference electrode was selected for detection of the carbohydrates.

Separation

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. Therefore, under alkaline conditions (pH > 12) carbohydrates can be separated by means of HPAEC [11]. Due to the extreme alkaline conditions, only polymeric anion-exchange columns are suitable for separation of the carbohydrates. The separation of the 10 carbohydrates was carried out using SweetSep™ AEX20 columns (4 × 200 mm analytical column and 4 × 50 mm precolumn), which are anion-exchange columns containing highly monodisperse 5 µm ethyl vinylbenzene-divinylbenzene copolymer (80% crosslinked) coated with functionalized nanoparticles with dual ion ex-change sites (quaternary amine + tertiary amine). In addition, a borate ion trap column (4 x 50 mm) was installed in the solvent line between the pump and autosampler as precaution to eliminate borate ions from the mobile phase.

Table 1

HPAEC-ECD Conditions

HPLC system	ALEXYS™ Carbohydrate Analyzer
Columns	SweetSep™AEX20, 4 x 200 mm analytical column, 5 µm SweetSep™AEX20, 4 x 50 mm precolumn, 5 µm Borate ion trap, 4 x 50 mm column, 10 µm (all columns from Antec Scientific)
Mobile phase (MP)	A: DI Water B: 100 mM NaOH C: 100 mM NaOH + 100 mM NaOAc Eluents blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
Back pressure	about 240 bar
Injection	10 µL
Temperature	25 °C for separation, 45 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF (Pd/ H_2) 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	0.1 Hz
Range	2 µA/V

Table 2

Gradient program

Time (min)	*Mobile phase*	*%A*	*%B*	*%C*	*Description*
0	6 mM NaOH	94	6	0	Elution & detection
7	10 mM NaOH	90	10	0	
12	20 mM NaOH	80	20	0	
17—19	25 mM NaOH	75	25	0	
19 –24	100 mM NaOH + 100 mM NaOAc	0	0	100	Column clean-up and regeneration
24—50	6 mM NaOH	94	6	0	Equilibration, starting conditions

The gradient elution program described in Table 2 was employed for the separation of these carbohydrates. The gradient program started with linear gradient elution from 6 mM NaOH from t = 0 min to 10 mM NaOH at t = 7 min. This step is important to have a better separation of 3-O-methylglucose and rhamnose. Additionally, although resolution of the these two carbohydrates can be slightly improved by decreasing the initial concentration of NaOH to 5 mM, it turned out that separation of other later eluting compounds was compromised. Following the first gradient step, another linear gradient to 20 mM NaOH at t = 12 min was employed. A third linear gradient step to 25 mM NaOH at t = 17 min was executed, and this composition is kept until t = 19 min to facilitate faster elution of lactose and lactulose without compromising the resolution. After elution of all sugars, the mobile phase composition is changed to 100 mM NaOH + 100 mM NaOAc and kept until t = 24 min to elute any strongly retained components and to remove carbonate ions (CO_3^{2-}) build up on the column. After the clean-up step the column is equilibrated for 26 minutes to the starting conditions, resulting in a total run time of 50 minutes. The temperature for separation was set at 25°C for optimal separation.

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%). During analysis the mobile phase headspace is also blanketed with Nitrogen gas (0.2—0.4 bar N_2 overpressure) using the ET210. The inert gas atmosphere will prevent the introduction of CO_2 (from the air) into the mobile phase and the subsequent formation of CO_3^{2-} ions, ensuring reproducible analysis.

Detection

For the pulsed amperometric detection of the analytes, the Antec SenCell™ electrochemical flow cell is used. This flow cell [12] 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 45°C to improve detection sensitivity due to the initial low concentration of NaOH used in the gradient program. The cell current was typically 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 benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [13], resulting in less flow cell maintenance and system down time.

Preparation of standards, reagents and samples

Standards: stock standards of the 10 individual sugars were prepared by dissolving a known amount of the individual compounds in deionized (DI) water to a final concentration of 10 mg/mL. Stock standards under these conditions are approximately stable for more than a month in the freezer at -30°C. A combined stock standard solution with a concentration of 1 mg/mL for each carbohydrate was prepared by mixing 100 µL of each individual standards.

Working standards were prepared by serial dilution of the combined stock standards with DI water. The working standards were prepared in the concentration range of 0.1 - 5 µg/mL.

Samples: A urine sample was collected in the morning and processed within 24 hours following standard hygiene precautions. Proper handling of urine is crucial to ensure accurate results. Urine is a metabolic by-product containing a mixture of sugars, proteins, and many other nitrogen-rich compounds. Therefore, in this note urine sample is treated with a modified protein precipitation protocol using acetone and trichloroacetic acid (TCA) before analysis using HPAEC-PAD [14].

