Application Note

Biomass

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

Uronic acids & neutral sugars in biomass hydrolyzates

- **ALEXYS™ Carbohydrate Analyzer**
- **SweetSep™ AEX20 anion-exchange column**
- **Analysis of neutral sugars and uronic acids**
- **Wood, pulp and shrimp shell biomass**
- **'Green' method**

Summary

The increasing global population, resource depletion, and environmental concerns have driven the demand for sustainable and eco-friendly industrial development [1,2]. Biomass sources such as wood, pulp (lignocellulose) and shrimp shells (chitin) are particularly promising materials due to their abundance and high carbohydrate content (polysaccharides). These renewable carbohydrate - based biopolymers offer great potential for diverse applications in biofuels, pharmaceuticals, and biodegradable materials. However, to exploit their potential requires a detailed understanding of their chemical composition, particularly carbohydrate content. Therefore, there is a need for a fast and accurate analytical method to evaluate the carbohydrate composition of these biopolymers extracted from biomass.

Carbohydrates can be separated and detected using High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). In this application note a HPAEC-PAD method is presented for the compositional analysis of neutral sugars and uronic acids in biomass hydrolyzates using the ALEXYS Carbohydrate Analyzer in combination with the new microbore SweetSep™ AEX20 column. The use of a 2.1 mm ID microbore column minimizes solvent consumption and waste, and thus environmental impact. To demonstrate the applicability of the method several hydrolyzed biomass samples originating from wood, pulp and shrimp shells were successfully analyzed.

Introduction

Environmental degradation, resource scarcity, and the growing global population drive the need for a sustainable and green industrial development [1]. The study of cellulose, chitin and related biopolymers from biomass is therefore essential for the development of sustainable materials, industrial fiber production, and green chemistry applications.

Lignocellulosic biomass, derived from plant cell walls, is the most abundant sustainable materials available on Earth. Examples of lignocellulosic biomass are switchgrass, poplar trees and sugar cane. Lignocellulosic biomass consists of two kinds of carbohydrate polymers, cellulose and hemicellulose, and an aromatic polymer called lignin [3]. Cellulose is the main structural constituent of plant cell walls. It is composed of long, linear chain of D-glucose units linked through β -(1,4)-glycosidic bonds [4]. Hemicellulose is a branched polysaccharide consisting of short lateral monosaccharides such as xylose, rhamnose, glucose, mannose, and galactose, and uronic acids [5]. Chitin is the most abundant natural amino-polysaccharide polymer and it is primarily found in the insects, crustaceans and cell walls of fungi. Chitin obtained from for example shrimp shells can be converted into the well-known biopolymer chitosan through chemical deacetylation. Chitosan represents a group of acid-soluble heteropolysaccharides which mainly consist of N-acetylglucosamine and glucosamine linked through β -(1,4)-glycosidic bonds. Chitosan and it derivatives have a large range of applications in the field of medicine, pharmaceuticals, food, cosmetics, agriculture, the textile and paper industries and the energy industry [6,7].

Figure 1. ALEXYS Carbohydrate Analyzer consisting of the ET210 eluent tray (for N_2 blanketing), a P6.1L quaternary LPG pump, AS6.1L autosampler, CT2.1 column thermostat, and the DECADE Elite electrochemical detector.

Understanding the carbohydrate composition of the cellulose, lignocellulose and chitin extracted from biomass is crucial for developing its potential applications. Since biomass contains polysaccharides, analysis of the carbohydrate composition is only possible after breaking down the glycosidic linkages. Therefore, hydrolysis with a strong acid, such as sulfuric acid, hydrochloric acid or trifluoroacetic acid is required for this purpose [8]. Hydrolysis breaks down the complex polymeric structure into neutral sugar and uronic acid monomeric units, which can then be analyzed and quantified using HPAEC-PAD.

In this application note, a HPAEC-PAD method is presented for the compositional analysis of neutral sugars and uronic acids in biomass hydrolyzates, using the ALEXYS™ Carbohydrate Analyzer in combination with the new microbore SweetSep AEX20 anion-exchange column. To demonstrate the performance and the applicability of the method, several biomass hydrolyzates originating from wood, pulp and shrimp shells were analyzed.

Method

The analyses of biomass samples were performed using the Antec Scientific ALEXYS™ Carbohydrate Analyzer (figure 1) and the conditions specified in table 1. This high-pressure ion chromatography system with metal-free flow path consists of the ET210 eluent tray, P6.1L quaternary LPG pump, CT2.1 column thermostat, and DECADE Elite electrochemical detector. The ET210 eluent tray has an integrated gas distribution system to blanket the headspace of the eluent bottles with inert gas (Nitrogen or Helium), to avoid diffusion of CO2 into the eluents and the build up of carbonate ions (CO_3^{2-}) which act like a strong 'pushing agent' in anion-exchange chromatography.

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 for carbo-hydrate separation. The retention time of carbohydrates is inversely correlated with pKa value and increases significantly with molecular weight.

In HPAEC-PAD carbohydrate analysis, small concentrations (low -ppb level) of borate contaminants present in hydroxide mobile phases can negatively affect chromatographic performance due to peak tailing and loss of peak symmetry of specific sugars.

Table 1

HPAEC-ECD Conditions

HPLC system	ALEXYS™ Carbohydrate Analyzer
Columns	SweetSep™AEX20, 2.1 x 200 mm analytical column, 5 µm SweetSep™AEX20, 2.1 x 50 mm precolumn, 5 µm Borate ion trap, 2.1 x 50 mm column, 10 µm (Antec Scientific)
Mobile phase (MP)	A: 10 mM NaOH B: DI Water (resistivity 18.2 MOhm.cm and TOC < 5ppb) C: 200 mM NaOH D: 200 mM NaOAc Eluents blanketed with Nitrogen 5.0
Flow rate	0.18 mL/min
Back pressure	about 190 bar
Injection	3 µL
Temperature	27 °C for separation, 45 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF Palladium 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 - 0.5 µA
ADF	0.05 Hz
Range	10 µA/V

Table 2

Step-gradient program

Time (min)	*Mobile phase*	*%A*	*%B*	*%C*	*%D*	*Description*
0 - 9	5.4 mM NaOH	54	46	0	0	Elution & detection (neutral sugars)
9 - 14.5	70 mM NaOH	0	65	35	0	Elution & detection (cellobiose)
14.5 - 23	34 mM NaOH, 130 mM NaOAc	0	18	17	65	Elution, detection (uronic acids) & column clean-up/ regeneration
23 - 57	5.4 mM NaOH	54	46	0	0	Equilibration to the starting condition

Borate ions (BO_3^{-3}) can easily complex with vicinal hydroxyl groups present in some carbohydrates, for example, mannose, sugar alcohols, and fructose. Therefore, as a precaution, a borate ion trap column (50 × 2.1 mm ID) was installed between the pump and the injector. Another possible source of borate ions is glassware, which can leach borate especially at high pH. Therefore, all solutions were prepared in polypropylene bottles, and the samples were stored in a polypropylene vial.

The separations were performed at 27 °C on a SweetSep AEX20 analytical column (200 × 2.1 mm ID). A SweetSep AEX20 precolumn (50 × 2.1 mm ID) was installed in series upfront the analytical column for protection against contaminants and particulate matter. This stationary phase consists of highly monodisperse 5 µm ethylvinylbenzene-divinylbenzene copolymer (80% crosslinked) substrate particles coated with functionalized nanobeads containing dual ion exchange sites (quaternary amine and tertiary amine). The high uniformity and small particle size of the resin allow for a fast and high-resolution separation of neutral sugars and uronic acids without the need for post-column sodium hydroxide addition.

