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Rapid analysis of carbohydrates in aqueous extracts and hydrolysates of biomass using a carbonate-modified anion-exchange column

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ABSTRACT

Quantitative liquid-chromatography techniques used to characterize carbohydrates present in biomass samples can suffer from long analysis times, limited analyte resolution, poor stability, or a combination of these factors. The current manuscript details a novel procedure enabling resolution of glucose, xylose, arabinose, galactose, mannose, fructose, and sucrose *via* isocratic elution in less than 5 min. Equivalent conditions also enable analysis of cellobiose and maltose with a minimal increase in chromatographic run time (*ca.* 3 and 6 min, respectively). Noted chromatographic performance requires that a commercially available anion-exchange column be modified with carbonate prior to analysis. Analytical performance of a modified column was assessed over a 5-day period *via* repeated analyses of 4 samples, resulting from aqueous extraction or quantitative saccharification of a potential biofuel feedstock (i.e., corn stover or switchgrass). A simple solid phase extraction procedure was utilized to clean up each sample prior to analysis. Analytical accuracy of the extraction protocol was assessed by evaluation of matrix spike recoveries which typically ranged from 84% to 98%. The instrumental variability of measured concentrations in real samples over the 5-day period was generally less than 5% RSD for all detected analytes, independent of sample type. Finally, it is important to note that the modified column exhibited exceptional stability over approximately 800 injections of biofeedstock-based samples. These data demonstrate that a carbonate-modified anion-exchange column can be employed for rapid determination of carbohydrates in biomass samples of lignocellulosic origin.

1. Introduction

The primary technical hurdle facing the advanced biofuels industry is development of an optimized process by which lignocellulosic biomass can be efficiently converted to useful fuels. For biofuel research, determining the concentrations of sugars present in aqueous hydrolysates resulting from quantitative saccharification [1] or chemical pretreatment [2–5] of biomass is paramount to reliable technical or economic valuations of a given feedstock or conversion process. It has recently been demonstrated that knowledge of the water-soluble sugar content of biofuel feedstocks may also be important in such valuations [6–8]. Analytes of interest in biofuel research typically include the monomeric sugars glucose, xylose, arabinose, galactose, mannose, and fructose, as well as sucrose, maltose, and cellobiose dimers. Rapid analysis of these carbohydrates in complex samples has remained a challenge [9,10], not only for the biofuels industry, but also for food and beverage industries [11–14].

Although a number of carbohydrate separations have been successfully demonstrated in literature [15], previous experience in our laboratory and others that routinely conduct biomass analyses has proven that a majority of these separations are only feasible for relatively less-complex sample matrices. The quantitative techniques currently available to characterize the full spectrum of carbohydrates present in biomass samples can suffer from long analysis times, limited analyte resolution, poor stability, or all of these factors [9,14–17]. Gas chromatography

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(GC) has frequently been applied to carbohydrate determinations [18,19]. However, the polar nature of target analytes requires that samples be derivatized prior to analysis [20–22]. Derivatization can be both time and labor intensive and typically results in the presence of multiple chromatographic peaks for a single analyte [14,20,22], significantly complicating quantitative characterization of samples having unknown and variable compositions. As a result, liquid chromatography (LC) has become increasingly popular for carbohydrate analysis in the biofuels industry.

Both ligand-exchange chromatography with refractive index (RI) detection [23] and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [1,17,24,25] have been successfully applied to monitor carbohydrates. Although ligand-exchange chromatography, employing a Pb^{2+}- or H^+-mobilized stationary phase, offers reasonable resolution of most analytes of interest, analysis times typically range from 35 to 60 min [26]. In addition, RI detection (a universal detection technique) is susceptible to interference from co-eluting sample components. Analyses utilizing HPAEC-PAD can typically be accomplished with shorter run times (~10–20 min [11,27]) and benefit from the selectivity afforded by pulsed amperometric detection. However, HPAEC separations suffer from limited chromatographic resolution (in particular the ability to resolve sucrose), such that incorporation of mass spectrometry or other advanced analytical detection schemes is required to accurately identify and quantify the full range of target analytes present in a sample [16]. Additionally, the sensitivity of HPAEC stationary phases to trace amounts of carbonate can cause stability concerns when aqueous hydroxide is utilized as the mobile phase [28].

An alternative approach to analysis of carbohydrates in pretreatment samples, utilizing an amine-form LC column and evaporative light scattering (ELS) detection, was presented by Agblevor and coworkers [9,16]. These reports demonstrated that all monomeric and select dimeric carbohydrates of interest in biofuels research can be monitored with baseline resolution in 45 min when a delayed-gradient elution profile was utilized. When dimers were excluded from the analysis, baseline resolution of monomeric sugars was accomplished in approximately 25 min using alternative mobile phase conditions. However, analyte sensitivities can vary significantly when monitored by ELS. The ELS detection strategy is also susceptible to interference from co-eluting sample components, as evidenced by regions of high background signal in chromatograms observed for real samples [9,16].

