

## SIMULTANEOUS SEPARATION AND QUANTITATIVE DETERMINATION OF MONOSACCHARIDES, URONIC ACIDS, AND ALDONIC ACIDS BY HIGH PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY COUPLED WITH PULSED AMPEROMETRIC DETECTION IN CORN STOVER PREHYDROLYSATES

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A method for simultaneous separation and quantitative determination of arabinose, galactose, glucose, xylose, xylonic acid, gluconic acid, galacturonic acid, and glucuronic acid was developed by using high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). The separation was performed on a CarboPac™ PA-10 column (250 mm × 2 mm) with a various gradient elution of NaOH-NaOAc solution as the mobile phase. The calibration curves showed good linearity ( $R^2 \geq 0.9993$ ) for the monosaccharides, uronic acids, and aldonic acids in the range of 0.1 to 12.5 mg/L. The detection limits (LODs) and the quantification limits (LOQs) were 4.91 to 18.75  $\mu\text{g/L}$  and 16.36 to 62.50  $\mu\text{g/L}$ , respectively. Relative standard deviations (RSDs) of the retention times and peak areas for the seven consecutive determinations of an unknown amount of mixture were 0.15% to 0.44% and 0.22% to 2.31%, respectively. The established method was used to separate and determine four monosaccharides, two uronic acids, and two aldonic acids in the prehydrolysate from dilute acid steam-exploded corn stover within 21 min. The spiked recoveries of monosaccharides, uronic acids, and aldonic acids ranged from 91.25% to 108.81%, with RSDs ( $n=3$ ) of 0.04% ~ 6.07%. This method was applied to evaluate the quantitative variation of sugar and sugar acid content in biomass prehydrolysates.

*Keywords:* Uronic acid; Aldonic acid; Monosaccharide; High performance anion-exchange chromatography (HPAEC); Prehydrolysate from pretreated corn stover

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### INTRODUCTION

Biomass biorefineries have become research hotspots in recent years (Fairly 2011; Ragauskas *et al.* 2006). The composition of the raw materials and the changes in biological or chemical processing are the basic scientific questions being asked in the field, while the conversion and utilization of biomass polysaccharides are of great importance (Akin and Rigsby 2008). Cellulose, hemicellulose, lignin, and pectin account for most of the biomass in annual and perennial plants. In addition, higher plants contain

various amounts of uronic acid-containing hemicellulose and pectin. The cell wall polysaccharides consist mainly of different pentoses ( $\beta$ -D-xylose,  $\alpha$ -L-arabinose), hexoses ( $\beta$ -D-glucose,  $\beta$ -D-mannose,  $\alpha$ -D-galactose), and uronic acids ( $\beta$ -D-glucuronic acid,  $\alpha$ -D-galacturonic acid) (Sjöström 1993; Whistler 1993; Waldron and Faulds 2007). Furthermore, aldonic acids ( $\beta$ -D-gluconic acid,  $\beta$ -D-xylonic acid) commonly appear in pretreated biomass substrates as products from oxidized cellulose and hemicellulose. They are also produced from the peeling reaction of green liquor pretreatment (Lai and Sarkanen 1969). During processing, the pretreated liquor is further transformed as a mixture of monosaccharides, uronic acids, and aldonic acids. It should be taken under consideration that there will be significant differences when the raw materials are from different types, sources, and processes. Therefore, a rapid and accurate determination of the monosaccharides, uronic acids, and aldonic acids in pretreated biomass materials is of great significance.

Monosaccharides in hydrolysates are usually analyzed by gas chromatography with flame ionization detection (GC-FID) and gas chromatography with mass spectrometric detection (GC-MS) (Koivula and Hänninen 2001; Pitthard and Finch 2001). High performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are commonly used to separate monosaccharides (Lv *et al.* 2009; Guttman 1997; Dahlman *et al.* 2000), of which uronic acid is a derivative. Several methods have been used for quantitative analysis of uronic acids, such as the colorimetric method (Dubois *et al.* 1965), gas chromatography (GC) (Selvendran *et al.* 1979; Sundberg *et al.* 1996), high performance liquid chromatography (HPLC) (Lv *et al.* 2009), and capillary electrophoresis (CE) (Goubet *et al.* 2005).