The step-gradient profile described in Table 2 was employed for the separation of the neutral sugars and uronic acids. The neutral sugars were separated using isocratic elution with 5.4 mM sodium hydroxide (NaOH) from t = 0 min to t = 9 min. This is followed by a gradient step of 70 mM NaOH from t = 9 min to t = 14.5 min to elute cellobiose. Cellobiose is a glucose dimer which is more strongly retained on the column than the neutral monosaccharides. To elute the uronic acids, which are retained even stronger, a gradient step using 34 mM NaOH-130 mM sodium acetate (NaOAc) was applied between t = 14.5 to t = 23 min. This gradient step also serves as clean-up and regeneration step to wash off late-eluting components and removal of carbonate ion (CO_3^{2-}) build up on the column. After the clean-up step the column is equilibrated for 34 minutes to the starting conditions, resulting in a total run time of 57 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 18.2 MΩ.cm, TOC <5 ppb), which was sparged with Nitrogen 5.0 (purity 99.999%). During analysis the mobile phase headspace is blanketed with Nitrogen gas (0.2—0.5 bar N_2 pressure) using the ET210 eluent tray to ensure reproducible analysis.

Detection

The ALEXYS Carbohydrate Analyzer was equipped with a DECADE Elite electrochemical detector in combination with a SenCell™ electrochemical flow cell for the pulsed amperometric detection [9]. The SenCell has a confined wall-jet flow cell design and consists of a gold working electrode (WE), HyREF palladium reference electrode (RE), and stainless-steel auxiliary electrode (AE). The flow cell has an adjustable spacer

and was set to position 2, which corresponds to a 50 µm spacing and a 160 nL working volume. A 4-step potential waveform was applied: E1, E2, E3, and E4 were +0.10, –2.0, +0.6, and –0.1 V, respectively, with pulse duration of t1 0.40 s, t2 0.02 s, t3 0.01 s, and t4 0.07 s, resulting in a total pulse time of 0.5 s (corresponding to a data rate of 2 Hz). The signal (cell current) is acquired for 200 ms at t1 between 0.20 - 0.40 s. This particular 4 -step waveform has several benefits: (1) long-term reproducible response factor for all analytes of interest and (2) minimal electrode wear [10]. The detection temperature was set to 45°C to improve detection sensitivity at low NaOH concentration in the eluent. Under the conditions specified, the typical cell current was 0.2 µA. The filter setting for the detection was set to 0.05 Hz.

Preparation of standards, reagents and samples

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 by serial dilution of the stock standards with DI water. The molar concentration range corresponds to 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 µg/mL for galactose, glucose, mannose, and fructose, 0.3 - 34.2 µg/mL for sucrose and cellobioses, and 0.2 - 19.4 µg/mL for the uronic acids.

Samples: In total 5 samples were analyzed. two samples, one wood (lignocellulosic biomass) and 1 commercial chitin hydrolyzate (originating from shrimp shells) were kindly provided by BOKU university (Vienna, Austria). The wood sample (amount not disclosed) was dried and digested with 0.5 mL 72% sulfuric acid. The chitin sample (20 mg) was hydrolyzed with 1 mL of 37% hydrochloric acid at 95°C for 1 hour. Both hydrolyzed samples were neutralized.

The three other biomass hydrolyzates, dissolved pulp (DP), paper grade pulp (PGP) and a non-wood experimental cellulose sample with an unknown origin (SJS-A), were kindly provided by Thüringisches Institut für Textil- und Kunststoff-Forschung (Rudolstadt, Germany). These samples (100 mg) were prepared by hydrolysis in 0.25 M sulfuric acid and filtered over a 0.45 µm nylon filter. Subsequently, the filtered hydrolyzate samples were neutralized.

Additionally, all five samples were diluted 100 times and filtered through a 0.22 µm polyethersulfone (PES) filter (25 mm Ø FFL/MLS). 3 µL of the filtered supernatant was injected into the LC system and analyzed.

Results

A chromatogram obtained with the 3 µL injection of a 100 µM standard mix in DI water is shown in Figure 2. All 12 neutral sugars and 5 uronic acids in the chromatogram are baseline separation (resolution ≥ 1.5). The monosaccharides eluted within 13 minutes, followed by the disaccharide cellobiose eluting around 17.5 minutes. The uronic acids eluted between

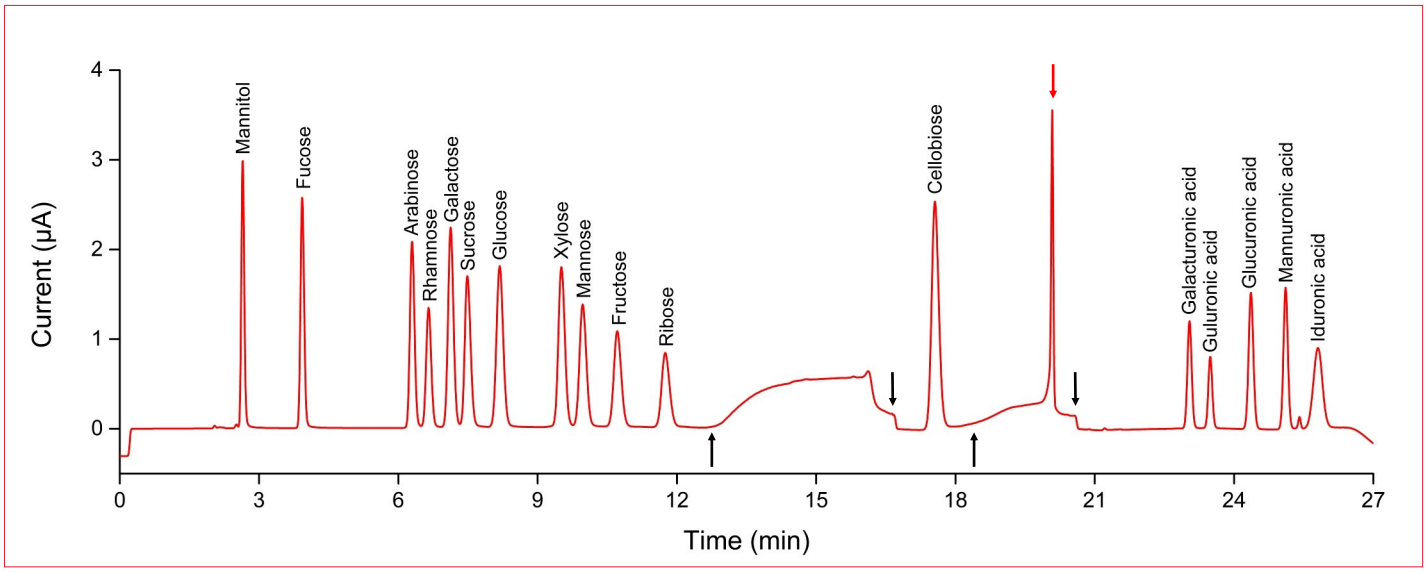


Figure 2. The chromatogram obtained from a 3 µL injection of the 100 µM sugar standard mix in DI water. The black ↑ arrows in the chromatogram indicate the start of a baseline elevation due to the changes in mobile phase composition (step gradient). Black ↓ arrows indicate an Autozero to remove the baseline 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.

