Determination of Uronic Acids and Wood Sugars in Wood-Based Hydrolysates

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Biofuel, Galacturonic Acid, Glucuronic Acid, Monosaccharides, Dionex CarboPac PA200 Column, Dionex CarboPac PA20 Column

Introduction

Wood, grass, and other lignocellulosic biomass are emerging as the preferred sources for biofuel and biochemical production. These sources are considered more sustainable and are expected to become a competitive commercial alternative to fuel made from corn and other food sources by 2020. Typically, the cellulosic biomass—starches and sugars in the plant matter—are broken down by the application of heat and/or chemicals (pretreatment), followed by enzymatic digestion and fermentation. There is continued research on economic ways to break down the cellulose and the hemicelluloses into sugars that are fermentable by yeast and other organisms. Compositional analysis of plant-derived material is crucial for maximizing the yield of biofuel production.[1-3]

Wood hydrolysates contain a variety of hemicellulosic sugars (including glucose, xylose, mannose, arabinose, and cellobiose) and sugar acids (e.g., galacturonic and glucuronic acids). Uronic acids are a class of sugar acids with both the carbonyl and the carboxylic acid functional groups. Uronic acids are present in plant cell walls and are also formed during biofuel processing. Accurate determination of both mono- and disaccharides and the uronic acids in biomass materials is important because compositional analysis enables evaluation of conversion yields, and carbohydrate content is directly proportional to bioalcohol yield.

Several methods have been used for determination of wood sugars and uronic acids, including the calorimetric method, gas chromatography, high-performance liquid chromatography, and capillary electrophoresis.[4-6] Use of liquid chromatography, including high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), has been reported for the determination of carbohydrates (monosaccharides, uronic acids, and aldonic acids) in biomass hydrolysate samples. Several members of the Thermo Scientific™ Dionex™ CarboPac™ column family (i.e., the Dionex CarboPac PA1, PA10, PA20, and PA100 columns) have been used for these analyses.[7-14]

This study presents the analysis of wood hydrolysate samples using the Dionex CarboPac PA20 and PA200 columns. A single method—Method 1, which uses the Dionex CarboPac PA20 column—can be used for the simultaneous determination of mono- and disaccharides and uronic acids. However, Method 1 has a long run time and multiple dilutions of samples must be analyzed to quantify all the analytes. In contrast, the Dionex CarboPac PA200 column is specially designed to provide high-resolution separation of charged and neutral oligosaccharides and is appropriate for analysis of the charged uronic acids. Method 2 uses the Dionex CarboPac PA200 column to determine uronic acids, and can be paired with a fast (<10 min) method using the Dionex CarboPac SA10 column to determine mono- and disaccharides as an alternative to Method 1.[15,16]

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Goal

To develop robust methods for the determination of uronic acids in wood hydrolysates, two approaches are explored. Method 1, a single HPAE-PAD method, uses the Dionex CarboPac PA20 column for wood sugars and uronic acids. Method 2, a faster method, uses the Dionex CarboPac PA200 column that is specifically designed for the determination of uronic acids.

Equipment

- A Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ system, capable of supporting high-pressure ion chromatography, including:
  - SP Single Analytical Gradient Pump with Degas (075924) or DP Dual Analytical Gradient - Isocratic Pump with Degas (P/N 075928)
  - DC Detector/Chromatography Compartment
  - ED Electrochemical Detector (without cell, P/N 072042)
  - ED Cell (no reference or working electrode, P/N 072044)
  - Gold (Au) on Polytetrafluororethylene (PTFE) Disposable Electrode (P/N 066480)
  - pH, Ag/AgCl Reference Electrode (P/N 061879)
- Thermo Scientific Dionex AS-AP Autosampler
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software

Consumables

- Vial Kit, 0.3 mL Polypropylene with Caps and Septa (P/N 055428)
- Thermo Scientific™ Nalgene™ MR75 Series Sterile Disposable Culture Filter Units, 1000 mL, 0.2 µm (VWR P/N 28198-514)

Reagents and Standards

Reagents

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better, filtered through a 0.2 µm filter immediately before use
- Sodium Hydroxide Solution, 50% w/w/Certified (Fisher Scientific P/N SS254-500)
- Sodium Acetate Salt (Fisher Scientific P/N 059326)*

*Use of this electrochemical-grade sodium acetate reagent is strongly recommended for carbohydrate analysis.

