



The finest HPAEC-PAD applications for carbohydrate analysis

Food and Beverage

Mono- and disaccharides
Sugars in meat & fish
Carbohydrates in food according to AOAC
Carbohydrates in instant coffee
Carbohydrates in Dutch candy
Carbohydrates in honey
Oligo- and Polysaccharides in honey

Prebiotics Food Additives

Analysis of Maltodextrin in Syrups
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

Biomass

Uronic acids
Neutral sugars

Uronic acids & neutral sugars in seaweed hydrolysates

- **ALEXYS™ Carbohydrate Analyzer**
- **SweetSep™ AEX20 anion-exchange column**
- **Compositional analysis of biomass polysaccharides**
- **Brown seaweed - *Ascophyllum nodosum***

Summary

Biomass is organic material, originating from for example plants, wood, and agricultural waste, that can be used as a renewable energy source or raw materials in various industrial processes [1]. Among various biomass sources, seaweed has emerged as a promising biomass source due to its rapid growth, high carbohydrate content, and minimal need for land and freshwater resources. Sugars in biomass need to be extracted, which is followed by conversion to the final product [2, 6, 7]. To determine the quality efficiency of the extraction of the carbohydrates from biomass, a sensitive analysis method is required to quantify the amount and composition of sugars in biomass hydrolysates.

In this application note, a HPAEC-PAD method is presented for the compositional analysis of neutral sugars and uronic acids in biomass hydrolysates, using the ALEXYS™ Carbohydrate Analyzer in combination with the new SweetSep AEX20 anion-exchange column. The method enables fast and sensitive analysis of neutral sugars and uronic acids in one single run. To demonstrate the applicability of the method one hydrolyzed brown seaweed sample from *Ascophyllum nodosum* was analyzed.



Introduction

The decrease of fossil resources and growing environmental challenges, such as climate change caused by greenhouse gas emission from the consumption and production of fossil-based fuels and products, increase the need to use plant biomass [3]. Plant biomass, mostly consist of lignocellulose and is a viable alternative feedstock for the production of the historically presented six 'Fs': food, feed, fuel, feedstock, fiber and fertilizer [4]. Seaweed, grasses, crops, and trees are reliable plant biomasses and are produced via photosynthesis. Biomass is considered to be carbon neutral source [3].

Seaweed has gained importance as a promising feedstock for production of food, feed, fuel, and biomaterials which is growing worldwide [5, 10]. Seaweed has a potential environmental benefit, which is that large scale farming of seaweed, does not compete with the agricultural crops for water, land and agricultural inputs (pesticides, fertilizers, etc.). Besides, seaweed has a higher growth rate and higher polysaccharide content than land crops [2, 6, 7].

The main components of lignocellulose are the polysaccharides hemicellulose & cellulose and lignin, a phenolic macro-molecule. Depending on the biomass species, the three major components in biomass are divided by 30 - 50% cellulose, 20 - 40% hemicellulose, and 10 - 35% lignin [3]. When hydrolyzed, hemicellulose can be broken down into neutral sugars such as xylose, glucose, mannose, galactose, arabinose and uronic acids like galacturonic and glucuronic acids [8]. Uronic acids, a type of sugar acid, play an important role in various biological

systems, such as contributing to the structural integrity and elasticity of connective tissues [9]. Brown seaweed does not consist of hemicellulose, but consist of cellulose, alginate and fucoidan. Alginate is an anionic linear polysaccharide and is composed of the two uronic acids: mannuronic acid and guluronic acid. Fucoidan is a sulphated fucose-based heteropolymer, which is composed of neutral sugars like, fucose, xylose, galactose etc. and uronic acids [10,11]. To determine the amount and composition of these sugars in the heteropolysaccharides extracted from biomass a selective and sensitive analysis method is required.

In this application note, a HPAEC-PAD method is presented for the compositional analysis of neutral sugars and uronic acids in biomass hydrolysates, using the ALEXYS™ Carbohydrate Analyzer in combination with the new SweetSep AEX20 anion-exchange column.

Method

The analysis was performed using the ALEXYS™ Carbohydrate Analyzer (Figure 1) equipped with the DECADE Elite electrochemical detector. The SenCell™ with Au working electrode and HyREF (Pd/H₂) reference electrode was selected for sensitive detection of the sugars and uronic acids. The method conditions are specified in table 1.