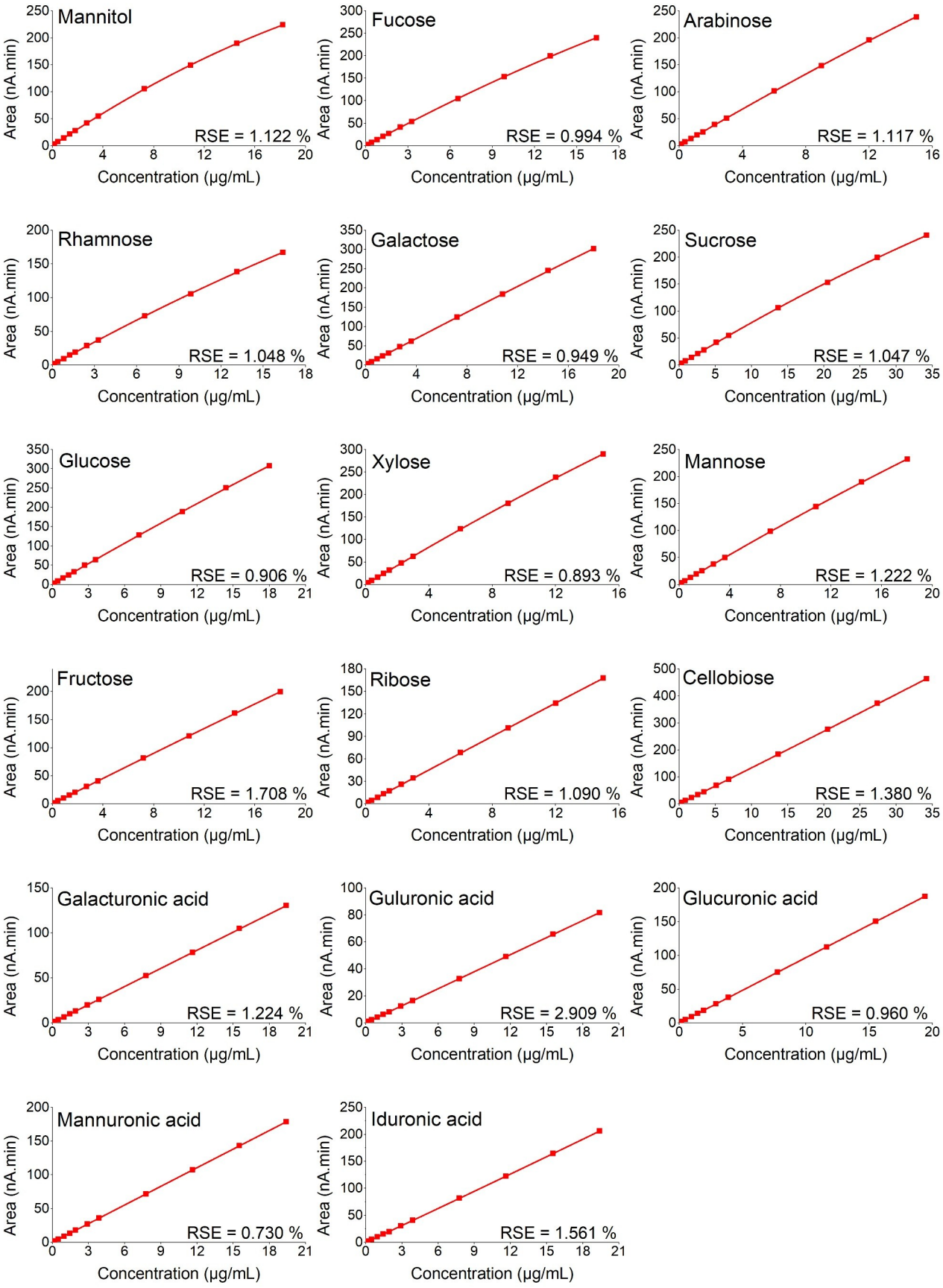
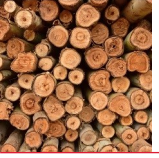


Figure 3. Calibration curves (11 calibration levels) of the 12 neutral sugars and 5 uronic acids in the range of 0.2 - 18.2 µg/mL for mannitol, 0.2 - 16.4 µg/mL for fucose and rhamnose, 0.2 - 15.0 µg/mL for arabinose, xylose, and ribose, 0.2 - 18.0 mg/mL for galactose, glucose, mannose, and fructose, 0.3 - 34.2 µg/mL for sucrose and cellobioses, 0.2 - 19.4 µg/mL for galacturonic acid, guluronic acid, glucuronic acid and mannuronic acid. For all compounds a quadratic fit was applied, ignoring the origin and using with a weight factor of 1/amount^2. The relative standard error (RSE) was <3% for all compounds.



23 and 26 minutes. Note that the changes in NaOH concentration, and addition of NaOAc in the mobile phase during the step-gradient run, results in an increase in cell current. In figure 2 the onset of the rise in background current due to changes in mobile compositions (and thus pH) are marked with black ↑ arrows. To compensate for the introduced baseline offset an autozero was executed at the time points marked with black ↓ arrows.

Calibration

The linearity was investigated in the concentration range of 1 – 100 µM (11 calibration levels) for all analytes. This corresponds to the following mass concentration range:

- Mannitol: 0.2 -18.2 µg/mL
- Fucose and rhamnose: 0.2 - 16.4 µg/mL
- Arabinose, xylose, and ribose: 0.2 - 15.0 µg/mL
- Galactose, glucose, mannose, and fructose: 0.2 - 18.0 µg/mL
- Sucrose and cellobiose: 0.3 - 34.2 µg/mL
- Uronic acids: 0.2 - 19.4 µg/mL

The calibration curves of all 17 analytes are shown in Figure 3. Electrochemical detection of carbohydrates has been known to have a broad linear dynamic range. However, the calibration curve may deviate from linearity at a high analyte concentration, and therefore a quadratic fit was used for curve fitting of the calibration levels for all analytes. The quadratic fitting was applied ignoring the origin and using a weighting factor of 1/concentration^2 [11,12]. The relative standard error (RSE) and the relative residual error (RRE) were calculated to assess the goodness-of-fit of the calibration curves. The relative standard error of the calibration curves obtained with the quadratic fit method are excellent and < 3% for all analytes, see figure 3. In addition, the relative residual errors for the calibration curves were < 5% for all analytes across all calibration levels. This demonstrates that the quantification based on the quadratic calibration curves has a good accuracy over the complete calibration range. The calibration curves in Figure 3 were used for the actual quantification of the carbohydrates in the biomass samples.

