Short application note

Biomass

High-throughput neutral sugars & uronic acids in biomass

Keywords: ALEXYS™ High-Throughput Carbohydrate Analyzer, SweetSep™ AEX20, HPAEC-PAD, biomass, seaweed, wood, chitin

Introduction

Lignocellulosic and marine biomasses are key renewable resources. Biomass utilization requires understanding of their carbohydrate content. However, analyzing neutral sugars and uronic acids in biomass hydrolysates is challenging due to the complex mixture of similar sugars, epimers, and co-extracted substances like organic acids and phenolics that can interfere with analysis.

High Performance Anion-Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD) is a widely used technique for carbohydrate analysis. It offers selective separation of carbohydrate isomers without the need for time-consuming sample derivatization steps. Typically, HPAEC-PAD method involves elution steps followed by column rinsing and equilibration (reconditioning). As a result, these column reconditioning steps increase the overall cycle time per analysis and limit sample throughput in laboratories that handle large numbers of samples.

This application note describes a new method using an ALEXYS High-throughput Carbohydrate Analyzer. The configuration allows two gradient pumps and two sets of columns to operate in parallel [1, 2], enabling analytical separation on one column while the second column undergoes reconditioning. The automated column switching ensures that each injection is directed to a fully reconditioned

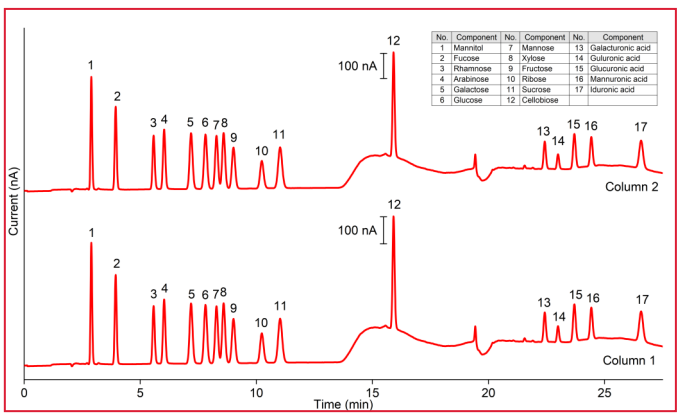

Fig. 1. Example chromatograms of each column injecting 2.5 µL of a 10 µM standard mix containing neutral sugars and uronic acids in DI water. Separation and detection was achieved using the HPAEC-PAD conditions and gradient program shown in Table 1 and 2, respectively.

Fig. 2. ALEXYS Carbohydrate Analyzer.

Table 1. HPAEC-PAD conditions

HPLC	ALEXYS™ High-throughput Carbohydrate Analyzer (Antec Scientific)
Columns	2× SweetSep™AEX20, 2.1 x 50mm precolumn, 5µm 2× SweetSep™AEX20, 2.1 x 200 mm column, 5µm 2× Borate ion trap, 2.1 x 50 mm column, 10 µm (all columns Antec Scientific)
Mobile phase	A: DI water B: 100 mM NaOH C: 100 mM NaOH + 500 mM NaOAc Eluents blanketed with Nitrogen 5.0
Flow rate	0.18 mL/min
Backpressure	About 240 bar
Injection volume	2.5 µL
Temperature	5°C for sample cooling (AS6.1L), 20°C for separation (CT2.1) and 45°C for detection (DECADE Elite)
Flow cell	SenCell Au WE, HyREF Pd 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	10 µA/V
I-cell	About 0 - 0.2 µA
ADF	0.05 Hz

column. This method ultimately increases sample throughput without compromising separation quality, sensitivity, or data accuracy, providing an efficient solution for high-throughput carbohydrate analysis of biomass hydrolysates.