Table 1

LC-ECD conditions

HPLC	ALEXYS™ carbohydrate analyzer
Columns	SweetSep™ AEX20, 4 × 200 mm column, 5 μm SweetSep™ AEX20, 4 × 50 mm precolumn, 5 μm Borate ion trap, 4 x 50 mm column, 10 μm (Antec Scientific)
Mobile phase	A: 10 mM NaOH B: DI water C: 100 mM NaOH D: 100 mM NaOH + 100 mM NaOAc Eluents prepared & blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
System backpressure	About 240 bar
Temperature	25 °C for separation and 45 °C detection
Injection volume	10 μL
Pump piston wash	DI water (refresh weekly)
Flow cell	SenCell™ with 2 mm Au and HyREF (Pd/H ₂), AST position 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
ADF	0.05 Hz
I-cell	About 0.3- 0.6 μA



Figure 1. ALEXYS Carbohydrate Analyzer consisting of the ET 210 eluent tray (for N₂ blanketing), a P 6.1L quaternary LPG pump, AS 6.1L autosampler, CT 2.1 column thermostat, and the DECADE Elite electrochemical detector.



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. Due to the extreme alkaline conditions, only polymeric anion-exchange columns are suitable. The retention time of carbohydrates is inversely correlated with pKa value and increases significantly with molecular weight.

Table 2

Step- gradient program

Time (min)	Mobile phase composition	%A	%B	%C	%D	Description
0 - 11	3 mM NaOH	30	70	00	00	Elution of neutral sugars
11 - 19	70 mM NaOH	00	30	70	00	Elution of cellobiose
19 - 34	70 mM NaOH, 70 mM NaOAc	00	00	00	100	Elution of uronic acids & column clean-up/regeneration
34 - 68	3 mM NaOH	30	70	00	00	Equilibration to the starting condition

The separation of the neutral sugars & uronic acids was performed using SweetSep™ AEX20 columns (4 × 200 mm analytical column and 4 × 50 mm precolumn). The novel AEX20 stationary phase is based on a highly monodisperse 5 μm resin coated with nanoparticles functionalized with dual ion exchange sites (quaternary amine + tertiary amine). The resin is optimized for high-resolution separation of monosaccharides. In carbohydrate analysis, the peak shape of certain sugars, such as mannose, sugar alcohols and fructose, are deteriorated

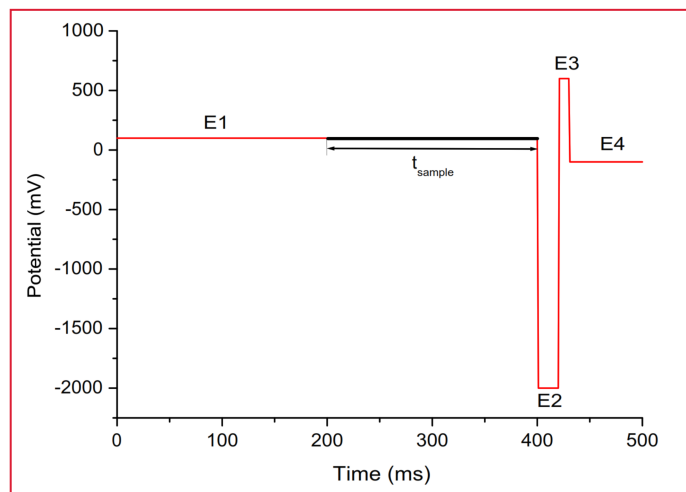


Figure 2. 4-step PAD potential waveform for the detection of carbohydrates.

when traces of borate are present in the mobile phase. Therefore, a borate ion trap column (4 × 50 mm) was installed in the solvent line between the pump and autosampler as a precaution to eliminate borate ions which might be present in the mobile phase.