Standards

- L(–)-Fucose (Fisher Scientific P/N AC22588-0010)
- D-Galactose (Fisher Scientific P/N S25334)
- D(+)-Mannose (Fisher Scientific P/N AC15060-1000)
- D-Fructose (Fisher Scientific P/N L96-500)
- D-Xylose (Fisher Scientific P/N X9-25)
- Sucrose (Fisher Scientific P/N S5500)
- D-Glucose (Fisher Scientific P/N D16-1)
- D-Arabinose (Fisher Scientific P/N S25650)
- D(+)-Cellobiose (Fisher Scientific P/N AC108460250)
- D(+)-Galacturonic Acid Monohydrate (Fisher Scientific P/N AC22782-0050)
- D(+)-Glucuronic Acid (Fisher Scientific AC20457-0250)

Conditions

Method 1

Columns:	Dionex CarboPac PA20 Guard, 3 × 30 mm (P/N 060144) Dionex CarboPac PA20 Analytical, 3 × 150 mm (P/N 060142)
Eluent:	A. DI water B. 200 mM sodium hydroxide C. 100 mM sodium hydroxide, 100 mM sodium acetate

Gradient:

Time (min)	A (%)	B (%)	C (%)
0.0	98.8	1.2	0
18.0	98.8	1.2	0
20.0	50	50	0
30.0	50	50	0
30.1	0	0	100
46.0	0	0	100
46.1	0	100	0
50.0	0	100	0
50.1	98.8	1.2	0
60.0	98.8	1.2	0

Flow Rate: 0.4 mL/min

Method 2

Columns:	Dionex CarboPac PA200 Guard, 3 × 50 mm (P/N 062895) Dionex CarboPac PA200 Analytical, 3 × 250 mm (P/N 062896)
Eluent:	A. 1 M sodium acetate, 100 mM sodium hydroxide B. 100 mM sodium hydroxide

Gradient:

Time (min)	A (%)	B (%)
0.0	2	98
18.0	2	98
18.1	50	50
22.0	50	50
22.1	2	98
30.0	2	98

Flow Rate: 0.5 mL/min

Methods 1 and 2

Injection Volume:	10 µL
Column Temperature:	30 °C
Cell Temperature:	30 °C
Backpressure:	~2500 psi
Detection:	PAD
Background:	30–70 nC
Working Electrode:	Au on PTFE Disposable
Reference Electrode:	
Mode:	Ag/AgCl mode
Noise:	30–60 pC

Carbohydrate Waveform
Carbohydrate 4-Potential Waveform for the Dionex ED

Time (s)	Potential (V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	–2.0	Off	On	Off
0.42	–2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	–0.1	Off	On	Off
0.50	–0.1	Off	On	Off

*Settings required in the Dionex ICS-3000/5000 systems but not used in older Dionex systems. Reference electrode in Ag/AgCl mode.

Preparation of Solutions and Reagents

Eluent Solution

Sodium hydroxide, 200 mM

Use high-quality water of high resistivity (18 MΩ-cm) containing as little dissolved carbon dioxide as possible. Biological contamination must be absent. Obtain source water using a water purification system consisting of filters manufactured without electrochemically active substances (i.e., glycerol). Perform prior filtration through 0.2 µm porosity nylon under vacuum to remove particulates and reduce dissolved air. It is important to minimize contamination by carbonate—a divalent anion at high pH that binds strongly to the column—which can cause a loss of chromatographic resolution and efficiency. Do not use commercially available sodium hydroxide pellets that are covered with a thin layer of sodium carbonate. A 50% w/w sodium hydroxide is much lower in carbonate and is the recommended source for sodium hydroxide.