Method

The high-throughput HPAEC-PAD conditions and gradient program for the analysis of neutral sugars and uronic acids are listed in Tables 1 and 2, respectively. The analysis was performed using the ALEXYS High-throughput Carbohydrate Analyzer (Fig. 2), a dedicated HPAEC-PAD system with a metal-free flow path optimized for the sensitive analysis of carbohydrates. The system consists of the ET210 eluent tray, two P6.1L quaternary LPG pumps, an AS6.1L autosampler, a CT2.1 column thermostat, a column switching valve, a DECADE Elite electrochemical detector, and a SenCell flow cell. The ET210 eluent tray has an integrated gas distribution system that blankets the eluents in the bottles with an inert gas (He or N_2) to prevent CO_2 diffusion into the eluents and minimize carbonate ion formation, ensuring reproducible analysis. Two sets of 2.1 × 200 mm Antec Scientific SweetSep^{TM}AEX20 analytical column, in combination with the corresponding precolumn (2.1 × 50 mm), were used for separation. The 2.1 mm ID microbore columns are operated at a low flow rate of 0.18 mL/min, minimizing mobile-phase consumption and waste and thereby reducing environmental impact.

As a precaution a 2.1 x 50 mm borate ion trap was installed in the solvent line between pump and injector to eliminate the presence of borate contaminants in the mobile phase. Borate ions can form complexes with some carbohydrates causing peak tailing and thus loss of peak symmetry.

A temperature of 20 °C was selected for the optimal separation of all target neutral sugars and uronic acids in the sample. The detection temperature was set to 45 °C to assure optimum detection sensitivity. The chromatographic system was configured according to application note 220_043 [1]. Data acquisition, processing, and instrument control were performed using DataApex Clarity Chromatography Software version 10.1.

Table 2. Gradient program

Analytical pump

Time (min)	*Mobile phase*	*%A*	*%B*	*%C*	*Description*
0—10	20 mM NaOH	90	10	0	Elution of neutral sugars
10—14	160 mM NaOH	20	80	0	Elution of cellobiose
14—24	40 mM NaOH, 140 mM NaOAc	66	6	28	Elution of uronic acids
24—33	20 mM NaOH	90	10	0	Flow path clean-up
32.98	Valve switches positions				

Rinsing pump (column reconditioning)

Time (min)	*Mobile phase*	*%A*	*%B*	*%C*	*Description*
0	20 mM NaOH	90	10	0	Initial conditions
0.02—5	50 mM NaOH, 250 mM NaOAc	50	0	50	Column clean-up
5—18	200 mM NaOH	0	100	0	Conversion to hydroxide form
18—33	20 mM NaOH	90	10	0	Equilibration to starting conditions

Sample preparation

A total of 8 biomass hydrolysate samples, consisting of seaweed, pulp, wood, and chitin samples, were analyzed. Pre-hydrolyzed biomass samples were kindly provided by several undisclosed collaborators. Hydrolysis was performed by either sulfuric acid or enzymatic treatment [3–6]. Sample-specific information is provided in the application notes 220_035 [7] and 220_037 [8]. No other sample preparation was performed, except for dilution with DI water to ensure the responses were within the calibration range followed by sample filtration with a 0.22 µm polyethersulfone (PES) syringe filter to the vials.

Results

Figure 1 shows an stacked overlay of two chromatograms, obtained from a 2.5 µL injection of a 10 µM standard mix containing neutral sugars and uronic acids on two column sets. The chromatograms demonstrate baseline separation for all components with a resolution (Rs) greater than 1.5, except for the mannose-xylose pair (Rs = 1.1). All neutral sugars eluted within 12 minutes, except for cellobiose, which eluted at 16 minutes. Uronic acids eluted within 27 minutes under the given conditions (Table 2) on both column sets. Before switching the fully reconditioned and equilibrated column into the analytical section of the system, the analytical flow path needs to be sufficiently flushed with the starting eluent (Table 2). This method ensures reproducible conditions and takes 33 minutes to complete, which is nearly half the time of a single-column approach [7, 8]. This results in a more than 80% increase in sample throughput.

The LOD was determined as the analyte response corresponding to 3 times the signal-to-noise (S/N) ratio, with noise measured in accordance with ASTM standards (average peak-to-peak baseline noise of segments of 0.5 minute). The noise was determined using a 2-minute section of the baseline of the 1 µM standard injection between t=11.5 min to 13.5 min. Detection limits for neutral sugars vary from 0.02

to 0.09 µM (equivalent to 3-22 µg/L) across both columns. Uronic acids have slightly higher detection limits, ranging from 0.06 to 0.32 µM (corresponding to 11-62 µg/L).