The analysis is based on isocratic elution of the neutral sugars using 3 mM NaOH, combined with a dual step gradient of 70 mM NaOH (start t = 11 min) and 70 mM NaOH + NaOAc (start t = 19 min), to elute cellobiose and the uronic acids, respectively. Cellobiose and the uronic acids are more strongly retained on the column and require a higher NaOH concentration and sodium acetate as a modifier (uronic acids). See Table 2 for the details of the multi-step gradient profile. In case that samples contain strongly retained high molecular

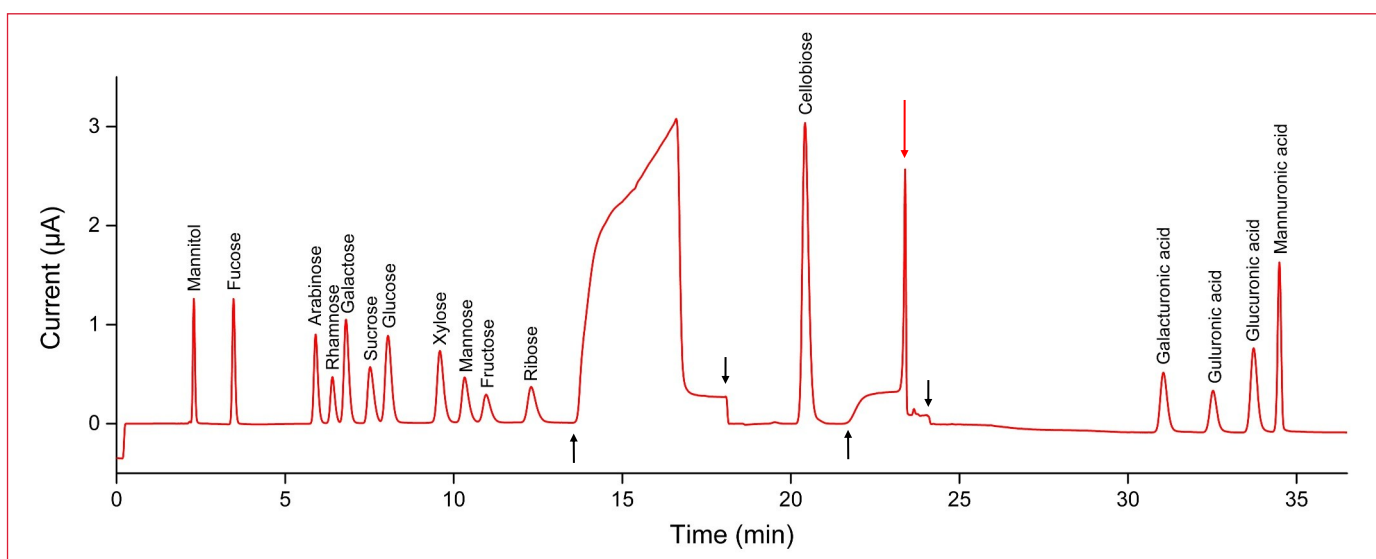


Figure 3. The chromatogram obtained from a 10 μL injection of the 100 μM sugar standard mix in DI water. The black ↑ arrows in the chromatogram indicate the start of a baseline elevation due to the changes in mobile phase composition (step gradient). Black ↓ arrows indicate an Autozero to remove the baseline current offset. The red arrow indicates a sharp OH⁻ peak due to displacement of a narrow zone of hydroxide ions from the column by the eluent containing acetate.



Neutral sugars & uronic acids in seaweed

weight polysaccharides or other contaminants a stronger wash step (100 mM NaOH + NaOAc, or higher concentration) might be necessary after elution of the uronic acids. The temperature for separation was set to 25°C

To minimize the introduction of carbonate ions (CO_3^{2-}) 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 type I DI water (resistivity >18.2 M Ω -cm, TOC < 5 ppb), which was sonicated and sparged with nitrogen 5.0 (purity 99.999%) prior to use. During analysis the ET210 eluent tray is used to pressurize the head space above the mobile phase with nitrogen gas (0.2–0.4 bar). The inert gas atmosphere will minimize the introduction of CO_2 in the mobile phase and the subsequent formation of CO_3^{2-} in the mobile phase and to ensure reproducible analysis.

Detection

For the PAD detection of the neutral sugars and uronic acids the Antec SenCell™ electrochemical flow cell (Figure 1) is used [12]. This flow cell with wall-jet design consists of a Au working electrode, palladium hydrogen (HyREF) reference electrode, and stainless steel auxiliary electrode. A 4-step potential

waveform is used on the Au working electrode, see Table 1 and Figure 2. The cell current was typically about 0.3 - 0.6 μA using these PAD settings under the specified method conditions. This particular 4-step waveform with a pulse duration of 500 ms has several benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [13]. The temperature for detection was set to 45°C.