The urine samples were prepared as follows: 1 mL of urine sample was mixed with 8 mL of ice-cold acetone. The mixture was stored in –30°C for 1 hours. Afterwards, the mixture was split into 6 Eppendorf tubes in an equal volume, followed by centrifugation for 30 minutes at 6000 rpm. The supernatant was further collected by decantation into a beaker. The beaker was put in a 50°C for 10 minutes to evaporate the remaining acetone. Into the remaining 1 mL sample, 250 µL solution of TCA (1 g/mL) was added, and the mixture was stored in 5°C for 1 hour. Next, the mixture was centrifuged for 30 minutes at 6000 rpm. The supernatant was collected and filtered over a 0.2 µm syringe filter. The supernatant was further diluted 100× and ready for injection. For validation purpose, the sample was also spiked with a known concentration of standards before the dilution step. The final concentration of standards spiked into the sample after dilution is 2.5 µg/mL. As negative control, DI water was subjected to the same sample preparation and injected.

Results

A chromatogram obtained with the 10 µL injection of the 2.5 µg/mL standard mix is depicted in Figure 2. The chromatogram shows good separation of all 10 compounds within 20 minutes. Under this condition, the resolution of all peaks are > 1.5 except for the 3-O-methylglucose and rhamnose pair, which exhibits slight coelution (R = 1.1). Note, that the use of multiple linear gradient steps resulted in a minor baseline drift.

Repeatability

The repeatability of the method was evaluated based on the relative standard deviations (RSDs) of the retention time and peak area. The RSDs were determined by 8 repetitive injections of the 2.5 µg/mL and 0.25 µg/mL standard mix in DI water. The retention times were stable as shown in Table 3, with RSD values ≤ 0.18% for all compounds for both concentrations.

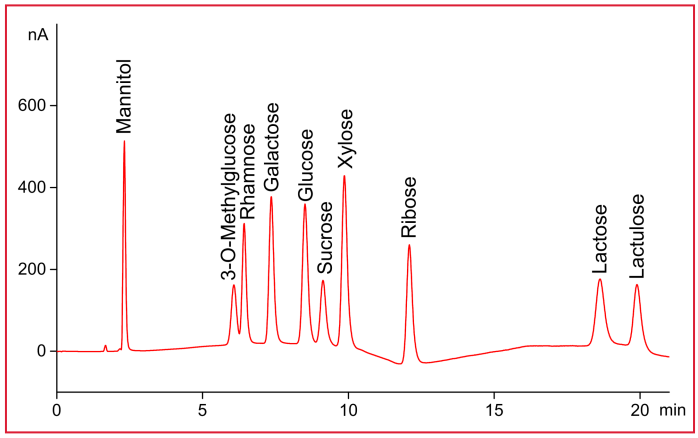

Figure 2. The chromatogram obtained from a 10 µL injection of 2.5 µg/mL standard mix in DI water.

The RSDs for peak areas were generally < 1%. A slightly higher peak area RSD of mannitol (1.19%) in the concentration of 2.5 µg/mL is likely due to the interference from a small, unidentified peak preceding the mannitol peak, which complicates peak integration. At a lower concentration (0.25 µg/mL), this interference was less pronounced, resulting in a significantly improved RSD (0.36%). Similar integration challenges were observed for 3-O-methylglucose (area RSD = 1.01%) and rhamnose (area RSD = 1.07%) in the concentration range of 2.5 µg/mL due to the slight coelution between the two peaks. Lastly, a slightly higher peak area RSD is observed for lactulose peaks at the concentration of 0.25 µg/mL (1.21%) due to baseline drift. Despite the slightly higher RSDs for several compounds, overall these data demonstrate good repeatability for the analysis of the 10 carbohydrates using the presented method.

Table 3

Repeatability of 10 µL injections of standard mix in DI water (n = 8)

	RSDs (%) 2.5 µg/mL		*RSDs (%)* 0.25 µg/mL	
Compound	t_R	*Area*	t_R	*Area*
Mannitol	0.13	1.19	0.17	0.36
3-O-methylglucose	0.09	1.01	0.09	0.64
Rhamnose	0.09	1.07	0.11	0.27
Galactose	0.07	0.63	0.08	0.40
Glucose	0.10	0.75	0.10	0.54
Sucrose	0.18	0.79	0.16	0.49
Xylose	0.09	0.59	0.10	0.71
Ribose	0.07	0.25	0.08	0.72
Lactose	0.09	0.18	0.08	0.49
Lactulose	0.09	0.27	0.07	1.21

Linearity

The linearity was evaluated for all 10 carbohydrate standards in the concentration range of 0.1—5 µg/mL. Excellent linearity was achieved with correlation coefficients based on the peak area better than 0.999 for all analytes. Correlation coefficients for all analytes are shown in Table 4.