The method's repeatability was tested with 10 consecutive injections of standards at 1 µM, 10 µM, and 100 µM in DI water. Excellent repeatability was achieved with retention time RSDs below 0.5% at the highest concentration and below 1.2% at the lowest concentration. Peak area RSDs were under 2.0% at 100 µM and under 5.8% for the 1 µM standard. Calibration was performed over the 1-100 µM range using 9 calibration levels for all analytes. The smallest relative standard errors (RSE [9]), between 0.7% and 7.5%, were achieved with a weighted ($1/x^2$) quadratic calibration curve, ignoring the origin. The average RSE was 1.8%, and the corresponding median value (1.7%) indicates high precision and reliability of the analytical method.

Sample analysis

A total of 8 hydrolyzed biomass samples were analyzed using the method presented. Example chromatograms of the wood and seaweed samples are shown in Figure 3. To ensure correct peak identification, the samples were spiked with the standard mix to a final concentration of 10 µM. Sample recoveries after spiking ranged from 88% to 113%, with an average of 99%.

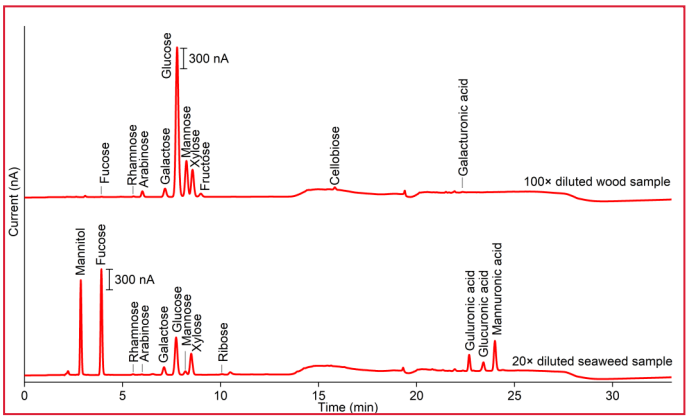

Fig. 3. Overlay of chromatograms obtained from 2.5 µL injections of the wood (100-fold diluted, top) and the seaweed hydrolysate (20-fold diluted, bottom).

Targeted analysis

If the analytical interest is only on neutral sugars or only on uronic acids, the method can be adapted to achieve faster cycle times. Figures 4 and 5 show that high-throughput separation of neutral sugars and uronic acids is achieved in 23 and 11 min, respectively, representing a 300% to nearly 500% increase in sample throughput compared to the conventional setup, which has a cycle time of about 60 min.

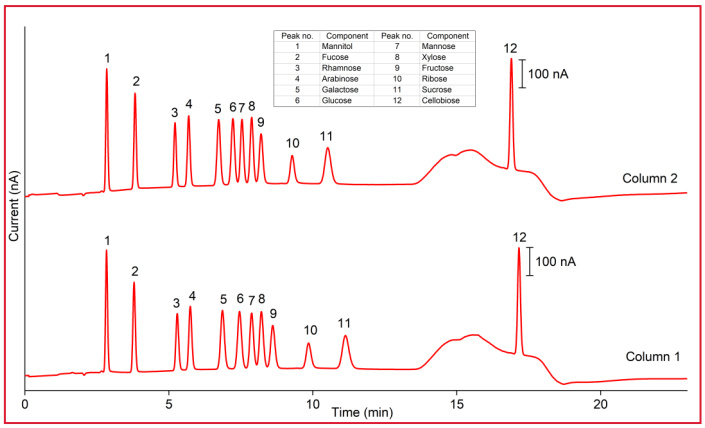

Fig. 4. Chromatograms of each column obtained from a 2.5 µL injection of 10 µM standard mix containing neutral sugars in DI water.