Sample preparation

Standards: 10 mM stock standards of the individual sugars were prepared in 95/5 (v/v%) water/acetonitrile to suppress bacterial and fungal growth and to prevent fast degradation. Stock standards under these conditions are approximately stable for more than a month in the fridge at 4°C. Working standards in the concentration range of 1 - 100 μM were prepared, which corresponds to 0.2 - 14.6 $\mu\text{g}/\text{mL}$ for mannitol, 0.2 - 13.1 $\mu\text{g}/\text{mL}$ for fucose and rhamnose, 0.2 - 12.0 $\mu\text{g}/\text{mL}$ for arabinose, xylose, and ribose, 0.2 - 14.4 $\mu\text{g}/\text{mL}$ for galactose, glucose, mannose, and fructose, 0.3 - 27.4 $\mu\text{g}/\text{mL}$ for sucrose and cellobioses and 0.2 - 15.5 $\mu\text{g}/\text{mL}$ for the four uronic acids. These standards were prepared by serial dilution of the stock standards with DI water.

Table 3

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

Compound	RSD (%)	
	t_R	Area
Mannitol	0.38	1.85
Fucose	0.18	1.44
Arabinose	0.20	1.52
Rhamnose	0.18	1.63
Galactose	0.14	1.34
Sucrose	0.23	1.94
Glucose	0.10	1.47
Xylose	0.15	1.53
Mannose	0.15	1.72
Fructose	0.11	1.85
Ribose	0.14	1.54
Cellobiose	0.04	0.93
Galacturonic acid	0.10	1.86
Guluronic acid	0.10	1.77
Glucuronic acid	0.10	1.80
Mannuronic acid	0.09	1.50

Table 4

Calculated Limit of Detection (LOD) and Limit of Quantification (LOQ)

Compound	LOD		LOQ
	nmol/L	ng/mL	ng/mL
Mannitol	41	8	25
Fucose	51	8	28
Arabinose	79	12	40
Rhamnose	150	25	82
Galactose	68	12	41
Sucrose	98	33	112
Glucose	82	15	49
Xylose	96	14	48
Mannose	160	29	96
Fructose	278	50	167
Ribose	199	30	100
Cellobiose	210	72	240
Galacturonic acid	1188	231	769
Guluronic acid	1697	329	1098
Glucuronic acid	808	157	523
Mannuronic acid	225	44	146