Some restrictions of the methods above should be mentioned. GC is an established method with superb resolution and high sensitivity, but neutral monosaccharide recovery can be affected by the formation of by-products during the derivatisation procedure, due to the complex procedure or an incomplete derivatisation. Moreover, derivatisation is labor-intensive and time-consuming and therefore not particularly suitable for routine analyses. A defect of the HPLC-H column is the difficulty in separating the three hemicellulosic sugars xylose, galactose, and mannose. The HPLC-P column can differentiate xylose, mannose, and galactose, but the incompatibility of the lead in the column with the high sulfate content in the extract samples is a problem. The colorimetric method cannot detect each specific type of uronic acid; it only determines the total amount of uronic acid. Reversed-phase HPLC separation of the uronic acid also uses pre-column or post-column derivative, then UV detection.

Most analytical techniques for detecting aldonic acids are the same as uronic acids, but little research has been reported. Some methods, such as thin-layer chromatography (Hay *et al.* 1963), gas-liquid chromatography (Lehrfeld 1985), and gas chromatography with mass spectrometric detection (Schadewaldt *et al.* 2004) require long development times, lack resolution or sensitivity, or are difficult to quantify. The most noteworthy problem is that every procedure is complicated by the tendency of a pure aldonic acid to form mixtures consisting of 1,4- and 1,5-lactones in addition to free acid (Theander 1980). The relative amounts of each component vary with the specific acid temperature, concentration, solvent, pH, and time. In the method developed, mixtures of 1, 4- and 1, 5-lactones are not a problem. Both are converted to the same

sodium aldinate form under alkaline conditions. In addition, biomass prehydrolysates pretreated by steam explosion or other techniques give lower amounts of aldonic acids, so the methods above are not sensitive enough.

In comparison, high performance anion-exchange chromatography has the advantages of speed, high specificity, and high sensitivity, generally not requiring sample derivatisation. Additionally, the described method was applied for the determination of lower amounts of carbohydrates in pretreated biomass substrates without preconcentrating the samples. At present, high performance anion-exchange chromatography is being used conventionally to determine a variety of monosaccharides, and some of the uronic acids can also be determined (Saeed *et al.* 2011; Liang *et al.* 2006), but the simultaneous separation and quantitative determination of monosaccharides, uronic acids, and aldonic acids has not been reported.

This study presents a novel, simple, and quick method for the simultaneous determination of four monosaccharides, two uronic acids, and two aldonic acids in biomass prehydrolysates by high performance anion-exchange chromatography with pulsed amperometric detection.

## EXPERIMENTAL

### Materials

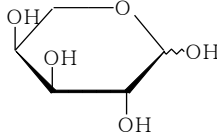
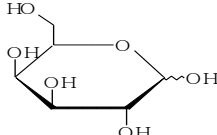
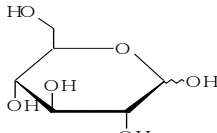
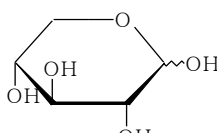
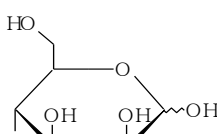
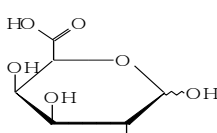
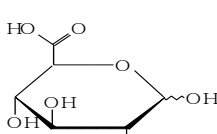
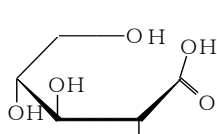
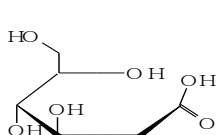
L(-)-Arabinose, D(+)-galactose, D(+)-xylose, D(+)-galacturonic acid, and sodium hydroxide (50% aqueous, w/w) were purchased from Fluka. D(+)-glucose, sodium gluconate, glucuronic acid, and sodium acetate were obtained from Sigma. Calcium xylonate was obtained from Toronto Research Chemicals, Inc. All were of analytical grade and served as reference compounds. Table 1 presents molecular formulas and weights, dissociation constants, and chemical structures of the sugars and sugar acids. Water was obtained from a Milli-Q purification system. All other chemicals were of the highest grade available.