The current manuscript details a novel approach to carbohydrate analysis that enables rapid monitoring of monomeric and dimeric sugars in aqueous extracts and hydrolysates of biomass. The approach relies on the use of a commercially available anion-exchange column and pulsed amperometric detection. However, the column is modified with carbonate prior to analysis, thus alleviating stability concerns common to HPAEC separations. Once modified, the column is capable of providing near-baseline resolution of monomeric sugars and sucrose (with isocratic elution) in approximately 5 min. Cellobiose and maltose can also be accommodated with a minimal increase in run time (*ca.* 3 and 6 min, respectively). The new approach offers a 3- to 4-fold reduction in analysis time, compared to current practice, with no significant compromise in chromatographic resolution. Moreover, the modified column exhibited exceptional stability during extended analyses of real samples. Herein, the column modification procedure is presented in detail, and analytical merits of developed methodology are evaluated using samples resulting from aqueous extraction and quantitative saccharification of potential biofuel feedstocks (i.e., corn stover, switchgrass, and hybrid poplar).

2. Experimental

2.1. Chemicals and reagents

All chemicals were reagent grade or better, purchased from commercial vendors, and used as received. Distilled water was purified and deionized to 18.2 M with a Barnstead Nanopure Diamond UV water polishing system. All stock solutions used to prepare calibration standards and spiked samples were prepared by dissolving the neat chemicals in water and were stored at 4 °C when not in use. Corn stover, switchgrass, and poplar wood samples were obtained from the National Renewable Energy Laboratory (NREL, Golden, CO).

2.2. Generation of aqueous extracts and hydrolysates

The procedure for generating aqueous extracts was adapted from the procedure reported by Chen et al. [6]. Briefly, dried feedstock was milled to achieve a particle size distribution of 20–80 mesh, and aqueous extracts of biomass were prepared *via* accelerated solvent extraction using a Dionex Accelerated Solvent Extractor (ASE, Model 200) with the following instrumental parameters: N_2 pressure at 1500 psi; temperature, 100 °C; preheat time, 0 min; heat time, 5 min; static time, 7 min; flush volume, 150%; purge time, 60 s; static cycles, 3. Following extraction, the collected liquid was quantitatively transferred into a 50-mL volumetric flask and diluted to volume with purified water. Extracts were subsequently stored at 4 °C until analysis.

Hydrolysates were generated according to the quantitative saccharification procedure described by NREL [29]. Briefly, 0.3 g of sieved biomass was transferred to a 16 × 125 mm test tube and incubated for 60 min with 3.0 mL 72% (w/w) sulfuric acid at 40 °C. Contents of each test tube were agitated with a glass stirring rod every 5–7 min. After 60 min the contents of each test tube were transferred to 100-mL serum bottles containing 64 mL of deionized water. The test tube was rinsed twice with 10 mL water, such that the total liquid volume in the serum bottle was 87 mL (*ca.* 0.07% (w/w) H_2SO_4). The bottles were subsequently sealed and autoclaved at 121 °C for 1 h. After cooling to room temperature, samples were stored at 4 °C until analysis. Note that some carbohydrate degradation is expected under the conditions employed for quantitative saccharification; however, analytical standards that enable one to correct for this factor were not included in this study. Accordingly, sugar concentrations reported in this work do not represent absolute values and should not be utilized to compare sugar content between tested feedstocks.

2.3. Analytical sample preparation

A 500 mg/6 mL Supelclean ENVI-Chrom P solid phase extraction (SPE) cartridge (Supelco, Bellefonte, PA) was connected in series to a 500 mg/4 mL Extract-Clean SAX SPE cartridge (Alltech, Deerfield, IL) and both cartridges were preconditioned with 8 mL methanol followed by 8 mL water. A 2-mL aliquot of aqueous standard, extract, or hydrolysate was then loaded onto the preconditioned cartridges and eluted with 8 mL water at a flow rate of approximately 8 mL/min. Resulting eluate was collected in a 10-mL volumetric flask and diluted to volume with water. Further dilution of each sample was carried out prior to analysis to bring sample concentrations into the linear range of the pulsed amperometric detector. Final dilution factors for aqueous extracts from corn stover, switchgrass, and poplar were 1:400, 1:400, and 1:200, respectively. A 1:1000 dilution was used to quantitate all carbohydrates in the cornstover and switchgrass hydrolysates except xylose, which was present at concentrations beyond the linear range of the detector. A separate dilution (1:2000) was required

to bring xylose into the detector's linear range. A single dilution of 1:400 was used for quantitation of all sugars present in hydrolysate from poplar. A constant amount of the monosaccharide fucose (1 ng/mL) was typically added to each sample as an internal standard during the final dilution process. However, in select instances where unidentified sample components were found to coelute with fucose, lactose (1.1 mg/mL) was utilized as an alternate internal standard. We note that analysis of a lactose standard exhibited only a single peak at the retention time of lactose, indicating alkaline hydrolysis was not occurring within the time frame of the separation (data not shown).