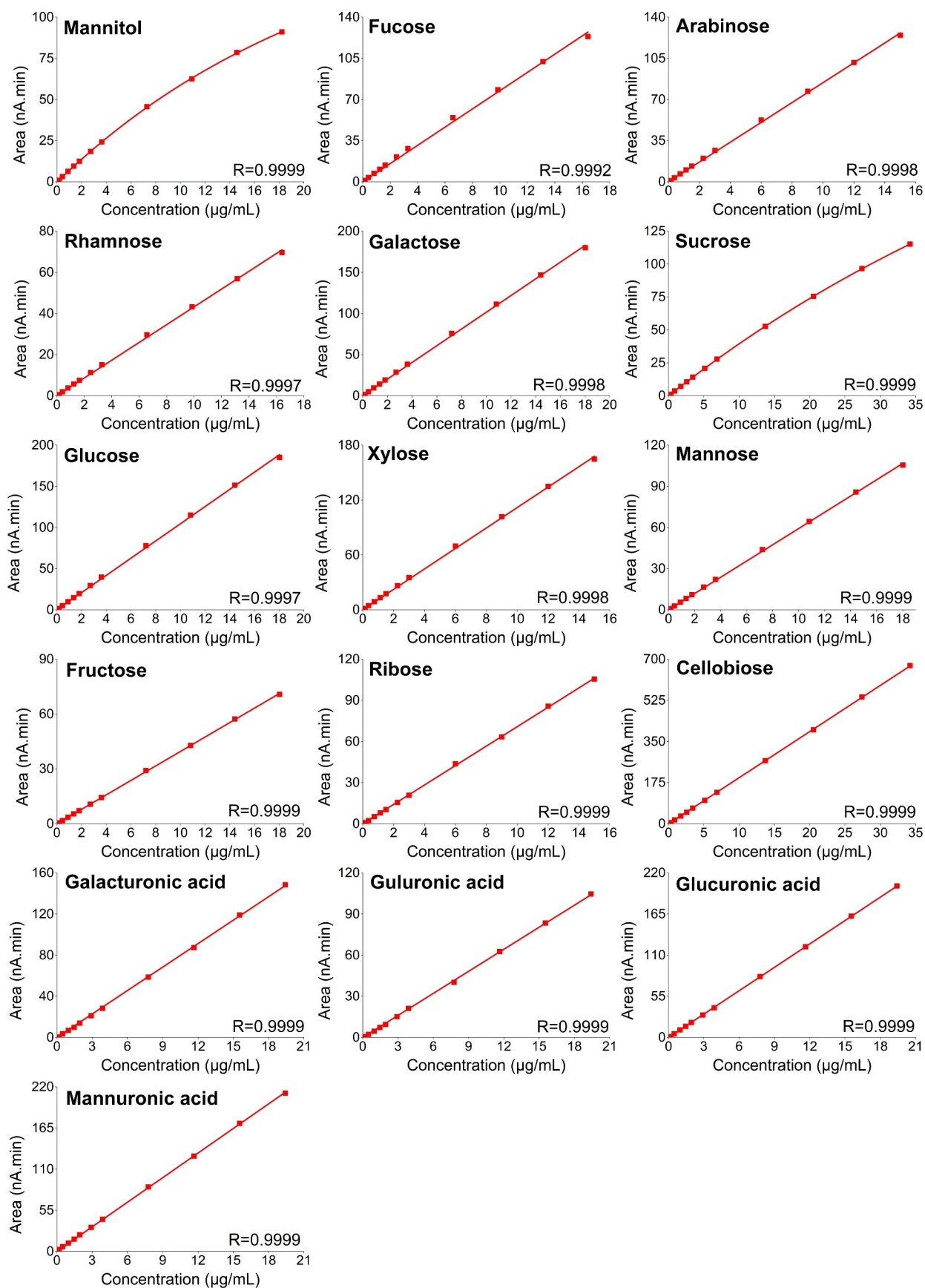


Figure 4. Calibration curves of the 12 neutral sugars and 4 uronic acids in the range of 0.2 - 18.2 µg/mL for mannitol, 0.2 - 16.4 µg/mL for fucose and rhamnose, 0.2 - 15.0 µg/mL for arabinose, xylose, and ribose, 0.2 - 18.0 mg/mL for galactose, glucose, mannose, and fructose, 0.3 - 34.2 µg/mL for sucrose and cellobioses, 0.2 - 19.4 µg/mL for galacturonic acid, gularonic acid, glucuronic acid and mannuronic acid. The fitted lines are extrapolated to the origin except for mannuronic acid. For all compounds (except mannitol and sucrose) a linear fit gave a good correlation ($r > 0.999$), for mannitol and sucrose a quadratic fit was applied.



