

# Determination of Carbohydrates in Acid Hydrolysates of Wood

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## Key Words

Dionex CarboPac SA10-4 $\mu$ m Column, 62 mil Gasket, 0.4  $\mu$ L Injection Valve, Dionex ICS-5000+ System, Rhamnose, Wood Liquor

## Introduction

Biofuels are now widely accepted as an alternative to fossil fuels.<sup>1,2</sup> Currently, biomass from corn and sugar cane are typically used to produce biofuel. Another promising source of biomass for biofuel production is wood, especially because it is a nonfood source. Compared to corn and other food crops, wood-based biofuels are considered more sustainable. Wood has other inherent advantages: trees can be grown with less irrigation and fertilizers, and can be harvested year round. Fuel made from wood is expected to become a competitive commercial alternative to corn-based fuel by 2020.<sup>3</sup>

The determination of carbohydrates in wood hydrolysates is of crucial importance during biofuel production. The breakdown of lignin and cellulose in wood into fermentable carbohydrates is monitored to maximize the efficiency of biomass-to-biofuel conversion, and is directly related to ethanol yield. Liquid chromatography, including high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), can be used to determine carbohydrates in the acid hydrolysates of wood.<sup>4,5</sup>

HPAE-PAD has been shown to deliver fast carbohydrate determinations in plant-derived samples using the Thermo Scientific™ Dionex™ CarboPac™ SA10 column.<sup>6,7</sup> Determination of carbohydrates in acid-hydrolyzed corn stover samples at 100-fold dilution was shown in Thermo Scientific Application Note 282.<sup>6</sup> The method uses electrolytically generated hydroxide eluent and a 15 mil gasket (as opposed to the standard 2 mil gasket) in the electrochemical flow cell to resolve the common biomass sugars (xylose, sucrose, arabinose, galactose, glucose, xylose, mannose, and fructose) in <8 min. More recently, two hardware modifications were applied in Thermo Scientific Application Update (AU) 192 for easy handling of high-concentration samples:

- Injection volume was reduced from 10 to 0.4  $\mu$ L.
- Thickness of the spacer gasket was increased to 62 mil in the electrochemical flow cell to reduce detection sensitivity.<sup>7</sup>



Due to the increased linear range of the modified method, carbohydrates present in acid-hydrolyzed corn stover can generally be measured using a 10-fold dilution, instead of the greater dilution (or multiple dilutions) needed with the 15 mil gasket.

This work develops methods for the determination of carbohydrates in acid hydrolysates of wood using the new Dionex CarboPac SA10-4 $\mu$ m column, along with the aforementioned modifications. This version of the column (which contains smaller resin particles) enables high-resolution, high-efficiency separation, particularly for the sugar pair rhamnose-galactose—sugars that have been challenging to resolve with existing methods. As expected, the smaller resin particles produce increased backpressure compared to the traditional Dionex CarboPac SA10 column. Therefore, the Dionex CarboPac SA10-4 $\mu$ m column must be configured with the new Thermo Scientific™ Dionex™ ICS-5000+ HPIC™ system, which is capable of high-pressure ion chromatography (IC). Method performance parameters (precision, accuracy, linearity) demonstrate that the described methods are fast, provide good sensitivity and consistent response, and can be routinely used to determine sugars in wood samples.

## Goal

To determine the individual carbohydrates in acidic hydrolysates of wood using a column with resin particles smaller than those of the standard column, and achieve high-resolution separation of sugars, especially those pairs that have been challenging to resolve using existing methods

## Equipment

- Dionex ICS-5000\* HPIC system, including:
  - SP Single Pump or DP Dual Pump with the vacuum degas conversion kit (P/N 063353) installed\*
  - DC Detector/Chromatography Compartment
  - 4-port Valve Rebuild Kit (P/N 074699), which includes a 0.4  $\mu$ L injection loop
  - ED Electrochemical Detector (without cell P/N 072042)
  - Electrochemical Cell with reference electrode (P/N AAA-061756)
  - Gold on Polytetrafluoroethylene (PTFE) Disposable Electrode (P/N 066480)
  - pH, Ag/AgCl Reference Electrode (P/N 061879)
  - High Concentration Carbohydrate Analysis Kit (includes 62 mil PTFE gasket and modified spacer block, P/N 085324)

- Thermo Scientific Dionex AS-AP Autosampler

- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software

\* Refer to Dionex ICS-3000 EG Vacuum Degas Conversion Kit Installation Instructions (Document No. 065067) for more information.