Table 4

Linearity of 10 µL injections of standards (0.1—5 µg/mL)

Compound	*Correlation coefficient (r)*
Mannitol	0.9999
3-O-methylglucose	0.9999
Rhamnose	0.9999
Galactose	0.9999
Glucose	0.9999
Sucrose	0.9995
Xylose	0.9999
Ribose	0.9999
Lactose	0.9999
Lactulose	0.9999

Limit of detection (LOD) and limit of quantification (LOQ)

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for all compounds are shown in Table 5. The LODs were calculated as the analyte response corresponding to 3× 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 from t = 45 minutes to t = 50 minutes. The response obtained with the 0.1 µM standard mix was used to calculate the LOD and LOQ. The LOQs were calculated in a similar way as the LODs, based on the 10× S/N ratio. The calculated LODs for all sugars range between 1.2 to 6.3 ng/mL, and the calculated LOQs range between 4 to 21.1 ng/mL, demonstrating the high detection sensitivity of the method.

Sample analysis

An overlay chromatogram of the analyzed samples is shown in Figure 3. DI water was used as a negative control and treated following the same sample preparation protocol. The chromatogram obtained from a negative control exhibited a peak at 1.8 min, which also appeared in the chromatogram of the samples and spiked samples. This peak may be attributed to the residual TCA in the sample. No sugars were detected in the chromatogram of this negative control.

Table 5

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

	Limit of detection		*Limit of quantification*	
Compound	*ng/mL*	*nM*	*ng/mL*	*nM*
Mannitol	1.2	6.6	4.0	22.0
3-O-methylglucose	4.4	22.7	14.7	75.7
Rhamnose	2.1	12.8	7.1	43.3
Galactose	1.7	9.4	5.7	31.6
Glucose	1.8	10.0	6.1	33.9
Sucrose	4.0	26.6	13.3	88.6
Xylose	1.5	4.4	5.0	14.6
Ribose	2.3	15.3	7.7	51.3
Lactose	4.2	12.3	13.9	40.6
Lactulose	6.3	18.4	21.1	61.6

The chromatogram of the urine sample in Figure 3 shows the presence of several sugars. To confirm the peak identities, the urine sample was spiked with known amount of standard mix. Comparison of the non-spiked and the spiked urine sample confirmed the presence of glucose, sucrose, xylose, ribose, and lactulose in the non-spiked urine sample. The presence of the detected sugar is considered normal as they are associated with common dietary source [6].

Quantification of the sugars was done based on the calibration curve. The amount of sugars in the undiluted urine sample is listed in Table 6. Glucose is the most abundant sugar found in this particular urine sample. For non-diabetic person, the glucose level found in urine is normally below 1 g/L. Lactulose is the second most abundant sugar found in the sample, followed by sucrose, xylose, and ribose. The amount of these sugars in urine may vary depending on the diet and the health condition.

The sample recovery is calculated based on the average amount of the analytes in the sample, spiked sample, and the amount of standard mix added to the spiked sample.