Neutral sugars & uronic acids in seaweed

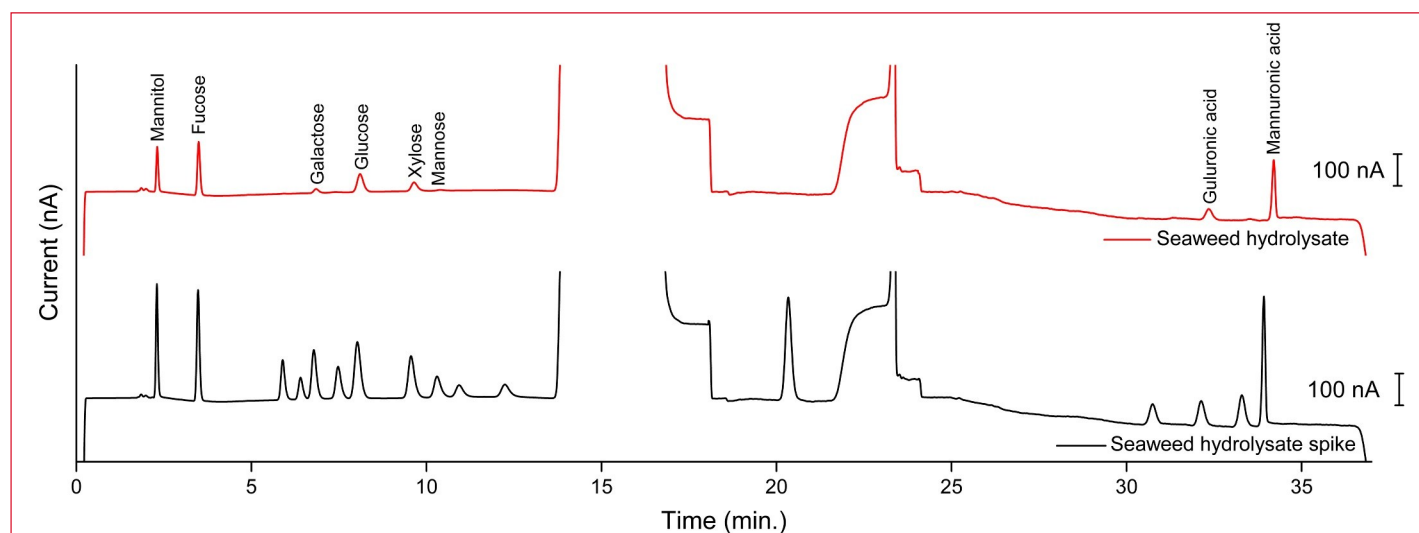


Figure 5. Chromatograms obtained from a 10 μL injection of the 100x diluted seaweed hydrolysate (red trace) and spiked 100x diluted seaweed hydrolysate spike (black trace).

Sample: The hydrolyzed brown seaweed sample (*Ascophyllum nodosum*) was kindly provided by Wageningen University & Research (WUR). No detailed sample preparation info was provided but it is assumed that the hydrolysis was performed with a similar protocol as described in reference [10]. The main components expected in the hydrolyzed seaweed sample are mannuronic acid, guluronic acid, glucose, fucose and mannitol (info obtained from WUR).

Results

A chromatogram of a 10 μL injection of the 100 μM sugar standard mix in DI water is shown in Figure 3. The sugar standard mix consist of 12 neutral sugars: mannitol, fucose, arabinose, rhamnose, galactose, sucrose, glucose, xylose, mannose, fructose, ribose and cellobiose and 4 uronic acids: galacturonic acid, guluronic acid, glucuronic acid and mannuronic acid. All neutral sugars and uronic acid were eluted within 35 minutes. Under these conditions all neutral sugars and uronic acids are baseline separated (baseline resolution > 1.5). It should be note that for mannuronic acid significant peak sharpening is observed under these step-gradient elution conditions.

Linearity

The linearity was investigated in the concentration range of 1-100 μM for all 16 compounds. 1-100 μM corresponds to 0.2 - 14.6 $\mu\text{g}/\text{mL}$ for mannitol, 0.2 - 13.1 $\mu\text{g}/\text{mL}$ for fucose and rhamnose, 0.2 - 12.0 $\mu\text{g}/\text{mL}$ for arabinose, xylose, and ribose, 0.2 - 14.4 $\mu\text{g}/\text{mL}$ for galactose, glucose, mannose, and fructose, 0.3 - 27.4 $\mu\text{g}/\text{mL}$ for sucrose and cellobioses and 0.2 - 15.5 $\mu\text{g}/\text{mL}$ for the four uronic acids. The calibration curves of all 16 compounds are shown in Figure 4. For 14 of the 16 sugars the linearity was excellent with correlation coefficients $r > 0.999$.

For mannitol and sucrose the linearity was slightly worse at higher concentrations ($r = 9955$ and 0.9983 , respectively), and therefore a quadratic fit was applied. The calibration curves in Figure 4 are used for the actual quantification of samples. It was observed that a very small interference was coeluting with the mannuronic acid peak. This small unidentified peak was also observed in the blank, its response is small but constant from run-to-run, therefore it is assumed that it has a neglectable effect on quantification of mannuronic acid.

Repeatability

The relative standard deviations (RSDs) of the retention time and peak area were determined for 10 repetitive injections of the 10 μM standard mix in DI water. The results are listed in Table 4. The retention times were stable, with RSD values < 0.4 % for all sugars. The RSD for peak areas for all sugars was < 2%.