## Consumables

- Vial Kit, 0.3 mL Polypropylene with Caps and Septa (P/N 055428)
- Nalgene™ MF75™ Series Sterile Disposable Tissue Culture Filter Units, 1000 mL, 0.2  $\mu$ m (Fisher Scientific P/N 09-740-46)
- Thermo Scientific Dionex EGC III KOH Eluent Generator Cartridge
- Thermo Scientific Dionex CR-ATC Continuously Regenerated Anion Trap Column

## Reagents and Standards

### Reagents

Deionized (DI) water, Type I reagent grade, 18 M $\Omega$ -cm resistivity or better, filtered through a 0.2  $\mu$ m filter immediately before use

### 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)

## Conditions

### Method

Columns:	Dionex CarboPac SA10 Guard, 4 $\times$ 50 mm (P/N 074902) Dionex CarboPac SA10 Analytical, 4 $\times$ 250 mm (P/N 074641) Dionex CarboPac SA10-4 $\mu$ m Analytical, 4 $\times$ 250 mm (P/N 088233)
Eluent:	Potassium Hydroxide (KOH), 1 mM
Eluent Source:	Dionex EGC III KOH Eluent Generator Cartridge with Dionex CR-ATC Continuously Regenerated Anion Trap Column
Flow Rate:	1.5 mL/min (Method 1) 1.2 mL/min (Method 2)
Injection Volume:	0.4 $\mu$ L Internal Loop
Column Temperature:	45 $^{\circ}$ C (Method 1), 30 $^{\circ}$ C (Method 2)
Cell Temperature:	20 $^{\circ}$ C*
Detection:	PAD
Background:	30–70 nC
Working Electrode:	Gold on PTFE Disposable
Electrochemical Cell Gasket:	62 mil
Reference Electrode	
Mode:	Ag/AgCl mode
Noise:	30–60 pC

\* This application was run on a dual system with the detector compartment at 20  $^{\circ}$ C, which is optimal for the conductivity detector on the second system. This application can also be run with the detector compartment at 30  $^{\circ}$ C.

## Carbohydrate Waveform

### Carbohydrate 4-Potential Waveform for the 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 on the Dionex ICS-3000/5000 system but not used on older Dionex IC systems, reference electrode in Ag/AgCl mode

## Preparation of Solutions and Reagents

### Eluent Solution

#### Potassium hydroxide, 1 mM

Generate the KOH eluent on line by pumping high-quality degassed DI water through the Dionex EGC III KOH Eluent Generator Cartridge. Chromeleon CDS software tracks the amount of KOH used and calculates the remaining cartridge lifetime.

Although eluents can be prepared manually, if needed, it is best to run this application using eluents prepared by an eluent generator. Use of manually prepared eluents is not recommended, nor can the performance be guaranteed with manually prepared eluents. Consistent preparation of a 1 mM hydroxide eluent or a 10 mM hydroxide eluent (if proportioning is used) is difficult, due to variable carbonate contamination. The impact of carbonate contamination is significant when using low-concentration hydroxide eluents. If eluents must be prepared manually, use NaOH rather than KOH, and prepare according to the general instructions for hydroxide eluents in Dionex Technical Note 71.<sup>8</sup> For this application, electrolytic eluent generation delivers superior performance.

### Stock Standard Solutions

Dissolve solid standards in DI water to prepare a 200 mg/mL 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. The concentrations used for calibration will be in the range of 0.05 to 2.4 g/L (0.05, 0.1, 0.3, 0.5, 0.8, 1.0, 1.6, 2.0, and 2.4 g/L).

## Sample Preparation

### Wood Acid Hydrolysates

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

Centrifuge wood acid hydrolysate samples (liquor and rinsate from lodgepole pine) at 16,000 g for 10 min to ensure elimination of particulates, then inject at dilutions of 1/2 or 1/50 with DI water for analysis.