2.4. HPAEC-PAD analysis

All analyses were carried out on an ICS-3000 HPLC system (Dionex Corp., Sunnyvale, CA) equipped with an AS autosampler (10-μL sample loop), eluent generator, column oven, and integrated electrochemical flow cell and detector. All analyses of standards and real samples (unless otherwise noted) were conducted at 40 °C with isocratic elution (1.0 mM NaOH at 0.5 mL/min) using carbonate-modified CarboPac PA-20 (Dionex) guard (30 mm × 3 mm) and analytical (150 mm × 3 mm) columns connected in series. The procedure for modification of these columns with carbonate is detailed below. Eluted analytes were monitored at a disposable gold electrode using the following waveform: E_1 = 0.00 V (t_1 = 0.00 s), E_2 = +0.25 V (t_2 = 0.33 s), E_3 = −2.00 V (t_3 = 0.34 s), E_4 = +0.40 V (t_4 = 0.43 s), E_5 = 0.00 V (t_5 = 0.44 s), E_6 = 0.00 V (t_6 = 0.50 s) with integration between 0.20 and 0.33 s (all potentials are reported versus a Ag/AgCl reference electrode). The noted waveform is slightly modified from that recommended by the manufacturer. In our experience, the modified waveform resulted in longer electrode life (relative to the recommended protocol), fewer problems with electrode fouling, and modestly enhanced background noise (resulting in a similar decrease in sensitivity and linear range).

2.5. Column modification

Column modification with carbonate was accomplished through a two-step process (i.e., carbonate loading, followed by a NaOH wash). Throughout the carbonate loading step, a 1.0 mM NaOH mobile phase was passed continuously through the column at a flow rate of 0.5 mL/min. Carbonate was loaded onto the columns with 7 successive 10-μL injections of 40 mM $Na_2CO_3(aq)$ using the AS autosampler. Each injection of Na_2CO_3 briefly increased mobile phase pH from 11 to greater than 12 (pH was monitored *via* the Ag/AgCl reference electrode on the detector). Accordingly, a 10 min wait time between successive carbonate injections was employed to allow monitored pH to stabilize back to 11. Subsequent analysis of a calibration standard, containing all monomeric carbohydrates of interest plus sucrose, showed all analytes eluting from the column in under 3 min. The guard column was subsequently removed and the analytical column was washed with 50 mM NaOH at a flow rate of 0.5 mL/min for 6 min. After washing, the column was reequilibrated with 1 mM NaOH until the mobile phase pH stabilized at a value of 11. At this point, the calibration standard was reanalyzed to assess resolution and selectivity of the separation. This protocol resulted in optimized column performance (i.e., the resulting chromatogram shown in the inset of Fig. 1 where sucrose is resolved from galactose and arabinose). Once optimum separation was achieved, the guard column was reconnected and analyses were conducted without further modification. Although it may seem intuitive to include the guard column in wash cycles, numerous column preparations have demonstrated that removal of the guard column prior to washing results in sharper analyte peaks and improved resolution. A specific rationale for this observation is not apparent.

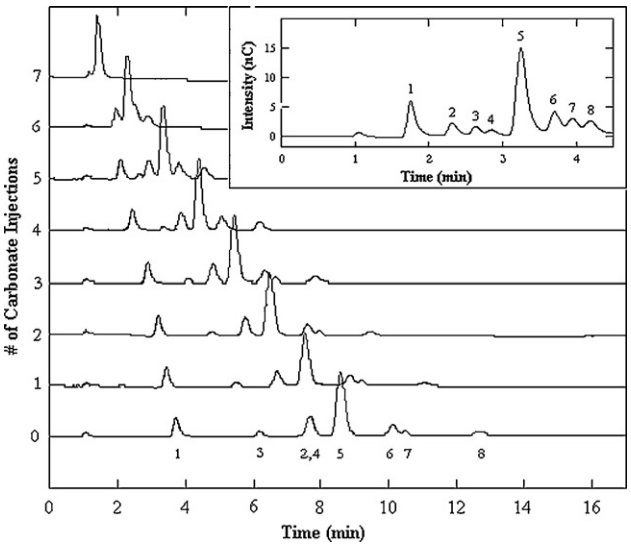

Fig. 1. Chromatograms illustrating the effect of carbonate addition to a PA-20 stationary phase on analyte retention for the following target analytes: (1) fucose (I.S.), (2) sucrose, (3) arabinose, (4) galactose, (5) glucose, (6) xylose, (7) mannose, and (8) fructose. Each successive trace represents observed analyte retention behavior after the indicated number of carbonate injections. The inset represents optimized carbohydrate separation, obtained upon washing the stationary phase with 50 mM NaOH after 7 carbonate injections. See Section 2.5 for additional details.

To aid the practitioner, it is important to note that if flow to the eluent generator was interrupted (e.g., stopped overnight), an equilibration period was required for the system to deliver eluent at the desired pH (i.e., initial pH was greater than its set value when resuming flow). In our experience, the initially high pH is characteristic of this generator when producing eluent at a concentration equal-to or less-than 50 mM (i.e., concentrations below the eluent generator's intended operating range). As a result of the initially high pH, carbonate was stripped from the PA-20 stationary phase and remodification of the analytical and guard columns was required prior to resuming sample analysis. To circumvent this inconvenience in subsequent experiments, eluent flow was diverted from the modified column arrangement until the pH stabilized to 11 whenever eluent generation was stopped for any period of time.