Limit of detection (LOD)

The limit of detection (LOD) and limit of quantification (LOQ) for all sugars are shown in Table 4. The LODs were calculated as the analyte response corresponding to 3x the ASTM noise (average peak-to-peak baseline noise of 20 segments of 0.5 min). The noise was calculated based on a 10 minute section of the baseline at 3 mM NaOH, 70 mM NaOH, and 70 mM NaOH + 70 mM NaOAc. The LOQs were calculated in a similar way to LODs, with a 10x S/N ratio. The response of an injection obtained with the 1 μM standard mix in DI water was used to calculate the LODs and LOQs for all sugars. The calculated LODs ranged from about 10 - 70 ng/mL for the neutral sugars up to about 330 ng/mL for Guluronic acid.



Sample analysis

The hydrolyzed brown seaweed sample (*Ascophyllum nodosum*) was diluted 100x and filtered over a 0.2 µm polyethersulfone (PES) syringe filter before injection. To ensure correct peak identification, the sample was spiked with the standard mix. The spike concentration was 10 µM for all components in the final sample.

Table 5

Neutral sugars & uronic acid content in seaweed hydrolysate

Compound	Seaweed hydrolysate (mg/mL)
Mannitol	0.12
Fucose	0.16
Arabinose	-
Rhamnose	-
Galactose	0.02
Sucrose	-
Glucose	0.10
Xylose	0.04
Mannose	0.01
Fructose	-
Ribose	-
Cellobiose	-
Galacturonic acid	-
Guluronic acid	0.14
Glucuronic acid	-
Mannuronic acid	0.18

The chromatograms of the sample and spiked sample are shown in Figure 5. The contents of neutral sugars and uronic acids in the seaweed sample were calculated based on the calibration curves of the standards (1-100 µM) shown in fig 4. The calculated amounts of neutral sugars and uronic acid are listed in Table 5. Furthermore, the content was also calculated using the standard addition method (single-point calibration) based on the spiked sample. There was a good correlation between the values in table 5 and the content calculated using the standard addition method (data not shown). The main components found in the sample are mannitol, fucose, glucose, guluronic acid and mannuronic acid, which corresponds with the information provided by the supplier of the sample. In addition some small amounts of other monosaccharides were found (galactose, xylose and mannose)

in the seaweed hydrolysate.

Point of attention: for neutral sugars which are separated at low sodium hydroxide concentrations small changes in the mobile phase composition can lead to significant shifts in retention times. The sensitivity of neutral sugars to small variations in the mobile phase composition therefore might require some tweaking of the method. Careful mobile phase preparation minimizes peak shifts and contributes to the robustness of chromatographic analysis.

Uronic acids only

When the focus is on the analysis of uronic acids only, it is possible to perform target analysis using isocratic separation using the conditions specified in table 2 (70 mM NaOH, 70 mM NaOAc). See figure 6 for an example chromatogram. The large front peak is due to the neutral sugars present in the standard

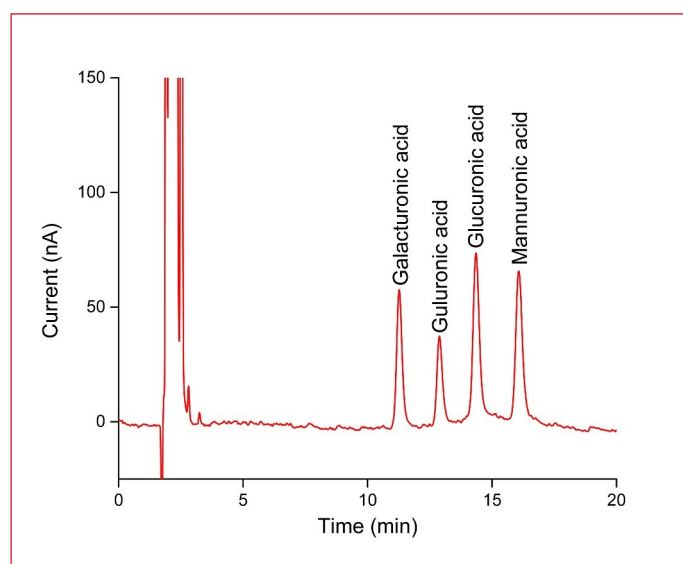


Figure 6. Chromatograms obtained from a 10 µL injection of a 10 µM standard mix of neutral sugars and uronic acids in DI water.