Table 3

Repeatability of 3 µL injections of a 10 µM standard mix in DI water (n=10)

	RSD (%) *10 µmol/L*	
Compound	t_R	*Area*
Mannitol	0.16	0.39
Fucose	0.21	0.29
Arabinose	0.22	0.29
Rhamnose	0.20	0.32
Galactose	0.23	0.51
Sucrose	0.28	0.65
Glucose	0.23	0.38
Xylose	0.19	0.43
Mannose	0.19	0.47
Fructose	0.21	0.45
Ribose	0.22	0.41
Cellobiose	0.24	0.40
Galacturonic acid	0.14	0.57
Guluronic acid	0.14	0.49
Glucuronic acid	0.13	0.48
Mannuronic acid	0.14	0.61
Iduronic acid	0.18	0.56

Table 4

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

Compound	*LOD*		*LOQ*
	nmol/L	*ng/mL*	*ng/mL*
Mannitol	15	3	9
Fucose	20	3	11
Arabinose	27	4	13
Rhamnose	39	6	21
Galactose	27	5	16
Sucrose	30	10	34
Glucose	31	6	18
Xylose	31	5	15
Mannose	41	7	24
Fructose	55	10	33
Ribose	69	10	34
Cellobiose	120	41	136
Galacturonic acid	426	83	276
Guluronic acid	571	111	369
Glucuronic acid	337	65	218
Mannuronic acid	297	58	192
Iduronic acid	658	128	426

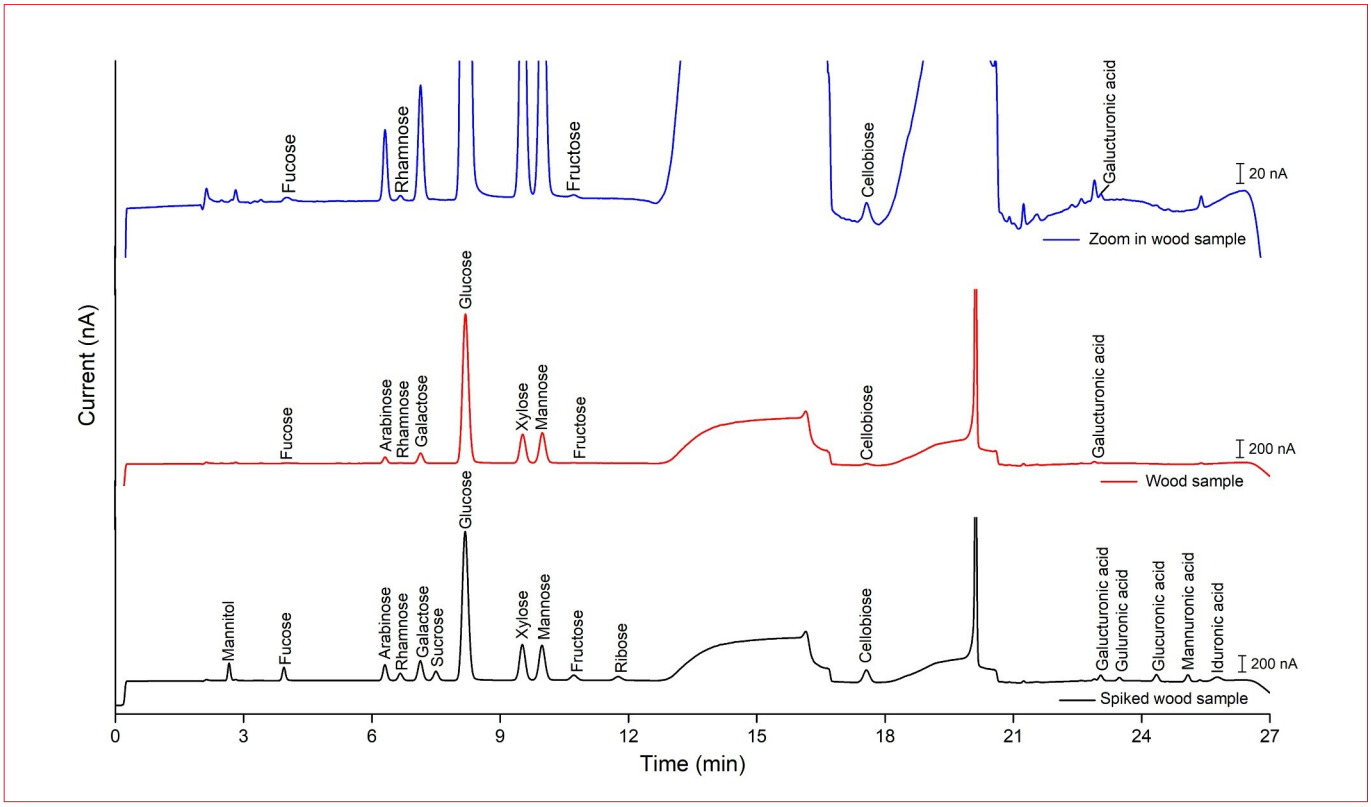


Figure 4. Overlay of chromatograms obtained from 3 µL injections of the wood sample spiked with 5 µM standards (bottom, black line), the wood sample (middle, red line), and the zoom into the baseline of the wood sample (top, blue line).

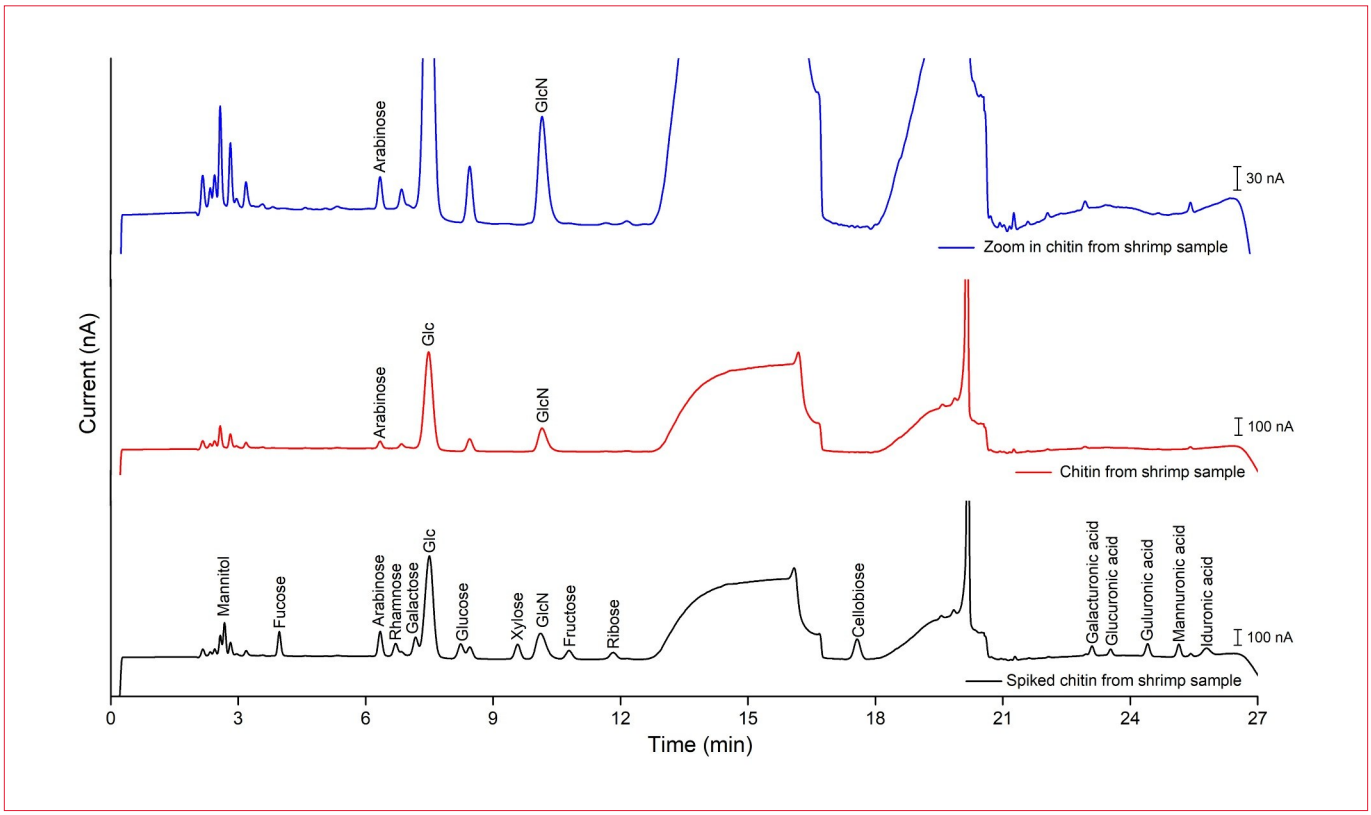


Figure 5. Overlay of chromatograms obtained from 3 µL injections of chitin from shrimp sample spiked with 5 µM standards (bottom, black line), the chitin from shrimp sample (middle, red line), and the zoom into the baseline of the chitin from shrimp sample (top, blue line).