### Pretreatment of Plant Samples

Corn stover obtained from Huhehaote, Inner Mongolia Autonomous Region, China, was immersed in 1.33% (w/v) sulfuric acid solution at 50 °C for 2 h and then air-dried to keep the solid content between 30% and 40%. The acid-impregnated corn stover was steam-exploded at 170 °C for 5.5 min, followed by washing with distilled water to a liquor/solid ratio of 7.5:1 (v/w). The pH of the mixture was adjusted to 5.0 with aqueous ammonia. The prehydrolysate was collected by filtration. The obtained prehydrolysate was stored at 4 °C and is referred to as steam-exploded prehydrolysate in this work. Prehydrolysate was diluted to the concentration of detection range and filtered with 0.22 µm filters after being centrifuged at 10000 rpm for 5 min. Finally, samples were transferred to an AS40 autosampler for analysis.

Black liquor was obtained from green liquor (GL)-pretreated corn stover. The prepared procedure was the same as described above.

**Table 1.** Molecular Formulas, Molar Masses, pKa Values (Zemann *et al.* 1997; Hummel *et al.* 2010), and Chemical Structures of the Monosaccharides, Uronic Acids, and Aldonic Acids

Compound	Molecular formula	MW (g/mol)	pK <sub>a</sub>	Chemical structure
L-(-)-Arabinose	C <sub>5</sub> H <sub>10</sub> O <sub>5</sub>	150.13	12.34	
D-(+)-Galactose	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	180.16	12.35	
D-(+)-Glucose	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	180.16	12.28	
D-(+)-Xylose	C <sub>5</sub> H <sub>10</sub> O <sub>5</sub>	150.13	12.15	
D-(+)-Mannose	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	180.16	12.08	
D-(+)-Galacturonic acid	C <sub>6</sub> H <sub>10</sub> O <sub>7</sub>	194.14	3.48	
D-(+)-Glucuronic acid	C <sub>6</sub> H <sub>10</sub> O <sub>7</sub>	194.14	3.20	
D-(+)-Xylonic acid	C <sub>5</sub> H <sub>10</sub> O <sub>6</sub>	166.13	3.65	
D-(+)-Gluconic acid	C <sub>6</sub> H <sub>12</sub> O <sub>7</sub>	196.16	3.70	

### Preparation of Standard Solution

Individual and mixed standard solutions (arabinose, galactose, glucose, xylose, xylonic acid, gluconic acid, galacturonic acid, and glucuronic acid) were prepared with deionized water. All the prepared solutions were stored in the dark at 4 °C until used.

### Anion-Exchange Chromatographic Method

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) used a Dionex ICS-3000 system with pulsed amperometric detector and a SP gradient pump with a degasser. The samples were filtered (0.22 µm membrane) and injected (10 µL) on a CarboPac PA-10 guard column (2 mm × 50 mm) attached to a CarboPac PA-10 anion-exchange analytical column (2 mm × 250 mm). The column was maintained at 30 °C in a cooler/heater. For the detection of carbohydrates, a Dionex ED40 Electrochemical Detector with a gold working electrode and an Ag/AgCl reference electrode was used in pulsed amperometry mode. Before each injection, the column was re-equilibrated by running for 15 min with 200 mM NaOH, followed by 10 min with 18 mM NaOH in order to achieve good repeatability. The eluent flow rate was kept at 0.3 mL/min. The pulse setting was the carbohydrates standard quadruple waveform, so named by Dionex. Data and graphs were collected and analyzed using Dionex Chromeleon 6.7 software. Three eluents were prepared in plastic bottles that were carefully purged using nitrogen. The eluents consisted of 18 mM NaOH (A), 200 mM NaOH (B), and 500 mM sodium acetate (C) containing 100 mM NaOH. The sugars and sugar acids were eluted using the programs given in Tables 2 and 3, respectively.