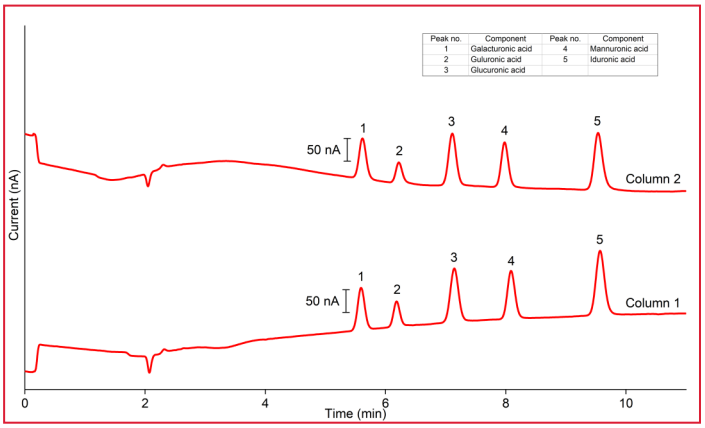

Fig. 5. Chromatograms of each column obtained from a 2.5 µL injection of 10 µM standard mix containing uronic acids in DI water.

This work is also published in the Journal of Chromatography A [10]. Contact Antec Scientific for more information about the experimental conditions.

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Conclusion

The ALEXYS High-throughput Carbohydrate Analyzer enables reliable separation and quantification of neutral sugars and uronic acids in diverse biomass hydrolysates at a cycle time of 33 minutes, corresponding to more than 80% faster than a conventional single-column setup without compromising analytical performance. The method showed excellent repeatability, detection limits down to 0.02 µM, and spike recoveries averaging 99%, demonstrating its accuracy and reliability for biomass carbohydrate analysis. For targeted analyses, cycle times as short as 11 minutes were achieved, making the approach well suited for high-throughput biomass characterization.

Ordering information

ALEXYS analyzer	
180.0059WA	ALEXYS High-throughput Carbohydrate Analyzer
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Columns	
260.0021	SweetSep™ AEX20, 2.1 x 200 mm column, 5 µm (2×)
260.0026	SweetSep™ AEX20, 2.1 x 50 mm precolumn, 5 µm (2×)
260.0031	Borate ion trap, 2.1 x 50 mm column, 10 µm (2×)
Software*	
195.0035	Clarity CDS single instr. incl. LC, AS module

*) The ALEXYS High-throughput Carbohydrate Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS and Agilent OpenLab CDS. Please contact Antec Scientific for more details.

Reagents, standards and sample prep accessories

NaOH (50% w/w), certified	Fisher Scientific, pn* SS254-500
Sodium acetate (NaOAc), 100%	Sigma Aldrich, pn 79714
DI water 18.2 MΩ·cm, TOC < 5 ppb	YoungIn Chromass Aquapuri Essence+ 393
Galactose	Sigma Aldrich, pn G0750
Fructose	Sigma Aldrich, pn F0127
Glucose	Sigma Aldrich, pn G8270
Sucrose	Sigma Aldrich, pn S9378
Cellobiose	BioSynth, pn OCO4040
Mannitol	Sigma Aldrich, pn 3340-100G
Fucose	Sigma Aldrich, pn F2252-5G
Arabinose	Sigma Aldrich, pn A3131
Rhamnose	Sigma Aldrich, pn W373011
Xylose	Sigma Aldrich, pn X1500
Mannose	Sigma Aldrich, pn M4625
Ribose	Sigma Aldrich, pn R7500
Mannuronic acid	Sigma Aldrich, pn SMB00280-10mg
Galacturonic acid	Merck, pn 48280
Glucuronic acid	Sigma Aldrich, pn G5269-10G
Guluronic acid	BioSynth, MG182938
Iduronic acid	BioSynth, MI08102
Syringe filter	0.22 µm PES (Polyethersulfone) 25 mm Ø FFL/MLS
Nitrogen 5.0 (purity 99.999%)	Messer Netherlands, pn 100542102

*) Part number (pn)

For research purpose only. The information shown in this short application note 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 and may be adjusted accordingly. Specifications mentioned are subject to change without further notice.

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