2.6. Standard preparation and quantitation

An aqueous stock solution of carbohydrates (containing sucrose, arabinose, galactose, glucose, xylose, mannose, and fructose) was utilized to prepare calibration standards and matrix spike samples that were analyzed over the course of the study. Six calibration standards spanning the concentration ranges specified in Table 1 were prepared by serial dilution. Spike concentrations added to aqueous extracts and hydrolysates were adjusted to achieve an analytical response in the upper third of the calibrated range for each analyte. All analytes were quantitated using multipoint, internal standard calibration curves. Analyte recoveries were determined as described previously [30].

3. Results and discussion

3.1. Effect of added carbonate on analyte retention and selectivity

Hydroxide-driven HPAEC separations typically suffer from carbonate interference. In fact, the gradual accumulation of carbonate on HPAEC columns is typically detrimental to analyte resolution and retention behavior to the extent that careful steps are recom-

Table 1
Quantitative performance metrics for analysis of carbohydrate calibration standards ($n = 6$).

Analyte	Investigated linear range (10^1 μg/L)	Line equation $y = mx + b$		r^2	LOD (10^1 μg/L)	LOQ (10^1 μg/L)
		m	b			
Sucrose	22–172	0.6616	−0.0003	0.9995	1.2	3.9
Arabinose	2–22	0.8893	−0.0037	0.9953	0.89	2.9
Galactose	3–24	0.7850	−0.0011	0.9969	1.1	3.8
Glucose	46–371	0.9071	−0.0484	0.9996	1.2	3.8
Xylose	3–25	0.6632	−0.0039	0.9978	1.1	3.2
Mannose	2–19	0.2708	0.0004	0.9997	2.2	7.2
Fructose	27–223	0.4494	0.0001	0.9977	2.5	8.0

mended to eliminate the presence of carbonate from mobile-phase composition [31]. However, Jeong and coworkers have demonstrated that the addition of a carefully controlled amount of carbonate to a hydroxide mobile phase can provide improved performance during HPAEC analysis of sugar phosphates without detrimental effects on column stability and analyte retention [32]. Furthermore, the addition of carbonate resulted in a significant decrease in chromatographic run time relative to an alkaline mobile phase that did not contain carbonate. However, the authors note that at lower concentrations of NaOH (e.g., 20 mM where peak-to-peak resolution appeared optimal for phosphorylated carbohydrates) the mobile phase had to be regularly degassed (*via* sonication and a helium purge) in order to stabilize analyte retention times. Although addition of carbonate to a hydroxide mobile phase provides an attractive option for separation of sugar phosphates, this methodology has not been explored for applications involving the target analytes or sample types typically encountered in biomass analyses. Moreover, addition of carbonate to a mobile phase is less practical for routine analyses where eluent generators are commonly employed.

The present study demonstrates an alternative approach to rapid, high performance separation of carbohydrates *via* direct addition of carbonate to the stationary phase of the analytical and guard columns. Chromatograms contained in Fig. 1 illustrate the effect of added carbonate on analyte retention and selectivity. Each carbonate injection results in a significant reduction of analyte retention (traces 0–7); presumably owing to carbonate occupation of anion-exchange sites on the column stationary phase. It is important to point out that the separation shown in the inset of Fig. 1 could not be achieved as a result of carbonate addition alone (i.e., optimized chromatographic performance could not be realized by a simple titration of the stationary phase with carbonate). It was only when retention behavior similar to that exhibited in trace 7 was achieved and the column was subsequently washed with 50 mM NaOH that sucrose was eluted between fucose and arabinose (Fig. 1, inset) rather than co-eluting with galactose. These observations suggest that selectivity for sucrose in this separation depends not only on the amount of carbonate present in the stationary phase, but also on additional factors (as described in Section 3.7).

The time required to modify a CarboPac PA-20 column was approximately 2 h. Although the modification time may be considered substantial, only a single modification is required before samples can be analyzed continuously (i.e., the column is not remodified between analyses). In contrast to traditional HPAEC separations, an equilibration/regeneration period between samples is also unnecessary. Overall, the modification time becomes negligible when compared to the time saved by the enhanced throughput afforded by presented methodology. For example, comparative retention times for analysis of a calibration standard before and after column modification are provided in Table 2. These data represent chromatogram 0 and the inset of Fig. 1, respectively. Note that sucrose is not resolved from galactose on the unmodified column, indicating that a separate methodology would be required to assess

Table 2
Carbohydrate pKa and retention times (min)[b] on modified and unmodified PA-20 columns.

Sugar	pKa[a]	Retention (min)	
		Modified	Unmodified
Fucose (IS)	–	1.73	3.59
Sucrose	12.71	2.23	7.76
Arabinose	12.43	2.58	6.14
Galactose	12.39	2.78	7.76
Glucose	12.28	3.14	8.58
Xylose	12.15	3.58	10.15
Mannose	12.08	3.76	10.53
Fructose	12.03	4.08	12.87

[a] Source: Refs. [24,29].
[b] The average relative standard deviation (RSD) for retention time ($n = 8$) was found to be less than 0.4% for all analytes.

these analytes in the absence of column modification. The modification protocol reported here resulted in an approximate 3-fold increase in throughput over an unmodified column while simultaneously enabling chromatographic resolution of each analyte, including sucrose. Other notable differences may also be observed in both retention time and elution order. In particular, analyte retention appears to be governed by pKa when analyzed on the carbonate-modified column. Moreover, the approach also enabled isocratic elution of cellobiose and maltose in approximately 7 and 10 min (Fig. 2). In comparison, these dimers elute at approximately 40 min when analysis is conducted on an unmodified column using similar chromatographic conditions (data not shown).