**Table 2.** Isocratic Program

Time (min)	A (%)	B (%)
0	100	0
10	100	0
10.1	0	100
25	0	100
25.1	100	0
40	100	0

A: 18 mM NaOH; B: 200 mM NaOH

**Table 3.** Gradient Program

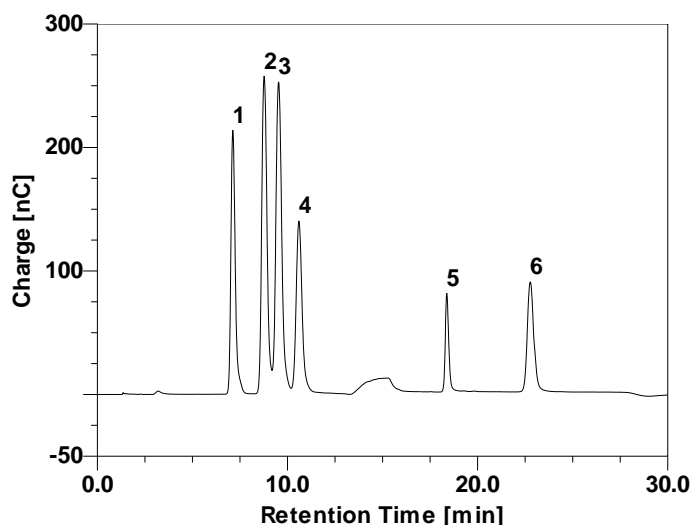
Time (min)	A (%)	B (%)	C (%)
0	100	0	0
10	100	0	0
10.1	0	90	10
20	0	60	40
20.1	0	100	0
40	0	100	0
40.1	100	0	0
50	100	0	0

A: 18 mM NaOH; B: 200 mM NaOH; C: 500 mM NaOAc containing 100 mM NaOH

## RESULTS AND DISCUSSION

### Optimization of Chromatographic Conditions

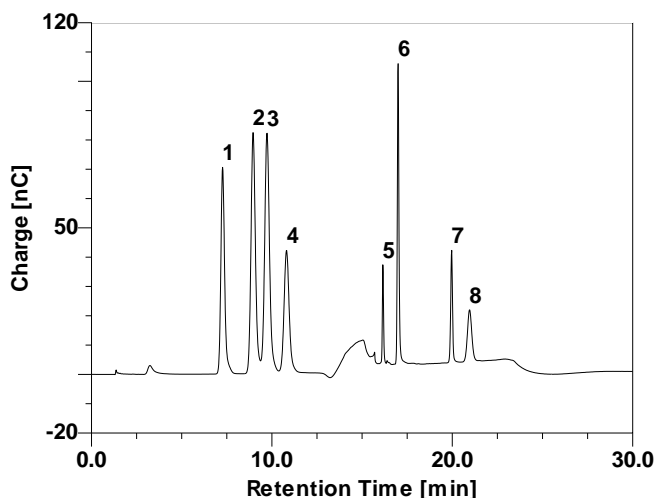
High performance anion-exchange chromatography (HPAEC) using polymer-based stationary phases and high pH in combination with pulsed amperometric detection (PAD) allowed direct quantification of underivatized carbohydrates. Elution at high pH value conditions allowed the carbohydrates to be present in their anion forms. Since the  $pK_a$  values of the neutral monosaccharides were in the range of 12 to 14 (Table 1), they acted as weak acids. At high pH, they were partially ionized, and the strong anion-exchange stationary phase could be used for separation. Gold electrodes were used for PAD, and in most instances the pH value of 0.1 M sodium hydroxide was high enough. A CarboPac PA-10 column was used in reducing mono- and disaccharide analysis. Monosaccharides were eluted using 18 mM sodium hydroxide between 0 and 10 min. However, xylose and mannose coelute on the PA-10 column in this method, so they could be modified using a CarboPac PA-1 analytical column if necessary (Lebet *et al.* 1997). Aldonic acids elute with 200 mM sodium hydroxide within 25 min (Fig. 1). However, uronic acids must be eluted with a linear gradient of 50 to 200 mM sodium acetate in continuously changing concentration of sodium hydroxide for 10 min because of their strong acidity (Fig. 2).