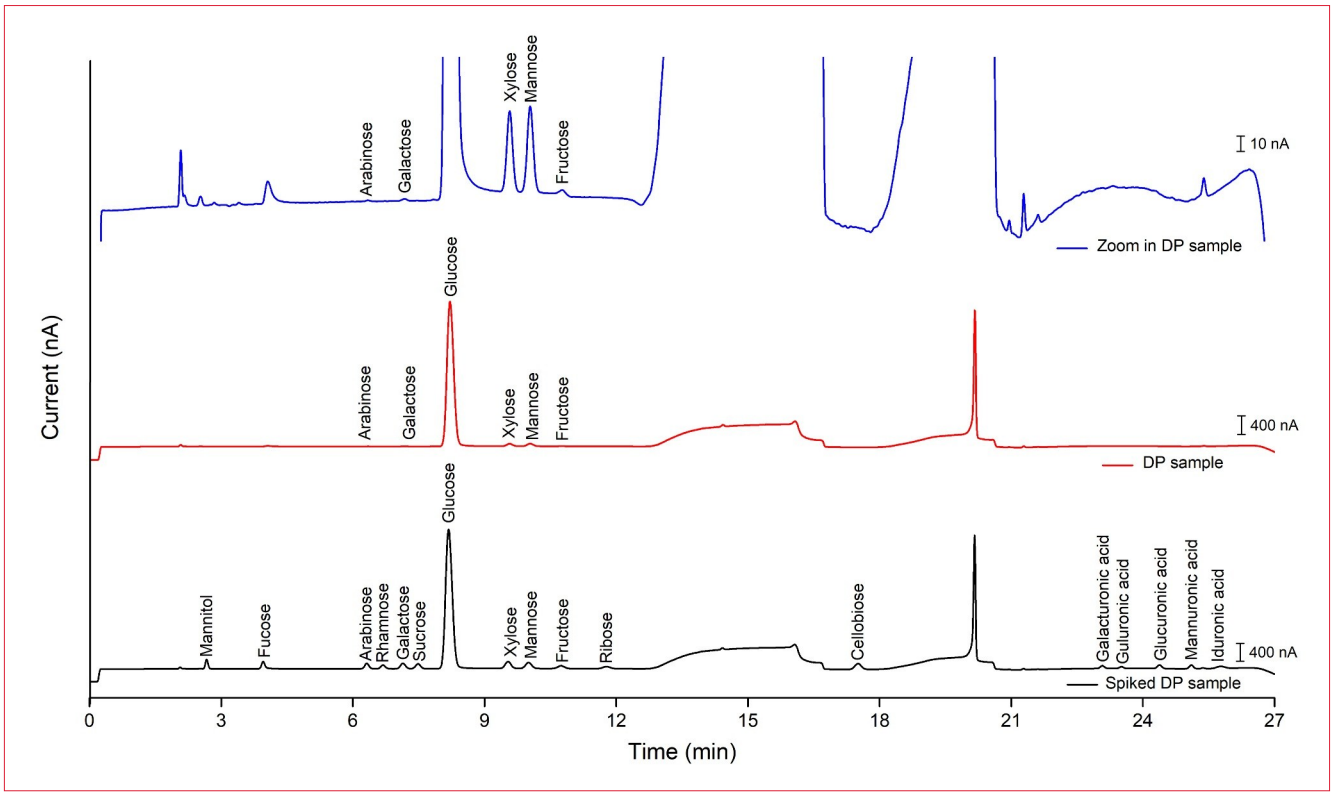


Figure 6. Overlay of chromatograms obtained from 3 µL injections of the DP sample spiked with 5 µM standards (bottom, black line), the DP sample (middle, red line), and the zoom in of the DP sample (top, blue line).

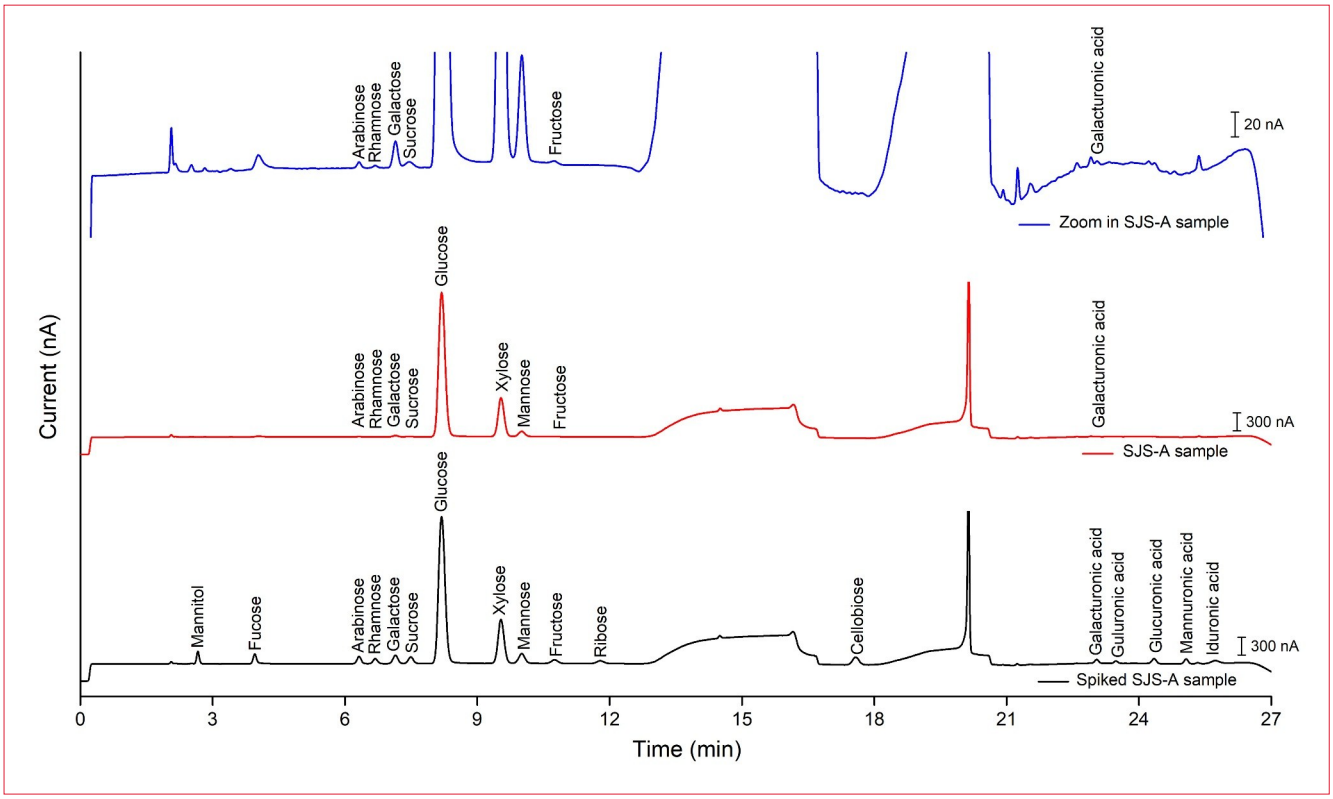


Figure 7. Overlay of chromatograms obtained from 3 µL injections of the SJS-A sample spiked with 5 µM standards (bottom, black line), the SJS-A sample (middle, red line), and the zoom in of the SJS-A sample (top, blue line).