Table 6

Calculated Limit of Detection (LOD) and Limit of Quantification (LOQ) for the target analysis of the uronic acids

Compound	LOD		LOQ
	nmol/L	ng/mL	ng/mL
Galacturonic acid	489	95	316
Guluronic acid	769	149	497
Glucuronic acid	415	80	268
Mannuronic acid	461	89	298



mix which elute in the front under these conditions. The LODs for the uronic acids were calculated based on the signal responses of a 10 μM standard mix in DI water, see table 6. It is evident that the sensitivity for the uronic acids under isocratic elution are slightly better compared to the method based on step-gradient elution (table 4).

Monosaccharides only

It is evident that in case the focus is on the analysis of the neutral sugars (monosaccharides and mannitol) only, it is possible to perform target analysis using isocratic separation using the conditions specified in table 2 (3 mM NaOH for 11 min) followed by a wash/regeneration step (100 mM NaOH and 100 mM NaOAc for 5 min) and equilibration using 3 mM NaOH (for 34 min). See figure 7 shown below for an example chromatogram.

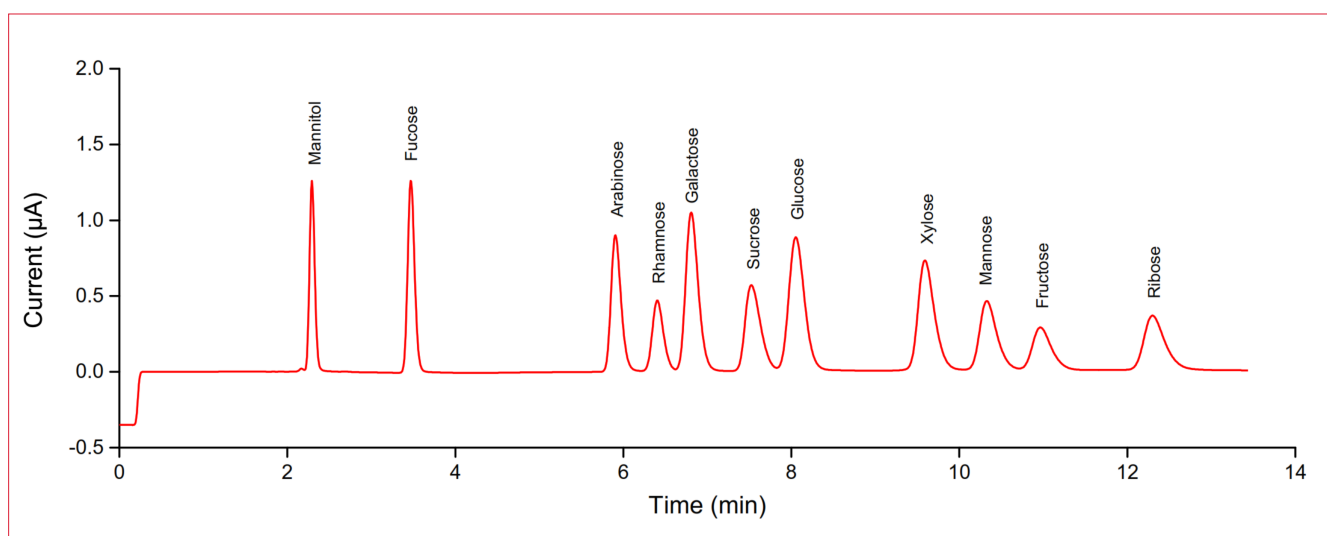


Figure 7. Chromatograms obtained from a 10 μL injection of a 100 μM standard mix of 11 neutral sugars in DI water.



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Conclusion

The ALEXYS™ Carbohydrate Analyzer in combination with the standard bore SweetSep™ AEX20 columns offers a selective and sensitive analysis solution for the compositional analysis of neutral sugars and uronic acids in biomass hydrolyzates, using HPAEC-PAD. Fast high-resolution separation ($r > 1.5$) within 35 minutes of all 16 analytes of interest was achieved with the developed method. In addition, also the targeted analysis of the neutral sugars and uronic acids only is shown in this application note. The method applicability was demonstrated by successful analysis of a seaweed hydrolysate sample.



Neutral sugars & uronic acids in seaweed

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
Columns	
260.0020	SweetSep™ AEX20, 4 x 200 mm column, 5 µm
260.0025	SweetSep™ AEX20, 4 x 200 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.

Reagents and standards

NaOH 50%, carbonate –free	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

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 and may be adjusted accordingly. Specifications mentioned in this application note are subject to change without further notice.

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