Dilute 10.4 mL of 50% w/w sodium hydroxide in thoroughly degassed water to a final volume of 1000 mL, thereby yielding a 100 mM sodium hydroxide solution. Do not shake the 50% w/w sodium hydroxide stock. Pipette the required aliquot from the middle of the stock solution where sodium carbonate is least likely to have formed. Do not pipette from the bottom where sodium carbonate precipitate may have fallen. Keep the eluents blanketed under 34–55 kPa (5–8 psi) of nitrogen at all times to reduce diffusion of atmospheric carbon dioxide and minimize microbial contamination.

Sodium acetate, 100 mM/1 M

Dissolve 8.2 g (for 100 mM) or 82 g (for 1 M) of sodium acetate in 400 mL of water. Once the solid has dissolved, add 5.2 mL of 50% sodium hydroxide, and dilute the solution with an additional 400 mL of DI water. Filter degas the solution through a 0.2 µm nylon filter unit, transfer to a 1 L polypropylene volumetric flask, and bring it to volume. See Technical Note 71 for detailed eluent preparation requirements.[17]

Stock Standard Solutions

Dissolve solid standards in DI water to prepare a 1 g/L stock solution for each carbohydrate. Maintain the stock solution at –20 °C until needed.

Working Standard Solutions

Prepare working standards in DI water by diluting the stock solutions. Store working standards at 4 °C. Make all dilutions gravimetrically to ensure high accuracy. For uronic acid calibration, use concentrations in the range 0.01 to 10 mg/L (0.01, 0.1, 0.5, 1.0, 5.0, and 10 mg/L).

Sample Preparation

Wood Acid Hydrolysates

Samples were kindly donated by the National Renewable Energy Laboratory in Golden, Colorado. The liquor sample contained 1% sulfuric acid.

Centrifuge Lodgepole Pine acid hydrolysate samples (liquor and rinsate) at 16,000 g for 10 min to ensure elimination of particulates. For analysis, inject at the dilutions (with DI water) shown in the figures.

Precautions: Treated biomass samples have high concentrations of sugars (e.g., xylose, glucose, and galactose) that can cause carryover. Perform a syringe flush of 500 µL DI water between samples to reduce carryover. Replace the reference electrode every six months and the disposable working electrodes every four weeks.

Results and Discussion

Separation Using Method 1 on the Dionex CarboPac PA20 Column

The separation of 10 carbohydrates and two uronic acid standards (0.5 mg/L) using Method 1 on the Dionex CarboPac PA20 column is shown in Figure 1. In the initial 18 min, a low sodium hydroxide concentration (2.4 mM) was used to separate the neutral monosaccharides and disaccharide (Peaks 1–9). There was baseline resolution of all target sugars except for rhamnose and arabinose (Peaks 2 and 3). A stronger hydroxide concentration (100 mM) was needed for cellobiose (Peak 10), which eluted at ~30 min. The charged uronic acids are strongly retained and eluted with 100 mM sodium acetate and 100 mM sodium hydroxide at ~35 min (Peaks 11 and 12). This was followed by a column wash and re-equilibration, resulting in a total run time of 60 min.