3.2. Column modification and reproducibility

Three CarboPac PA-20 columns were utilized to assess the reproducibility of the carbonate-modification procedure. These three columns were modified and assessed independently to evaluate inter- and intra-column reproducibility of the carbonate-modification procedure. Three modifications of Column A were used to demonstrate intra-column reproducibility, and inter-

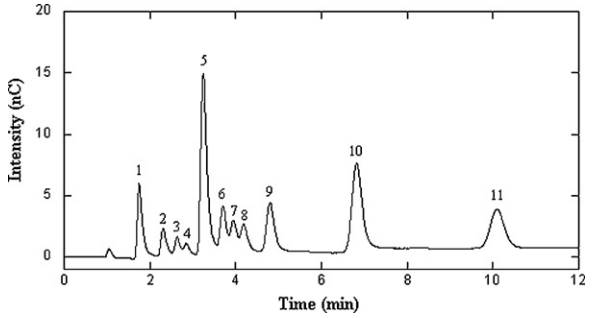

Fig. 2. Chromatogram demonstrating optimized separation of (1) fucose (I.S.), (2) sucrose, (3) arabinose, (4) galactose, (5) glucose, (6) xylose, (7) mannose, (8) fructose (9) lactose (I.S.), (10) cellobiose, and (11) maltose on a carbonate-modified PA-20 stationary phase.

Table 3
Inter- and intra-column reproducibility of the carbonate modification procedure. Retention times for each modification represent the first analysis of target analytes after each modification procedure.[a] Refer to Section 3.2 for additional details.

	Column A[b]			Column B[b]	Column C[c]
	Mod 1 Time (min)	Mod 2 Time (min)	Mod 3 Time (min)	Mod 1 Time (min)	Mod 1 Time (min)
Fucose	1.73	1.73	1.72	1.73	1.80
Sucrose	2.23	2.23	2.22	2.23	2.34
Arabinose	2.58	2.57	2.57	2.58	2.68
Galactose	2.78	2.78	2.78	2.78	2.90
Glucose	3.14	3.13	3.13	3.14	3.28
Xylose	3.58	3.57	3.56	3.58	3.73
Mannose	3.77	3.75	3.73	3.76	3.91
Fructose	4.09	4.08	4.08	4.08	4.26

[a] The average relative standard deviation (RSD) for retention time ($n = 8$) was found to be less than 0.4% for all analytes.
[b] Lot #006-23-017.
[c] Lot #004-27-105.

column reproducibility was assessed with the first modification of Column A, and a single modification of Columns B and C, respectively. Retention times of target analytes following each column modification are presented in Table 3 and are used as a metric of the variability in carbonate deposition between multiple modifications. The relative standard deviation (RSD) for intra-column analyte retention times varied from 0.0–0.5% for all analytes (Column A, Modifications 1–3). These data indicate excellent reproducibility for analyte retention when remodifying a given column, thus enabling this methodology for routine analyses. Although the RSD for inter-column analyte retention times was approximately 2.4%, we note that the greatest difference in retention times for all modifications shown in Table 3 is observed when assessing analytes eluting from the third carbonate-modified column (column C). In fact, retention for all analytes on column C is shifted to longer retention times than those obtained from columns A and B. A similar variation in analyte retention time was also observed when analyzing calibration standards on the unmodified columns (data not shown), indicating this difference in retention behavior was due to the inherent differences in production lots and not to the modification process (i.e., columns A and B are from Lot #006-23-017, and column C is from Lot #004-27-105).

3.3. Temperature effect on carbohydrate resolution

Although optimal carbonate loading is necessary to achieve baseline resolution of sucrose from galactose, additional improvements in the resolution of monomeric sugars were found to be dependent on column temperature. In general, as column temperature increased from 30 to 50 °C, the retention time for most analytes decreased by 4–8%, with minimal change in analyte resolution. However, changes in retention time for xylose, mannose, and fructose between 30 and 50 °C were found to significantly alter the resolution of these analytes. For example, Fig. 3 shows observed changes in retention time for xylose, mannose, and fructose between 40 and 50 °C. At lower column temperatures (e.g., 30 °C), mannose and fructose remain unresolved. As column temperature is increased, mannose experiences a greater shift in retention time than xylose and fructose, thus enabling its resolution from those analytes. Similar temperature dependence was also observed among other target analytes (data not shown); however, data in Fig. 3 represent the chromatographic region where changes in temperature were necessary to resolve coeluting components in samples pertinent to this study.

In practice, optimal column temperature is likely to depend upon sample type. Compare, for example, the chromatograms resulting from aqueous extraction and quantitative saccharification of various biomass feedstocks in Fig. 4. Notice that aqueous extracts typically contain high concentrations of fructose and essentially no xylose. In contrast, hydrolysates contain high concentrations of xylose and no fructose. Mannose is typically present at comparatively low levels (often below quantitation limits) in both samples. Thus, optimal column temperature for analysis of aqueous extracts would maximize resolution of mannose from fructose since xylose would be absent. This is favored by increased column temperature (i.e., closer to 50 °C). On the other hand, lower column temperatures (near 30 °C) would be preferred for analysis of hydrolysates where no fructose is present, thus promoting optimal resolution of mannose from xylose.