**Fig. 1.** Chromatograms of mixed standard solution by single NaOH conditions. Peak identifications: 1. arabinose; 2. galactose; 3. glucose; 4. xylose; 5. xylonic acid; 6. gluconic acid

On this basis, the elution mode, flow rate, temperature, and other chromatographic conditions were further optimized. The column efficiency, resolution, peak symmetry, column pressure (unpublished data), and other factors were considered, and optimal conditions were ultimately confirmed. The gradient program (Table 3), flow rate of 0.3 mL/min, and column temperature of 30 °C were selected during optimization. The results showed that it was possible for the method to achieve high efficiency and fast separation of eight standard components with resolutions of 1.5 within 21 min. In

addition, taking into account the cleaning requirements of the chromatographic system, ensuring full leaching out of the strong reservations in the sample, avoiding interference between the different samples, and extending the life of the analytical column, the total operation time should be extended to 50 min because the HPAEC-PAD system needs to be equilibrated with 18 mM sodium hydroxide.



**Fig. 2.** Chromatograms of mixed standard solution by optimal conditions. Peak identifications: 1. arabinose; 2. galactose; 3. glucose; 4. xylose; 5. xylonic acid; 6. gluconic acid; 7. galacturonic acid; 8. glucuronic acid.

### Method Validation

The established method was validated in terms of linearity, reproducibility, limits of detection (LODs), limits of quantification (LOQs), and precision. Linearity was verified by the analysis of six points in the range of 0.1 to 12.5 mg/L of standard sugars (glucose, xylose, galactose, arabinose) and sugar acids (glucuronic acid, galacturonic acid, gluconic acid, xylonic acid).

The linear regression parameters of the calibration curves are shown in Table 4. As a consequence, good linearity ( $R^2 \geq 0.9993$ ) between  $y$  (peak area of the standards) and  $x$  (concentration of the standards) was achieved in the tested range. Furthermore, LODs and LOQs were quantified from the threefold and tenfold signal-to-noise ratio, respectively. The results showed that the LODs of the four sugars and four sugar acids were in the range of 4.91 to 18.75  $\mu\text{g/L}$  (Table 4), indicating that the sensitivity of the method was satisfactory.