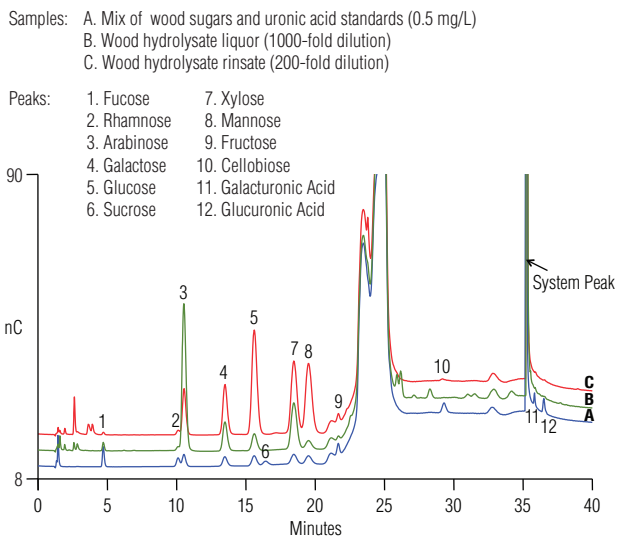

Figure 1. Separation of 10 wood sugars and two uronic acids using Method 1 on the Dionex CarboPac PA20 column in (A) a mix of standards and (B and C) wood hydrolysate samples.

Figure 1, Chromatograms B and C show the separation of the target compounds in wood-based acid hydrolysate samples (200- and 1000-fold dilutions for rinsate and liquor, respectively). These dilutions are appropriate for quantitating some sugars (e.g., fucose, galactose, and fructose), but higher/multiple dilutions may be needed for sugars that are present at high concentrations (e.g., arabinose, glucose, xylose, and mannose). In contrast, to quantify the uronic acids (Peaks 11 and 12 in Figures 2 and 3), samples must be analyzed at 10-fold less dilution. In the less-diluted samples, the more concentrated sugars overload the column (Peaks 3–8 in Figure 2). Thus, it is clear that when using Method 1 on the Dionex CarboPac PA20 column, samples must be run at multiple dilutions to quantitate all the wood sugars and uronic acids. Therefore, an alternative to this analysis is presented here.

For carbohydrate determinations in biomass samples (e.g., corn stover and wood hydrolysates), faster HPAE-PAD-based methods have been reported using the Dionex CarboPac SA10 and SA10-4µm columns to separate common biomass sugars (fucose, sucrose, arabinose, galactose, glucose, xylose, mannose, fructose, and cellobiose) in <10 min.[15,16] Additional hardware modifications (i.e., a thicker gasket in the electrochemical cell, and a reduced injection volume of 0.4 µL using an injection valve with an internal loop) allow for increased linear range, enabling easier handling of high-concentration samples. However, this method cannot be used for analysis of uronic acids in high-concentration biomass samples (data not shown).

Uronic acids can be analyzed on the Dionex CarboPac PA200 column, which is specially designed for high-resolution separation of charged oligosaccharides. Figure 4 shows the separation of uronic acid standards and wood hydrolysate samples using Method 2 on the Dionex CarboPac PA200 column. The uronic acids were separated with 20 mM sodium acetate in 100 mM sodium hydroxide. Galacturonic and glucuronic acids eluted at ~14 and 16 min, respectively. This was followed with a higher concentration of acetate (500 mM) to wash the column before equilibrating to initial conditions, resulting in a total run time of 30 min.

The amounts of uronic acid in the samples measured using both methods (corrected for their respective dilutions) are summarized in Table 1. Both methods yielded similar amounts of both uronic acids. The amount of uronic acid in these samples ranged from ~4 to 50 mg/L.

Table 1. Uronic acid quantitation by Method 1 (using the Dionex CarboPac PA20 column) and Method 2 (using the Dionex CarboPac PA 200 column) in wood hydrolysates.

Uronic Acid	Sample 1		Sample 2	
	Method 2	Method 1	Method 2	Method 1
	Amount Present Corrected for Dilution (mg/L)			
Galacturonic	9.40	9.76	47.2	46.2
Glucuronic	4.26	4.39	23.0	21.6