3.4. Analytical performance metrics for a carbonate-modified stationary phase

Six calibration standards were analyzed to demonstrate baseline quantitative performance of a carbonate-modified CarboPac PA-20 stationary phase. Data characterizing the resulting calibration curves are shown in Table 1. In general, the observed response for calibration standards was linear over approximately 1 order of magnitude for all analytes, with correlation coefficients (r^2) exceeding 0.995. Although limits of detection (LOD; S/N = 3) and limits of quantitation (LOQ; S/N = 10) are useful in evaluating method performance for trace analysis, carbohydrate concentrations in

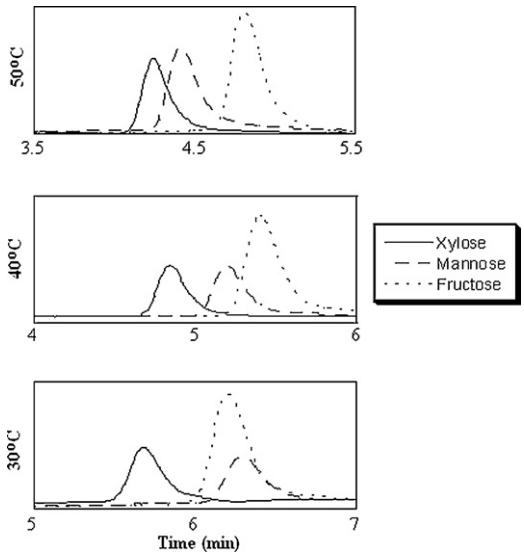

Fig. 3. Representative chromatograms illustrating the effect of temperature on retention of xylose, mannose, and fructose when separated on a carbonate-modified PA-20 stationary phase. Flow was reduced to 0.380 mL/min during these studies to minimize excessive back pressure at low temperatures. All other chromatographic conditions were as described in Section 2.4.