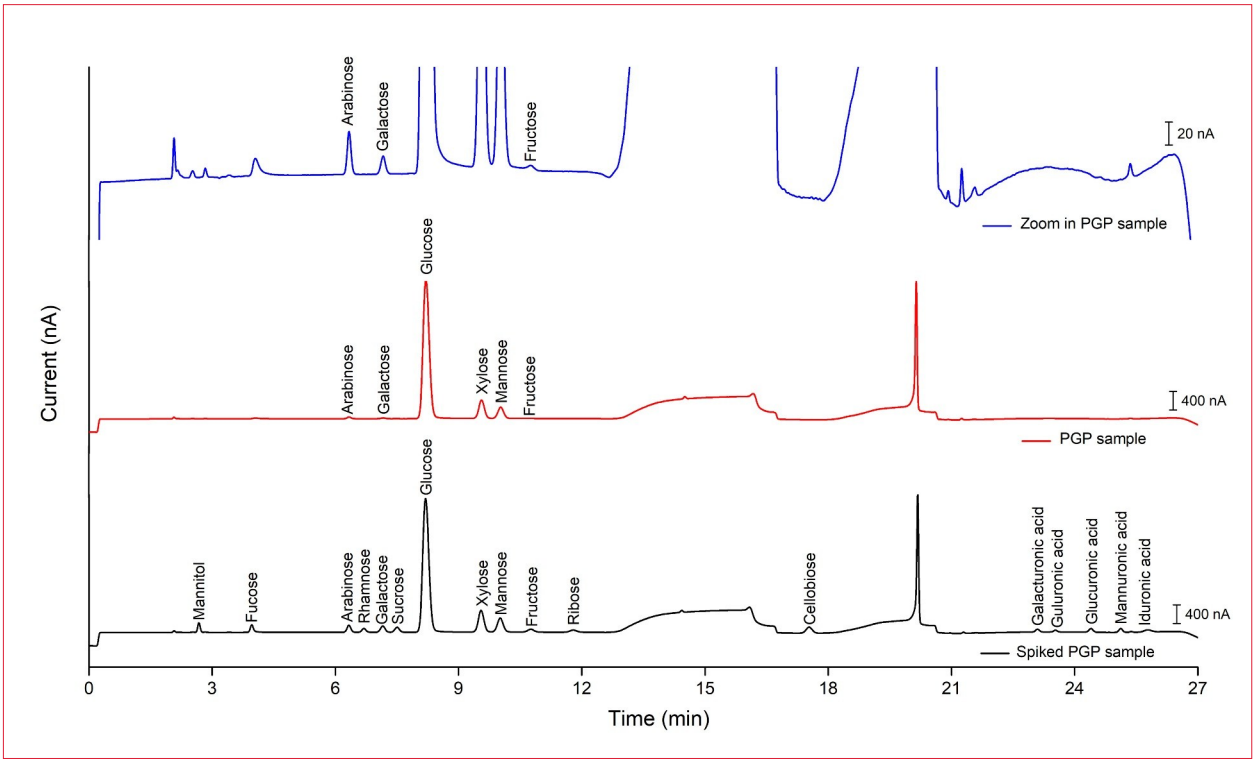


Figure 8. Overlay of chromatograms obtained from 3 µL injections of the PGP sample spiked with 5 µM standards (bottom, black line), the PGP sample (middle, red line), and the zoom in of the PGP sample (top, blue line).

Table 5

Neutral sugars & uronic acid content in hydrolyzed biomass samples (mg/mL)

Compound	*Wood*	*Chitin*	*PGP*	*SJS-A*	*DP*
Mannitol	-*	-	-	-	-
Fucose	0.002	-	-	-	-
Arabinose	0.046	0.026	0.025	0.003	0.0003
Rhamnose	0.003	-	-	0.002	-
Galactose	0.090	-	0.012	0.015	0.001
Sucrose	-.	-	-	0.008	-
Glucose	1.674	-	3.048	2.354	3.200
Xylose	0.246	-	0.310	0.518	0.041
Mannose	0.412	-	0.295	0.102	0.069
Fructose	0.003	-	0.006	0.003	0.005
Ribose	-.	-	-	-	-
Cellobiose	0.023	-	-	-	-
Galacturonic acid	0.003	-	-	0.003	-
Glucuronic acid	-	-	-	-	-
Guluronic acid	-	-	-	-	-
Mannuronic acid	-	-	-	-	-
Iduronic acid	-	-	-	-	-

* - = not detected

Repeatability

The repeatability of the method was evaluated with a 10 µM standard mix in DI water. The relative standard deviations (RSD) of the retention time and peak area were determined based on 10 repetitive injections (see table 3). Low RSD values were obtained for both retention time (<0.28%) and peak area (<0.65%) for all compounds. These low RSD values for peak area demonstrate the excellent precision of the method.

LOD & LOQ

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for all sugars are summarized in Table 4. The LODs were calculated as the analyte response corresponding to 3× the ASTM noise (average peak-to-peak baseline noise of 20 segments of 0.5 min). The ASTM noise was determined for each section of the chromatogram with different separation conditions (5.4 mM NaOH, 70 mM NaOH and 34 mM NaOH + 130 mM NaOAc) using a blank injection. The peak heights obtained with an injection of the lowest concentration standard (1 µM) were used to calculate the LODs for all analytes. The LOQs were calculated in a similar way to LODs, using a 10× signal-to-noise (S/N ratio). The LOQ for all monosaccharides ranges from 9 – 34 ng/mL, demonstrating the high sensitivity of the method. The LOQs for cellobiose and the uronic acids are higher, in the range of 136—426 ng/mL, due to a higher noise and/or lower response factor under these specific elution conditions.

Sample analysis

A total of five hydrolyzed samples were analysed: (1) wood, (2) chitin from shrimp shells, (3) dissolved pulp (DP), (4) paper grade pulp (PGP) and (5) a non-wood experimental cellulose from unknown origin (SJS-A). The chromatograms of these samples are shown in Figure 4 - 8. All samples were 100× diluted before injection. To ensure correct peak identification, the samples were spiked with the standard mix resulting in a final concentration of 5 µM of the analytes in the spiked sample. The contents of neutral sugars and uronic acids in the samples were calculated based on the calibration curves of the standards shown in fig 3. The quantified amounts of neutral sugars and uronic acid are listed in Table 5.

Lignocellulosic biomass samples

It is evident from table 5 that glucose is the monosaccharide with the highest abundance in the hydrolyzed lignocellulosic biomass samples (wood, PGP, SJS-A and DP). The relative amount of glucose, compared to all sugars and uronic acids detected in the samples, ranged from 67% (wood) to 96% (DP).

In the hydrolyzed wood sample, cellobiose was detected. Cellulose is a homopolymer of glucose, which is formed by beta 1-4 linkage of D-glucose monomer units. Cellobiose is a glucose dimer. The presence of cellobiose in the sample is therefore a marker for incomplete/partial hydrolysis of cellulose [13].

Xylose and Mannose are the other two dominant mono-saccharides present in the 4 lignocellulosic biomass samples (wood, PGP, SJS-A and DP). These monomers are released from the hydrolyzed hemicellulose portion present in the samples. In addition small amounts of galactose, arabinose, rhamnose, fucose and fructose are found originating from the hemi-cellulose in the samples.