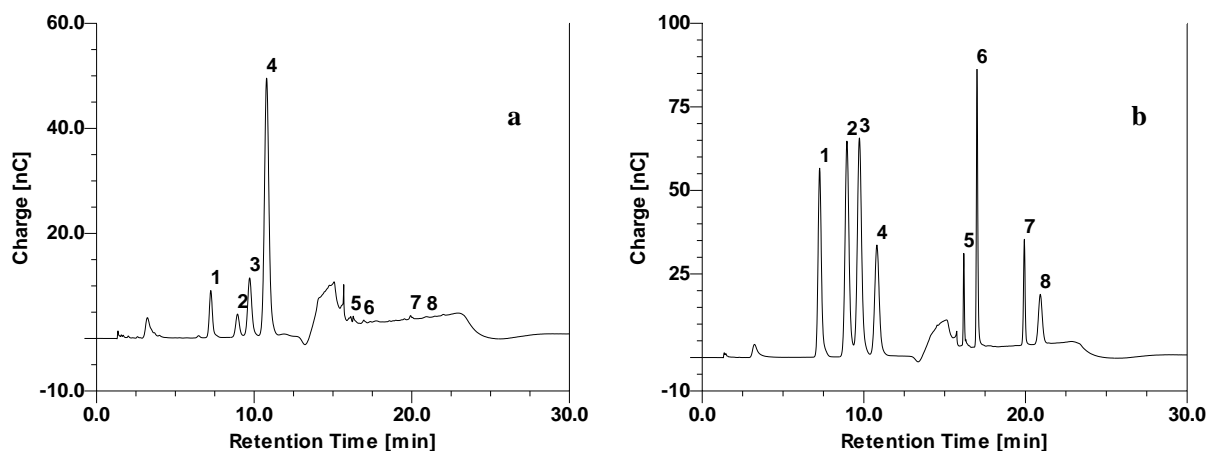
Repeatability (relative standard deviations, RSD %) for the migration time (MT) and the peak area (PA) are also given in Table 4. The repeatability was checked by repetitive injection of the unknown amounts of mixture sample ( $n = 7$ ). RSD values for MT and PA were less than 0.44% and 2.31%, respectively. In comparison with other accounts (Saeed *et al.* 2011; Liang *et al.* 2006; Lebet *et al.* 1997), the parameter values in this study were similar or better, indicating that the method precision was satisfactory.

**Table 4.** Linearity Range, Retention Time, Correlation Coefficient, Regression Equation, LOD, LOQ, and Repeatability

Compound	Linearity Range/ (mg/L)	Ret. Time/ min	Coeff. Det./ %	Regression Equation	LOD/ ( $\mu\text{g/L}$ )	LOQ/ ( $\mu\text{g/L}$ )	Repeatability (RSD)/ % (n = 7)	
							Time	Area
Arabinose	0.1-12.5	7.23	99.93	$Y=2.74X+0.17$	8.93	29.76	0.39	0.22
Galactose	0.1-12.5	8.92	99.98	$Y=3.78X+0.13$	7.44	24.80	0.39	0.49
Glucose	0.1-12.5	9.68	99.97	$Y=4.11X+0.03$	7.62	25.41	0.43	1.44
Xylose	0.1-12.5	10.77	99.97	$Y=2.16X+0.12$	13.69	45.62	0.44	1.69
Xyonic Acid	0.1-12.5	16.15	99.98	$Y=0.43X+0.02$	14.53	48.45	0.22	2.31
Gluconic Acid	0.1-12.5	16.98	99.99	$Y=1.76X+0.04$	4.91	16.36	0.15	2.24
Galacturonic Acid	0.1-12.5	19.93	99.99	$Y=0.74X+0.06$	12.50	41.67	0.16	0.71
Glucuronic Acid	0.1-12.5	20.93	99.98	$Y=0.80X+0.02$	18.75	62.50	0.17	1.67

### Application and Recovery

Recovery experiments were performed in order to investigate the accuracy of the method. Known amounts of each sugar and sugar acid solutes were added to the sample, detected, and the resulting spiked sample was subjected to the entire analytical sequence (Fig. 3). Each solute was spiked at a close concentration with the sample and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate (Table 5). The results show that the recoveries of all eight components ranged between 91.25% and 108.81%, and the RSD values fell within the range 0.04 to 6.07%. Such results further demonstrated that this method is precise and practical for the analysis of dilute acid steam-exploded prehydrolysate of corn stover.