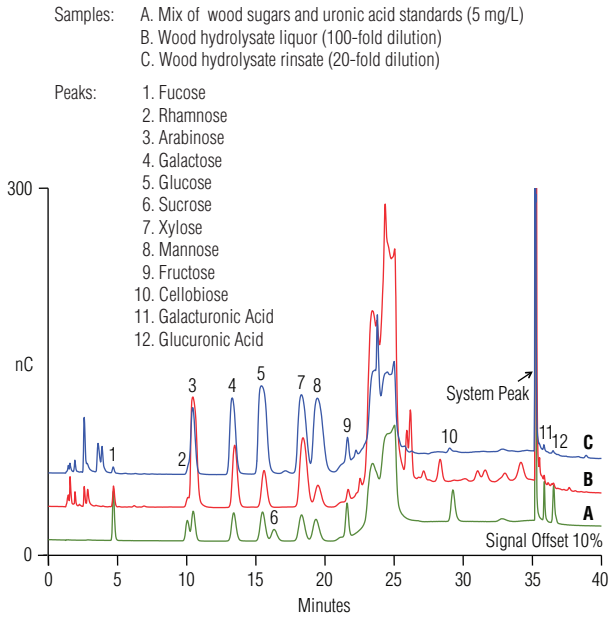

Figure 2. Separation of 10 wood sugars and two uronic acids using Method 1 on the Dionex CarboPac PA20 column in (A) a mix of standards and (B and C) wood hydrolysate samples.

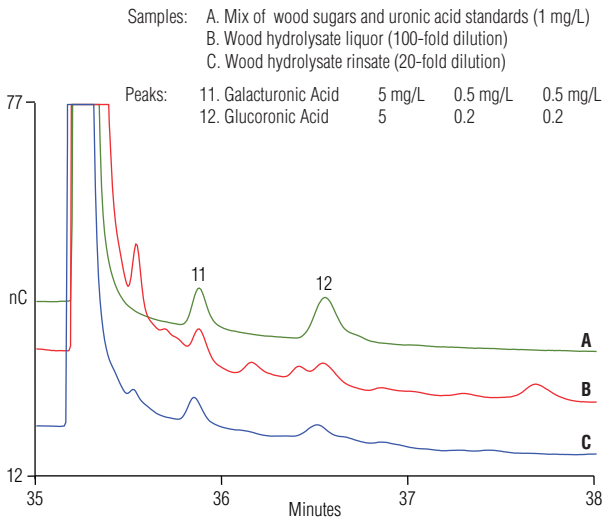

Figure 3. Separation (35–38 min) of two uronic acids using Method 1 on the Dionex CarboPac PA20 column in (A) a mix of standards and (B and C) wood hydrolysate samples. Conditions are the same as in Figure 2.

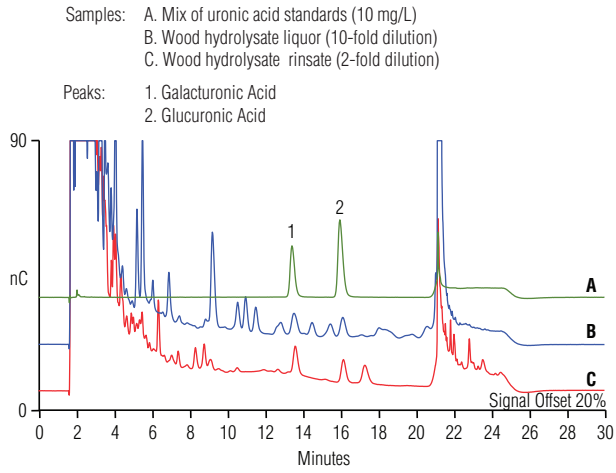

Figure 4. Separation of uronic acids using Method 2 on the Dionex CarboPac PA200 column in (A) a mix of standards and (B and C) wood hydrolysates.

Precision

The precision of an analytical procedure is usually expressed as the RSD of a series of measurements. For both methods, the peak area and retention time (RT) precisions were determined for six replicate injections of a mixture of sugar and/or uronic acid standards (Table 2). The concentrations used for precision injections were 0.5 mg/L on the Dionex CarboPac PA20 column and 1.0 mg/L on the Dionex CarboPac 200 column. For the hydrolysate samples, the RT precisions (RSD) were <0.1% and the peak area precisions ranged from 0.7 to 5.0%. The high precisions indicate that both methods can be used for analysis of carbohydrates in wood hydrolysates.