### Precautions

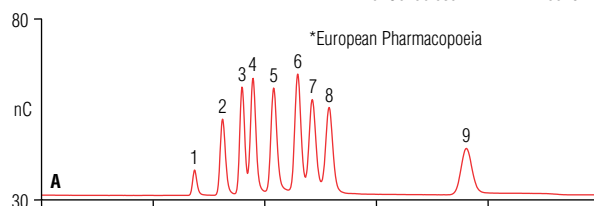
Carryover was initially observed because the treated biomass samples have high concentrations of sugars such as xylose, glucose, and galactose. A syringe flush of 500  $\mu$ L DI water will reduce carryover and is recommended between samples. A column wash at 100 mM KOH for 20 min is recommended if retention time (RT) shifting is observed. Typically, perform a column wash once every five to six days (~300 injections) to maintain RT and peak area RSD <8%. The application of 100 mM KOH changes the system equilibrium; therefore, re-equilibrate at 1 mM for 30 min after a column wash to achieve high precision. Replace the reference electrode every six months and the disposable working electrodes every four weeks.

## Results and Discussion

### Separation

The carbohydrates of interest in acid hydrolysates of wood are fucose, sucrose, arabinose, galactose, rhamnose, glucose, xylose, mannose, fructose, cellobiose, and maltose. Figure 1 compares the separation of these carbohydrates on the 6  $\mu$ m (standard) and the new 4  $\mu$ m version of the Dionex CarboPac SA10 column using Method 1 (i.e., flow rate of 1.5 mL/min and column temperature of 45 °C). The 4  $\mu$ m format enables highly efficient separations and higher signal-to-noise ratios (S/Ns) than the standard column. The smaller particle-size column results in higher system backpressure, and the analysis is supported on a Dionex ICS-5000<sup>+</sup> HPIC system capable of handling pressures up to 5000 psi. Although the run time is similar for the two columns, there is a >40% increase in peak efficiencies with the 4  $\mu$ m column that allows for higher S/N and higher resolution.

Columns:	Dionex CarboPac SA10 Guard (4 $\times$ 50 mm)	
	Dionex CarboPac SA10 Analytical (4 $\times$ 250 mm)	
Flow Rate:	1.5 mL/min	Plates (EP*)
Temperature:	45 °C	1. Fucose 5968
Backpressure:	2300 psi	2. Sucrose 5417
		3. Arabinose 7208
		4. Galactose/Rhamnose 6733
		5. Glucose 6756
		6. Xylose 7543
		7. Mannose 6861
		8. Fructose 7332
		9. Cellobiose 6913



Columns:	Dionex CarboPac SA10 Guard (4 $\times$ 50 mm)	
	Dionex CarboPac SA10-4 $\mu$ m Analytical (4 $\times$ 250 mm)	
Flow Rate:	1.5 mL/min	Plates (EP)
Temperature:	45 °C	1. Fucose 7733
Backpressure:	4300 psi	2. Sucrose 7390
		3. Arabinose 10255
		4. Galactose/Rhamnose 9939
		5. Glucose 9894
		6. Xylose 11405
		7. Mannose 10363
		8. Fructose 10818
		9. Cellobiose 10946

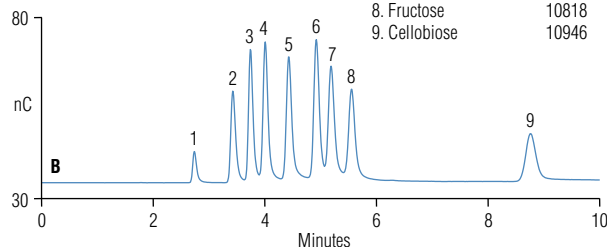


Figure 1. Separation of sugar standards on the Dionex CarboPac SA10 (A) and SA10-4 $\mu$ m (B) columns using Method 1.