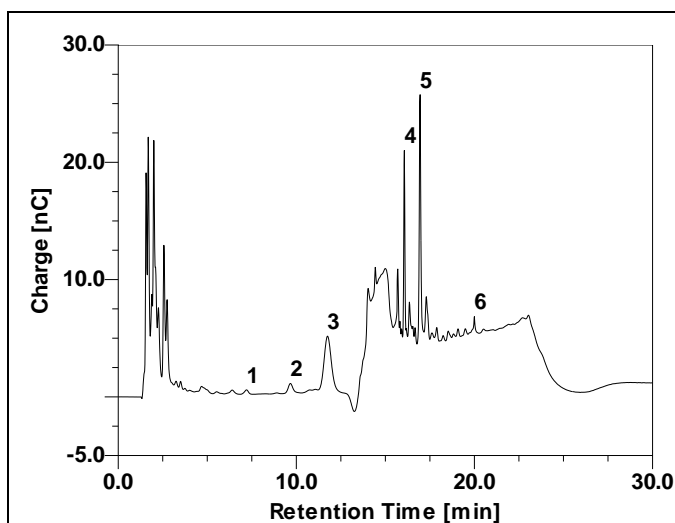
**Fig. 3.** Chromatograms of (a) corn stover steam-exploded prehydrolysate and (b) spiked prehydrolysate.

Peak identifications: 1. arabinose; 2. galactose; 3. glucose; 4. xylose; 5. xyonic acid; 6. gluconic acid; 7. galacturonic acid; 8. glucuronic acid.



**Table 5.** Recoveries of Eight Components in Corn Stover Steam-Exploded Prehydrolysate (n = 3)

Compound	Background/(mg/L)	Spiked/(mg/L)	Found/(mg/L)	Recovery/%	RSD/%
Arabinose	0.362	0.461	0.857	107.29	0.51
	0.362	1.488	1.873	101.56	0.16
	0.362	5.002	5.301	98.75	0.08
Galactose	0.146	0.464	0.629	104.09	0.32
	0.146	1.443	1.601	100.85	0.71
	0.146	4.897	5.001	99.14	0.04
Glucose	0.416	0.472	0.880	98.21	0.70
	0.416	1.453	1.807	95.71	2.62
	0.416	4.968	5.183	95.95	1.18
Xylose	3.812	0.471	4.294	102.31	0.09
	3.812	1.459	5.328	103.91	1.16
	3.812	4.944	8.543	95.70	0.09
Xyonic Acid	0.150	0.391	0.518	94.01	2.92
	0.150	1.205	1.461	108.81	3.74
	0.150	4.482	4.840	104.64	1.33
Gluconic Acid	0.017	0.465	0.478	99.07	0.96
	0.017	1.472	1.472	98.85	1.08
	0.017	4.930	4.695	94.89	0.23
Galacturonic Acid	0.046	0.506	0.551	99.87	0.90
	0.046	1.505	1.395	93.67	0.66
	0.046	4.875	4.341	91.25	3.25
Glucuronic Acid	0.049	0.525	0.574	100.10	0.57
	0.049	1.383	0.863	97.52	6.07
	0.049	4.627	4.039	91.22	3.11

**Fig. 4.** Chromatograms of black liquor.

Peak identifications:

1. arabinose;
2. glucose;
3. xylose;
4. xyonic acid;
5. gluconic acid;
6. glucuronic acid.

Furthermore, black liquor from green liquor-pretreated corn stover has also been tested by the described method. As a result, relatively large amounts of aldonic acids were detected (Fig. 4). They may have been produced from the peeling reaction during green liquor pretreatment (Lai and Sarkanen 1969).

## CONCLUSIONS

Preferred methods have been used to successfully identify and quantify arabinose, galactose, glucose, xylose, xylonic acid, gluconic acid, galacturonic acid, and glucuronic acid in corn stover prehydrolysates within a 21 min time period. The method is water-based with a simple sample preparation. Compared with the analytical methods reported previously, the method proved to be superior with respect to sensitivity, selectivity, and speed of analysis. According to the test results, the method showed good applicability in determination of prehydrolysates of corn stover.

## ACKNOWLEDGMENTS

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