In the wood and SJS-A sample a small amount of Galacturonic acid was quantified (3 µg/mL, relative amount 0.1 %). In the other samples no uronic acids were detected. The reason for the low abundance of uronic acids in the samples is related to the strong hydrolysis method required to release the sugars in cellulose. Hydrolysis with a high concentration of strong acids such as sulfuric acid or hydrochloric acid will result in degradation of the uronic acids in the sample. Therefore, the approach based on hydrolysis with sulfuric acid is unsuitable for the compositional analysis of uronic acids. Milder approaches using hydrolysis based on acid methanolysis with trifluoric acid (TFA) gives the possibility to quantify the uronic acids in biomass samples [14,15]. Note, that under these milder conditions cellulose will be partially hydrolyzed, and therefore no complete neutral sugar profile can be obtained for cellulose. In this case, fast targeted analysis of the uronic acids, as demonstrated in the next paragraph (page 11) can be a suitable workflow when using acid methanolysis.

Dissolved pulp has a high cellulose content, as confirmed by the quantified glucose content by HPAEC-PAD, and can be used in the lyocell process to produce textile due to its high purity. The Lyocell process is a method to produce cellulosic fibers by dissolving pulp in N-Methylmorpholine N-oxide (NMMO) and (dry jet-) wet spinning it into fibers [16] . The Lyocell process is favored over the Viscose process to produce fibers, because it requires less energy, uses less toxic chemicals which are recycled in a closed-loop process. Paper grade pulp is used for paper products and contains more hemicellulose than cellulose. The difference in pulp, due to chemical and mechanical processes, explains the difference in carbohydrates content [17].

Chitin biomass sample

Chitin is the most abundant aminopolysaccharide polymer occurring in the nature. It gives strength to the exoskeletons of

crustaceans, insects and cell walls of fungi. Chitin has the same structure as cellulose except that it is composed of N-acetylglucosamine, an amide derivative of D-glucose, in place of D-glucose in cellulose. The acid hydrolysis of chitin involves two reaction: (1) hydrolysis of the glycosidic bond leading to depolymerization, and (2) hydrolysis of the N-acetyl bond (de-N -acetylation). The resulting monomers are N-acetylglucosamine (GlcN) and glucosamine (Glc), respectively. Under the conditions used in this method for the analysis of neutral sugars and uronic acids, glucosamine and N-acetylglucosamine coelute with sucrose and mannose, respectively (data not show here). Therefore, the peaks eluting at t = 7.5 min and t = 10.2 minutes in the chromatogram shown in figure 5 are with a high probability glucosamine and N-acetylglucosamine, respectively.

Uronic acids only

For applications focusing solely on uronic acids, a targeted analysis based on a method using isocratic separation conditions specified in Table 2 (34 mM NaOH, 130 mM NaOAc) can be applied. A chromatogram of an the 3 µL injection of a 100 µM uronic acid standard mix in DI water is shown in Figure 6. All uronic acids eluted within 11 minutes under this conditions.

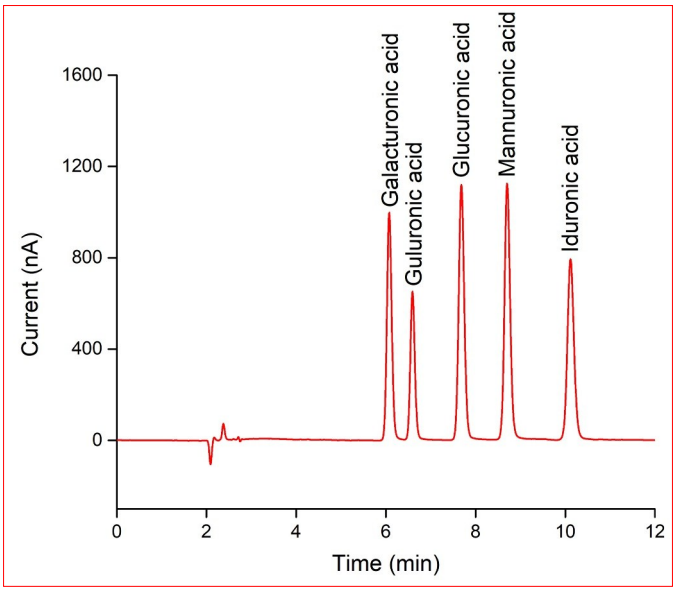


Figure 6. Chromatograms obtained from a 3 µL injection of a 100 µM mix of 5 uronic acids in DI water.

The calibration was evaluated in the concentration range of 0.2 - 19.4 µg/mL (1-100 µM) for all uronic acids, using a quadratic fitting method as described in a previous section (page 6). Also for this targeted method accurate calibration were obtain with relative standard errors RSE <2.3% (Table 6). The repeatability of the method was assessed with a 10 µM mix of uronic acid standards in DI water. The results are listed in Table 7.

The precision was excellent with RSD values for retention time and peak area of < 0.18% and < 0.41% respectively for all uronic acids.

Table 6

Calibration of 3 µL injections of standards (1 - 100 µM)

Compound	*RSE(%)*
Galacturonic acid	0.761
Guluronic acid	0.501
Glucuronic acid	2.158
Mannuronic acid	2.221
Iduronic acid	2.025

Table 7

Repeatability of 3 µL injections of 10 µM uronic acid mix standard in DI water (n=10)

	RSD (%) *10 µmol/L*	
Compound	t_R	*Area*
Galacturonic acid	0.14	0.31
Guluronic acid	0.16	0.40
Glucuronic acid	0.18	0.41
Mannuronic acid	0.13	0.34
Iduronic acid	0.17	0.35

Table 8

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	219	43	142
Guluronic acid	321	62	208
Glucuronic acid	160	31	103
Mannuronic acid	193	37	125
Iduronic acid	175	34	114

The LOD and LOQ of the uronic acids for this method were calculated based on the signal response of a 1 µM standard mix in DI water, see Table 8. It is evident from the LOD and LOQ values that the method has excellent sensitivity. Notably, the LOD and LOQs for the isocratic elution of the uronic acid are significantly lower than of the step-gradient method (table 4).