Figure 2 presents the determination of carbohydrates in two wood acid hydrolysate samples (a rinsate and a liquor). Samples were analyzed after a 2- or 50-fold dilution and mainly contained arabinose, glucose, xylose, and mannose. The total run time for this analysis was 14 min. Under these conditions, however, galactose and rhamnose coelute.

This galactose-rhamnose pair can be resolved by lowering the temperature to 30 °C. Backpressure increases as temperature lowers, hence the flow rate was reduced to 1.2 mL/min. Under these conditions (Method 2), 11 wood sugars were separated, as shown in Figure 3. This figure shows separation on both versions of the Dionex CarboPac SA10 column and, as expected, the 4 µm column gives a higher-resolution separation than the 6 µm column. Under these conditions, however, sucrose-arabinose and fructose-mannose are no longer resolved. Hence, two methods are proposed, with Method 1 being used for all wood sugars except rhamnose while Method 2 is used for samples in which rhamnose is expected.

Figure 4 shows the presence of 0.14 g/L (corrected for dilution) rhamnose in a wood liquor sample.

*Note: Chromatographic performance is not affected by the presence of a high concentration of sulfate in the acid-hydrolyzed samples; neutralization or removal of sulfate is not needed prior to injection. The concentration of acid in the samples is typically ~70% during the early stages of hydrolysis and ~1–4% at later stages.<sup>4</sup> Dilute samples 2- or 50-fold for this application.*

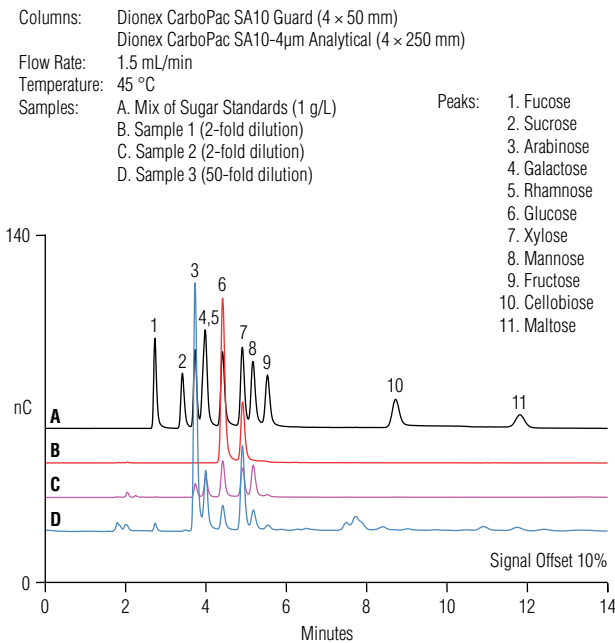


Figure 2. Separation of wood sugars on the Dionex CarboPac SA10-4µm column using Method 1.

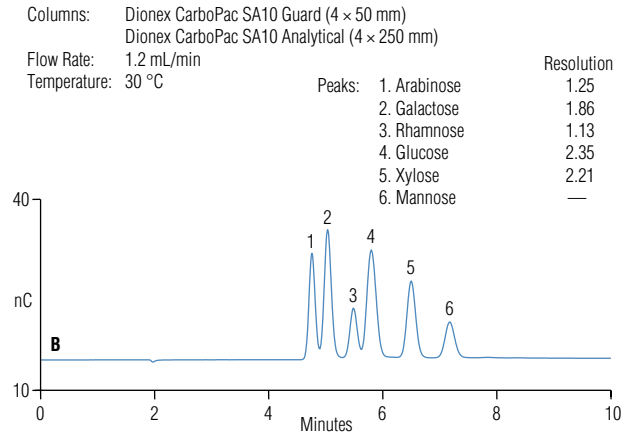
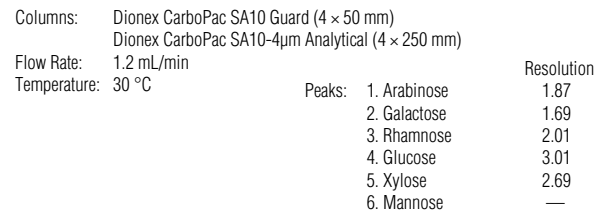


Figure 3. Comparison of the Dionex CarboPac SA10-4µm (A) and SA10 (B) columns for rhamnose determination using Method 2.