Linear Range

In various wood acid hydrolysates, the major sugars are present in concentrations ranging from 50 to 100 g/L, whereas the minor components are present in the 0.01 to 10 g/L range. Samples were diluted so that the analyte concentration fell within the linear range. Linearity for the two uronic acids was determined by injecting calibration standards ranging from 0.01 to 10 mg/L in triplicate. Galacturonic acid was linear, with the coefficients of determination being 0.9997 and 0.9982 on the Dionex CarboPac PA20 and PA200 columns, respectively. Glucuronic acid was also linear, with the coefficients of determination being 0.9999 and 0.9990, respectively, on the two columns.

Accuracy

The accuracy of the uronic acid method on the Dionex CarboPac PA200 column (Method 2) was verified by determining recoveries of uronic acids in spiked wood acid hydrolysate samples (Sample 1 was a rinsate and Sample 2 was a liquor) over three consecutive days (Table 3). Samples were spiked with 5 mg/L for both uronic acids. Recoveries were calculated from the difference in response between the spiked and unspiked samples. The average recovery for the uronic acids ranged from 80 to 90%, indicating that Method 2 can accurately determine uronic acids in these complex acid hydrolysate samples.

Table 2. Method precisions.

Analyte	RT (min)	RT RSD	Area (nC * min)	Peak Area RSD
Method 1 Using the Dionex CarboPac PA20 Column				
Fucose	4.71	0.07	0.860	2.08
Rhamnose	10.10	0.03	0.665	1.11
Arabinose	10.53	0.06	0.998	0.96
Galactose	13.49	0.05	0.968	0.68
Glucose	15.59	0.06	1.044	3.53
Sucrose	16.44	0.08	0.385	1.80
Xylose	18.44	0.05	1.253	2.35
Mannose	19.49	0.04	1.024	1.32
Fructose	21.66	0.02	0.556	1.63
Cellobiose	29.36	0.03	0.915	2.49
Galacturonic Acid	35.89	0.09	0.296	1.98
Glucuronic Acid	36.57	0.08	0.593	1.79
Method 2 Using the Dionex CarboPac PA200 Column				
Galacturonic Acid	13.73	0.08	0.500	2.16
Glucuronic Acid	16.34	0.06	0.899	1.38

Table 3. Recovery of uronic acids using Method 2 on the Dionex CarboPac PA200 column.

Sample 1 (2-Fold Dilution)							
Analyte	**Amount Present (mg/L)**	**Spike 1 Measured (mg/L)**	**Recovery (%)**	**Spike 2 Measured (mg/L)**	**Recovery (%)**	**Spike 3 Measured (mg/L)**	**Recovery (%)**
Galacturonic Acid	4.70	9.40	94	9.00	86	9.40	94
Glucuronic Acid	2.13	6.65	90	6.40	85	6.16	81
Sample 2 (10-Fold Dilution)							
Analyte	**Amount Present (mg/L)**	**Spike 1 Measured (mg/L)**	**Recovery (%)**	**Spike 2 Measured (mg/L)**	**Recovery (%)**	**Spike 3 Measured (mg/L)**	**Recovery (%)**
Galacturonic Acid	4.72	9.20	90	9.50	96	9.20	90
Glucuronic Acid	2.30	6.50	84	6.80	90	6.30	80

Conclusion

This study describes two HPAE-PAD-based methods for the determination of uronic acids in acid-hydrolyzed wood samples. Method 1, which uses the Dionex CarboPac PA20 column, separates common sugars as well as uronic acids. This method has a relatively longer run time (60 min), and samples must run at multiple dilutions to quantitate all the carbohydrates of interest. Method 2, as described in AN 282 and AU 192, uses the Dionex CarboPac PA200 column for accurate determination of uronic acids. For a complete analysis (i.e., wood sugars and uronic acids), the rapid sugar method on the Dionex CarboPac SA10 column can be used in conjunction with the uronic acid method (Method 2) on the Dionex CarboPac PA200 column.

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