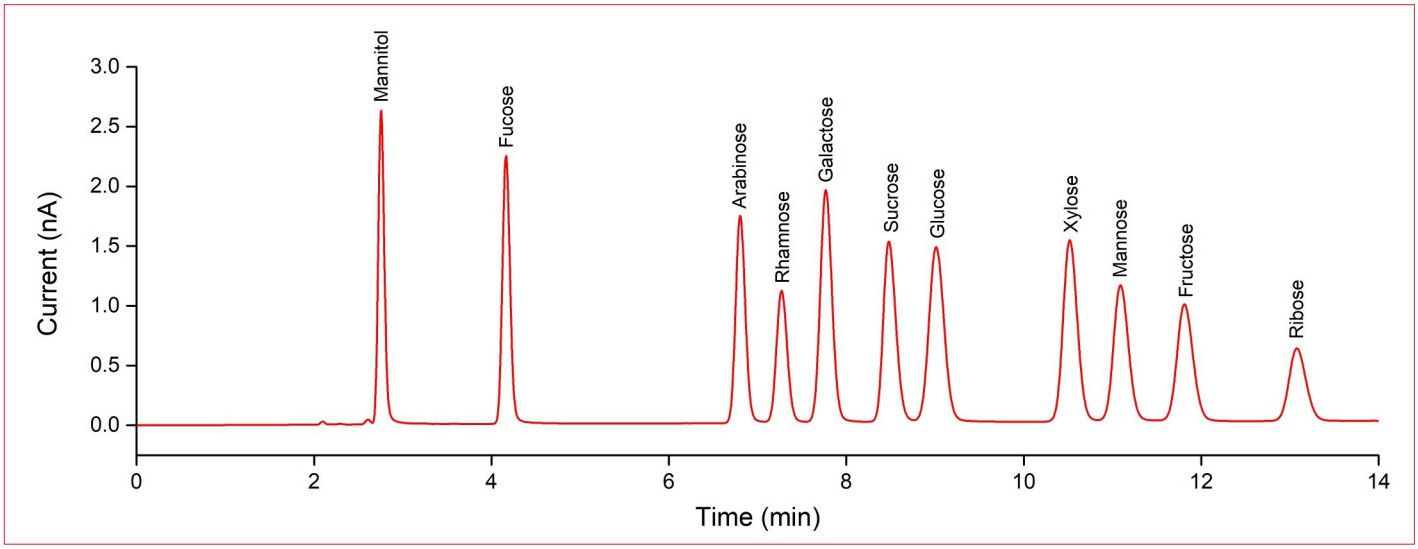


Figure 8. Chromatogram obtained from a 3 µL injection of the 100 µM standard mix of 10 monosaccharides and mannitol in DI water.

Monosaccharides only

For applications focused on the monosaccharides only, a targeted analysis can be used based isocratic elution of the sugars, directly followed by a wash/regeneration step and re-equilibration to starting conditions. See the example chromatogram in figure 8. In this case the optimized gradient program listed in table 9 is applied. The separation temperature was set to 25°C.

Table 9

Step-gradient program - Monosaccharides only

Time (min)	*Mobile phase*	*%A*	*%B*	*%C*	*%D*	*Description*
0 - 10	5 mM NaOH	50	50	0	0	Elution & detection (monosaccharides)
10 - 15	100 mM NaOH, 100 mM NaOAc	0	0	50	50	column clean-up/ regeneration
15 - 34	5 mM NaOH	50	50	0	0	Equilibration to the starting condition

Conclusion

The ALEXYS™ Carbohydrate Analyzer in combination with the microbore 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 26 minutes of all 17 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 several biomass samples from wood, pulp and shrimp. A four-fold reduction of mobile phase usage was achieved by using a 2 mm ID column instead of a standard bore version (4 mm ID).

References

1. Wang, J., & Azam, W., Natural resource scarcity, fossil fuel energy consumption, and total greenhouse gas emissions in top emitting countries, *Geoscience Frontiers*, 2023, 15(2), 101757 https://doi.org/10.1016/j.gsf.2023.101757
2. Carafa, R. N., Foucher, D. A., Sacripante, G. G., Biobased polymers from lignocellulosic sources*, Green Chem Lett Rev*, 2023, 16(1), 1 – 22 https://doi.org/10.1080/17518253.2022.2153087
3. Cai, J. et. al., Review of physicochemical properties and analytical characterization of lignocellulosic biomass*., Renewable and Sustainable Energy Reviews*, 2017, 76, 309 –322 http://dx.doi.org/10.1016/j.rser.2017.03.072
4. Maleki, S. S., Mohammadi, K., & Ji, K., Characterization of cellulose synthesis in plant cells, *The Scientific World Journal*, 2016, 1–8 https://doi.org/10.1155/2016/8641373
5. Kaur, R., Sharma, M., Cereal polysaccharides as sources of functional ingredient for reformulation of meat products: A review, *Journal of Functional Foods*, 2019, 62, 103527. https://doi.org/10.1016/j.jff.2019.103527
6. Elieh-Ali-Komi,D., Chitin and Chitosan: Production and application of versatile biomedical nanomaterials, *Int J Adv Res (Indore), 2016*, 4(3), 411–427. https://pmc.ncbi.nlm.nih.gov/articles/PMC5094803/pdf/nihms774320.pdf
7. Gao, K., Qin, Y., Liu, S., Wang, L., Xing, R., Yu, H., Chen, X., A review of the preparation, derivatization and functions of glucosamine and N-acetyl-glucosamine from chitin, *Carbohydrate Polymer Technologies and Applications*, 2023, 5, 100296 https://doi.org/10.1016/j.carpta.2023.100296
8. Rupley, J. A., The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low-molecular-weight substrates for lysozyme, *Biochimica et Biophysica Acta*, 1964, 245(8), pp. 245–255 https://doi.org/10.1016/0926-6526(64)90001-1
9. Louw, H.J. Brouwer, N. Reinhoud, *Electrochemical* flowcell, *US patent 9310330*, 2016 https://patents.google.com/patent/US9310330B2/en
10. R.D. Rocklin, A.P. Clarke, M. Weitzhandler, Improved longterm reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform, *Anal. Chem*, (1998),70, 1496 – 1501 https://doi.org/10.1021/ac970906w
11. Jensen, D. , Enhancing Calibration Precision with Relative Standard Error and Weighted Least Squares Approximations in Chromatographic Analysis, *Thermo Fisher Scientific Technical Note,* 2021, No. 003551 https://appslab.thermofisher.com/App/4931/tn003551-enhanced-calibration-precision-leveraging-rse-wls-for-optimal-function-optimization
12. Danzer, K., Currie, L. A., Guidelines for Calibration in Analytical Chemistry Part 1: Fundamentals and Single Component Calibration (IUPAC Recommendations, *Pure & Appl. Chem.* , 1998, 70(4), 993–1014 http://dx.doi.org/10.1351/pac199870040993
13. Sluiter, A., et. al., Determination of structural carbohydrates and lignin in biomass, *NREL Laboratory Analytical Procedures*, NREL/TP-510-42618, 2012 https://www.nrel.gov/docs/gen/fy13/42618.pdf
14. Hemicellulose content and monosaccharide composition (methanolysis/total hydrolysis), Core Facility Analysis of Lignocellulosics, BOKU-Core Facilities, BOKU https://boku.ac.at/cf/alice/equipment-and-services/hemicellulose-content-and-monosaccharide-composition-methanolysis-total-hydrolysis
15. Sundberg, A. et.al., Determination of hemicelluloses and pectins in wood and pulp fibres by acid methanolysis and gaschromatography., Nordic *Pulp and Paper Research Journal,* 1996, 11 (4), 216-219 https://doi.org/10.3183/npprj-1996-11-04-p216-219
16. Wikipedia, *Lyocell*, https://en.wikipedia.org/wiki/Lyocell
17. Kultalahti, E. (2025). Analytical and testing methods for assessing pulp performance in Lyocell fiber production *Master thesis Aalto School of Chemical Engineering*, https://aaltodoc.aalto.fi/items/acbca931-ea70-4a0e-957a-ccc72ab46861

Ordering information

ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Column	
260.0021	SweetSep™ AEX20, 2.1 x 200 mm column, 5 µm
260.0026	SweetSep™ AEX20, 2.1 x 50 mm precolumn, 5 µm
260.0031	Borate ion trap, 2.1 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, standards and sample prep accessories

NaOH 50%, carbonate –free	Fisher Scientific, pn SS254-500
Sodium acetate trihydrate, HPLC grade	Fisher Scientific, pn 10122400
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

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.

Antec Scientific (USA)
info@AntecScientific.com
www.AntecScientific.com
T 888 572 0012

Antec Scientific (worldwide)
info@AntecScientific.com
www.AntecScientific.com
T +31 (172) 268888