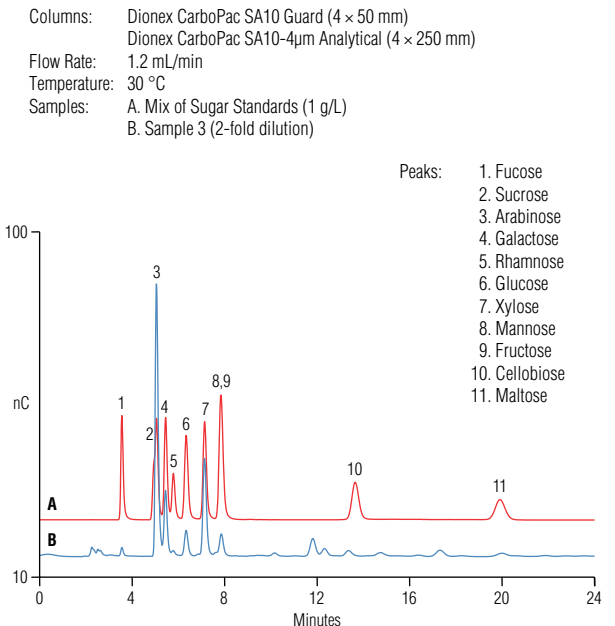


Figure 4. Determining rhamnose in a wood acid hydrolysate using Method 2.

## Precision

The precision of an analytical procedure is typically expressed as the RSD of a series of measurements. For both methods, the peak area and RT precisions were determined for six replicate injections of a mixture of sugar standards (Table 1). The concentration used for precision injections was 1.0 mg/mL for each of the sugars. The RT precisions (RSD) were <0.3 and the peak area precisions ranged from 2.0 to 3.4. The RT and peak area RSDs in a wood hydrolysate sample were <0.5 and 1–3.8, respectively. The high RT precisions were attributed to consistent generation of high-purity KOH using the eluent generator. With manually prepared mobile phases, the precisions—especially RT precision—will almost certainly not be as low as with eluent generation.

## Linear Range

In various wood acid hydrolysates, the major sugars were present in concentrations ranging from 50 to 100 g/L, whereas the minor components were present in the 0.1 to 10 g/L range (Table 2). Using the modifications suggested in AU 192—reduced sample volume and a thicker working electrode gasket—samples can be analyzed after a 2-, 10-, or 50-fold dilution. Linearity for Method 1 was determined by injecting calibration standards in triplicate ranging from 0.05 to 2.4 g/L. The sugars were linear, with the coefficient of determination ranging from 0.9980 to 0.9998. Similar linearity results were obtained for Method 2 (not shown).

Table 2. Linear range for Method 1.

Concentration Range 0.05–2.4 g/L	
Analyte	r <sup>2</sup> (Coefficient of Determination)
Fucose	0.9991
Sucrose	0.9980
Arabinose	0.9992
Galactose	0.9985
Glucose	0.9990
Xylose	0.9986
Mannose	0.9980
Fructose	0.9996
Cellobiose	0.9998
Maltose	0.9984

Table 1. Precision of wood sugars on the Dionex CarboPac SA10-4 $\mu$ m column.