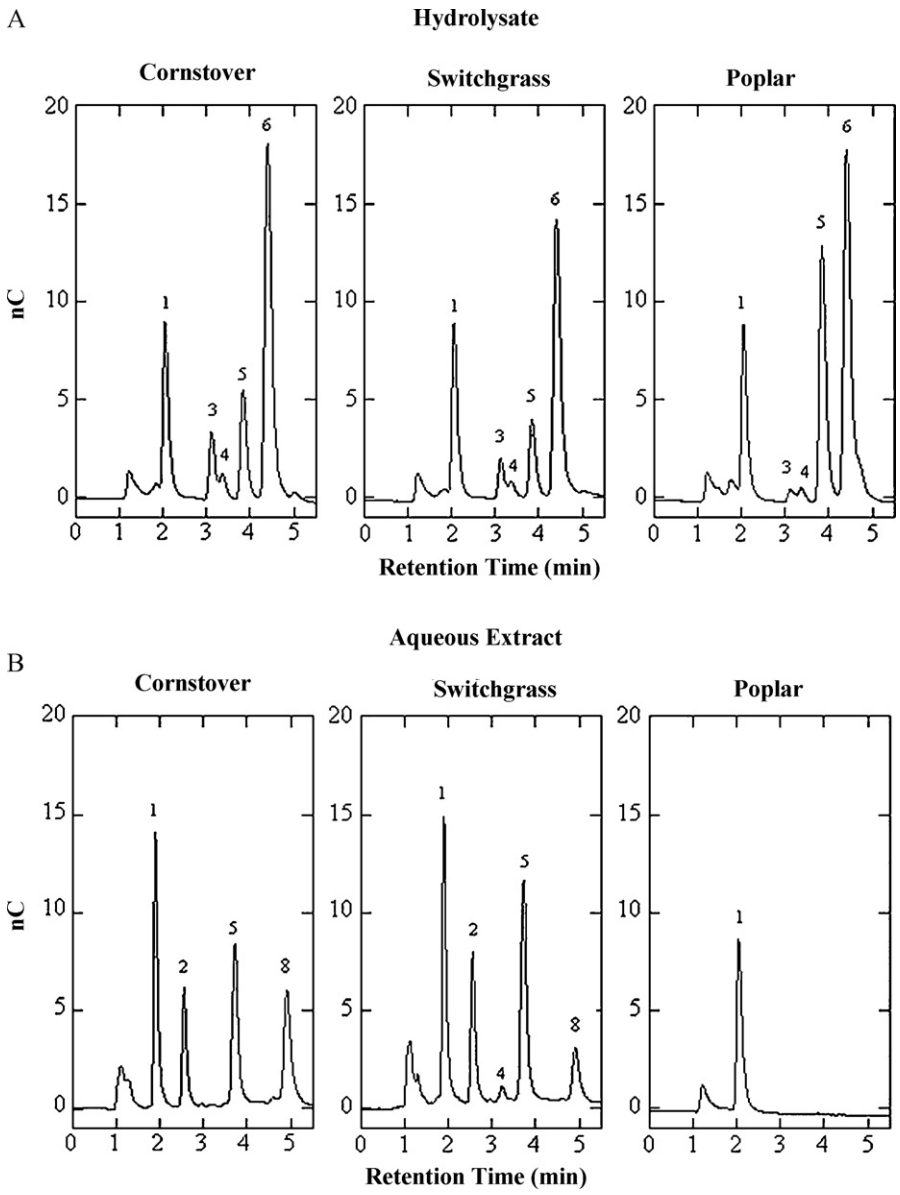

Fig. 4. Representative chromatograms resulting from carbohydrate analysis of (A) hydrolysates and (B) aqueous extracts derived from three leading biofuel-feedstock candidates on a carbonate-modified PA-20 stationary phase. (1) fucose (I.S.), (2) sucrose, (3) arabinose, (4) galactose, (5) glucose, (6) xylose, and (8) fructose.

aqueous extracts and hydrolysates resulting from biomass feedstocks are typically well above the values noted in Table 1. However, LOD and LOQ were used in this study to experimentally establish lower limits of linear range for each analyte and also to provide the reader with a relative comparison of analyte sensitivities.

3.5. Analysis of aqueous extract and hydrolysate samples resulting from representative biofuel feedstocks

Samples derived from three leading feedstock candidates (i.e., corn stover, switchgrass, and poplar wood) were analyzed in initial work. Representative chromatograms showing near baseline resolution for all carbohydrates detected in these samples are contained in Fig. 4. These data qualitatively demonstrate the potential of developed methodology for analysis of carbohydrates in complex matrices of diverse origin. Despite the relative increase in sample complexity that may be expected for biomass hydrolysates compared to aqueous extracts, observed chromatographic resolution and peak shape were essentially constant for all samples. Additionally, average retention times observed for each detected analyte in aqueous extracts and hydrolysates of biomass varied by less than 1% compared to retention times observed in the analysis of calibration standards using the same column preparation. Note that carbohydrates were not detected in aqueous extracts of poplar wood. This result can be attributed to a more general absence of water-soluble sugars in this feedstock candidate.

3.6. Extended reproducibility of retention and calibration data during analysis of real samples

Samples derived from corn stover and switchgrass (i.e., hydrolysates and aqueous extracts) were further utilized to investigate the analytical merits and robustness of developed methodology. Inter- and intra-day accuracy and precision were assessed *via* repeated analyses of selected samples over 5 days. During this assessment, subsequent sample injections were performed *via* autosampler and immediately after concluding the previous run without shutting-down or otherwise disrupting the

Table 4
Concentrations and recoveries[a] of carbohydrates quantified in aqueous extracts and hydrolysates of corn stover and switchgrass. All data were derived from samples analyzed during a 5-day evaluation period. See Section 3.6 for details.

Aqueous extract				Hydrolysate			
Sugar	Conc. (mg/g)[b]	Recovery (%)	RPD (%)[d]	Sugar	Conc. (mg/g)[c]	Recovery (%)	RPD (%)[d]
Corn stover				*Corn stover*			
Sucrose	9.5 ± 0.5	88	0.4	Sucrose	nd	–	–
Arabinose	nd[e]	–	–	Arabinose	23.5 ± 0.5	84	5
Galactose[e]	–	–	–	Galactose	6.6 ± 0.2	86	7
Glucose	8.8 ± 0.8	91	3	Glucose	334 ± 7	88	0.1
Xylose	nd	–	–	Xylose	272 ± 6	86	3
Mannose	nd	–	–	Mannose	–	–	–
Fructose	17.5 ± 0.8	95	4	Fructose	–	–	–
Switchgrass				*Switchgrass*			
Sucrose	7.04 ± 0.08	91	8	Sucrose	nd	–	–
Arabinose	nd	–	–	Arabinose	20.4 ± 0.3	97	5
Galactose	0.48 ± 0.02	83	1	Galactose	8.8 ± 0.4	98	1
Glucose	7.27 ± 0.02	97	2	Glucose	324 ± 15	96	8
Xylose	nd	–	–	Xylose	314 ± 29	98	7
Mannose	nd	–	–	Mannose	nd	–	–
Fructose	5.2 ± 0.1	98	4	Fructose	nd	–	–

[a] Concentrations (Conc.) and recoveries are reported as the mean value plus or minus 1 standard deviation. See Section 3.6 for details.
[b] The number of replicates (n) is equal to 155 independent determinations for each unspiked aqueous extract of corn stover and switchgrass sample.
[c] The number of replicates (n) is equal to 54 independent determinations for each unspiked hydrolysate of corn stover and switchgrass sample.
[d] Relative percent difference is determined as $(|(\bar{X}_{MS} - \bar{X}_{MSD})|/\bar{X}) \times 100\%$ where X is the determined concentration, and MS and MSD represent the matrix spike and matrix spike duplicate samples, respectively. The number of replicates (n) for each matrix spike and matrix spike duplicate is 11.
[e] Analyte was not detected.