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

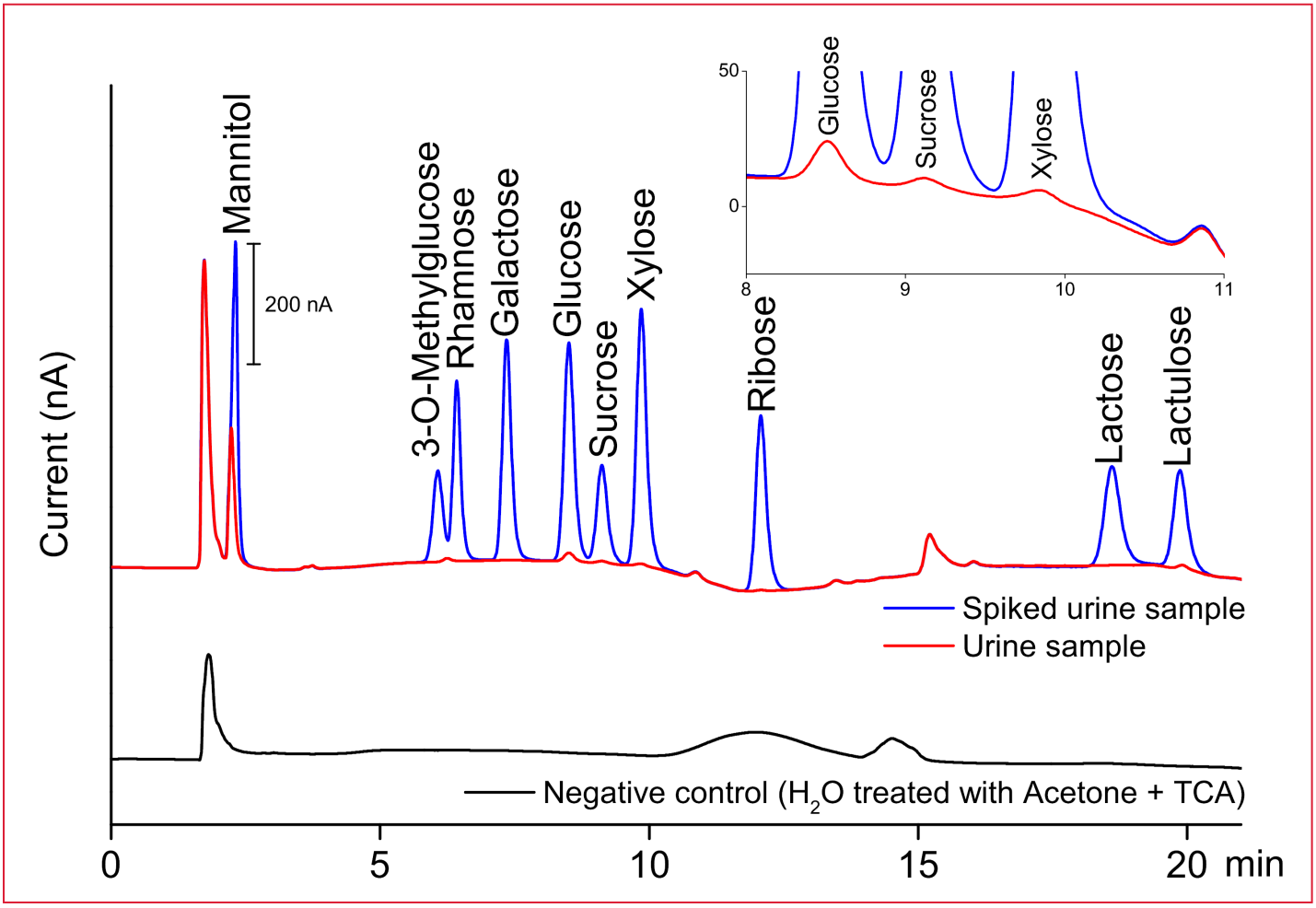

Figure 3. Overlay chromatogram obtained from a 10 µL injection of a negative control (H2O treated with acetone + TCA, black trace), urine sample (100x diluted, red trace), and spiked urine sample (100x diluted, the final spike concentration is 2.5 µg/mL, blue trace). Inset shows a zoom-in overlay chromatogram of the urine sample and spiked urine sample from t = 8 min to t = 11 min.

Table 6

Average carbohydrates content and sample recovery in urine sample (n = 3)

Compounds	*Amount in urine sample (µg/mL)*	*Recovery (%)*
Mannitol	n.d.	102.9
3-O-methylglucose	n.d.	103.8
Rhamnose	n.d.	103.1
Galactose	n.d.	102.1
Glucose	12.2	102.2
Sucrose	8.4	100.2
Xylose	5.9	102.2
Ribose	1.0	101.2
Lactose	n.d.	100.0
Lactulose	10.7	100.2

*n.d. = not detected

The sample recoveries are also listed in the Table 6. The sample recoveries found for all samples ranged between 100% - 105%, indicating excellent recoveries for all compounds analyzed in urine using this presented method. Lastly, the spiked sample chromatogram shows that sugars in the treated urine matrix can be detected and quantified accurately.

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Conclusion

The ALEXYS™ Carbohydrate Analyzer in combination with the new SweetSep™ AEX20 provides a tailored solution for the trace analysis of carbohydrates in various matrices. In this application note a mix of 10 carbohydrates were evaluated which are used as intestinal permeability probes or which might be present as endogenous carbohydrates in urine. The 10 carbohydrates were successfully separated and eluted within 20 minutes on the SweetSep™ AEX20. The method demonstrates excellent linearity, good repeatability and high detection sensitivity, as evidenced by the low Limit of Detection (LOD) for all 10 carbohydrates. The method applicability is demonstrated by successful analysis of a human urine sample using the presented method.

Ordering information

Detector only	
176.0035B	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Column	
260.0020	SweetSep™ AEX20, 4 x 200 mm column, 5 µm
260.0025	SweetSep™ AEX20, 4 x 50 mm precolumn, 5 µm
260.0030	Borate ion trap, 4 x 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 not for use in diagnostic procedures. 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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