Analyte	Standard				Sample			
	RT (Min)	RT (RSD)	Peak Area (nC * Min)	Peak Area (RSD)	RT (Min)	RT (RSD)	Peak Area (nC * Min)	Peak Area (RSD)
<b>Method 1</b>								
Fucose	2.735	0.12	3.2884	3.17	2.732	0.52	0.2592	3.28
Sucrose	3.418	0.10	2.3940	2.95	—	—	—	—
Arabinose	3.736	0.12	3.1008	2.60	3.714	0.13	9.6025	1.57
Glucose	4.417	<0.01	3.9936	2.20	4.395	0.22	0.9562	1.08
Xylose	4.909	<0.01	4.0176	2.81	4.881	0.10	3.7952	2.76
Mannose	5.174	0.07	3.7785	2.26	5.15	0.16	0.966	2.86
Fructose	5.54	0.11	3.3406	3.23	5.539	0.09	0.1744	3.18
Cellobiose	8.72	0.08	2.4227	3.28	—	—	—	—
Maltose	11.81	0.09	1.5106	3.41	—	—	—	—
<b>Method 2</b>								
Fucose	3.563	0.20	3.4421	1.77	3.566	0.18	0.0144	2.31
Galactose	5.45	0.08	4.0764	2.00	5.466	0.15	0.9642	3.12
Rhamnose	5.792	0.13	2.0400	2.25	5.791	0.19	0.0615	1.18
Glucose	6.342	0.07	4.1518	2.02	6.353	0.16	1.9097	3.50
Xylose	7.142	0.18	4.6737	2.15	7.154	0.16	1.6435	3.75
Cellobiose	13.674	0.27	3.4199	1.91	—	—	—	—
Maltose	19.95	0.21	2.6290	2.18	—	—	—	—



### Accuracy

Method accuracy was verified by determining recoveries of sugars in spiked wood acid hydrolysate samples (Sample 1 was a rinsate and Sample 2 was a liquor) over three consecutive days (Table 3). Sucrose is not stable in these acidic hydrolysate samples. Sucrose hydrolyzes into glucose and fructose (data not shown). Samples were cooled prior to spiking, then immediately injected for analysis.

*Note: If using a spiking solution containing a mix of sugars, do not add sucrose to the spiking solution because it will give erroneous results for glucose and fructose.*

Samples were spiked to yield an additional concentration of 0.4 g/L of each sugar. Recoveries were calculated from the difference in response between the spiked and unspiked samples. The average recovery for the sugars with both methods ranged from 71 to 103%, indicating that these methods can accurately determine the sugars of interest in these complex acid hydrolysate samples.

### Column Reproducibility

Columns from three different production lots were evaluated for column reproducibility. RSDs for arabinose efficiency, xylose-mannose resolution, and xylose RT for three separate columns with an injection of 1 g/L mix of sugar standards were <5%.

Table 3. Accuracy of carbohydrates in wood acid hydrolysates (Methods 1 and 2).

Analyte	Sample 1			Sample 2		
	Amount Present (g/L)	Amount Measured (g/L)	Recovery (%)	Amount Present (g/L)	Amount Measured (g/L)	Recovery (%)
<b>Method 1</b>						
Fucose	—	0.3860	97	0.0620	0.4630	100
Sucrose	—	0.3963	99	—	0.3984	100
Arabinose	0.1559	0.5413	96	3.2421	a	
Glucose	0.4216	0.7805	90	0.3158	0.6992	96
Xylose	0.3277	0.6829	89	1.0320	1.3177	71
Mannose	0.4150	0.7391	81	0.2974	0.6473	87
Fructose	0.0524	0.4052	88	0.1096	0.4587	87
Cellobiose	—	0.3129	78	—	0.3220	81
Maltose	—	0.3346	84	0.2376	0.5530	79
<b>Method 2</b>						
Fucose	0.0043	0.4143	103	0.0923	0.4281	90
Galactose	0.2377	0.6236	96	0.7750	1.0778	81
Rhamnose	0.0301	0.4142	96	0.1366	0.4784	93
Glucose	0.4729	0.4729	97	0.3200	0.7177	99
Xylose	0.3555	0.7299	94	1.0653	1.3556	78
Cellobiose	—	0.3606	90	b		
Maltose	—	0.3747	94	0.1339	0.4418	82

a. The arabinose concentration in this sample was at the upper limit of calibration.

b. There is an unknown peak in Sample 2 that interferes with cellobiose quantification.

## Conclusion

This study describes two rapid and robust HPAE-PAD methods for the accurate determination of common sugars in acid-hydrolyzed wood samples. Both methods use the Dionex CarboPac SA10-4 $\mu$ m column with electrolytically generated hydroxide eluent, reduced sample size, and the thicker gasket for the working electrode. The smaller resin particles used in the 4  $\mu$ m column allow for higher-efficiency separations compared to the standard column. In particular, Method 2 allows for better resolution of galactose and rhamnose compared to the standard column.

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