instrumentation. It is important that the reader recognize that a column-equilibration period is not employed between subsequent analyses. Sample volumes were refreshed once daily when the sample vial was not in use. A typical chromatographic run sequence progressed through the following analyses: (1) calibration standards, (2) a water blank, (3) high- and low-calibration check standards, and (4) twenty injections of an aqueous-extract or hydrolysate sample, including duplicate matrix spikes. Steps 2–4 were then looped for the remainder of the assessment period. Approximately 800 sample injections were made over 5 days, of which greater than 80% represented analysis of an aqueous extract or hydrolysate. Calibration curves were prepared from standards analyzed on day 1 (step 1 above) and used for all concentration determinations during the evaluation. Concentrations observed for each of the calibration check standards were within ±9% of the expected value over the 5-day period, justifying continued use of initial calibration curves.Quantitative data for detected analytes in aqueous extract and hydrolysate samples are reported as a cumulative average plus or minus one standard deviation from the mean in Table 4. Relative standard deviations (RSD) for measured concentrations of target analytes ranged from 0.3 to 9% in all cases, demonstrating a high level of precision, independent of sample type. Additionally, it is important to note that standard deviations obtained on day 1 were not significantly different than those obtained on day 5, indicating no progressive deterioration in performance with time. Matrix spike data (i.e., recoveries) were utilized to assess method accuracy. Mean recoveries, shown in Table 4 as % recovery, are limited to analytes present in the prepared samples. Aqueous extract samples exhibited recoveries between 88–95% for corn stover and 83–98% for switchgrass. Hydrolysate samples exhibited recoveries between 84 and 86% for corn stover and 96 and 98% for switchgrass. In general, all analyte recoveries were near quantitative and similar in each sample matrix, with the only notable outlier being galactose in aqueous extracts of switchgrass (i.e. 83% recovery compared to >90% for other detected analytes in that matrix). The lower than expected percent recovery observed for galactose is likely due to the relatively small concentration (0.48 mg/g) of this analyte in the switchgrass sample, which is near the limit of quantitation for galactose in the diluted samples. The most significant observation made over the 5-day assessment of precision and accuracy was that the carbonate-modified stationary phase exhibited exceptional stability. Stability was assessed by monitoring changes in analyte retention time and aqueous extracts and hydrolysates were compared independently. As demonstrated in Fig. 5, relative shifts in retention time were less than 3% for all detected analytes in hydrolysate samples. In contrast, modest shifts in retention time (up to 6%) were observed for some analytes present in aqueous extracts. Observed shifts were more

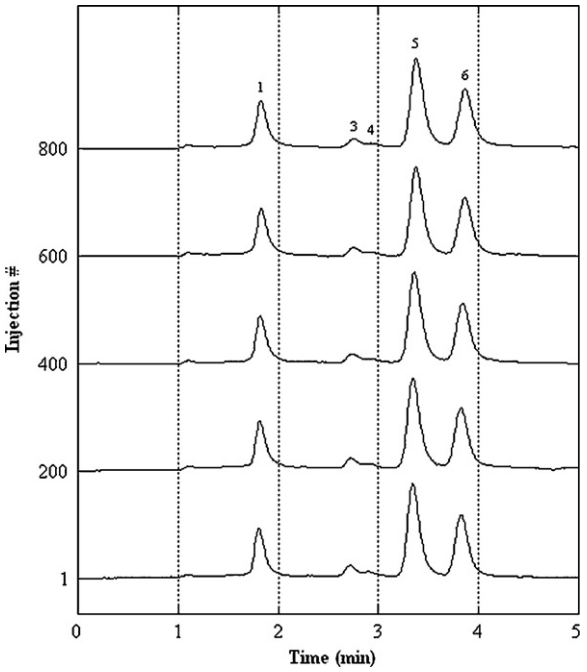

Fig. 5. Representative chromatograms of a biomass hydrolysate monitored over a 5-day evaluation period to assess the stability, accuracy, and precision of a carbonate-modified PA-20 stationary phase. See Section 3.6 for details. Peak labels are specified in Fig. 1.

pronounced in aqueous extracts at longer retention times; ranging from 0.04 min for fucose to 0.26 min for mannose over the 5-day period. However, these shifts did not compromise analyte resolution or quantitative accuracy in the analysis of either aqueous extracts or hydrolysates. A possible cause for the observed change in retention time may be gradual displacement of carbonate from the analytical column by hydroxide or other unidentified anionic components in the sample matrix. More definitive speculations to describe this behavior are not apparent at this time. We note that similar changes in analyte retention time will occur frequently when analyzing the same samples on an unmodified column, presumably owing to the complexity of the sample matrix, thus requiring the unmodified column to be washed periodically to maintain performance characteristics.

Continued use of the modified column beyond the 5-day evaluation period eventually resulted in a more pronounced deterioration in column performance, requiring that the analytical and guard columns be stripped of carbonate and remodified. Columns are typically remodified once the retention time for glucose has shifted by approximately 10%, or when co-elution of mannose and fructose becomes problematic. In our experience, it is not uncommon that a column be subjected to continuous use for a period of 1 month prior to remodification. Stripping of carbonate is typically accomplished by passing 100 mM NaOH through the system at 0.5 mL/min for 45 min. After the carbonate has been removed, the column is remodified using the procedure described in Section 2. Although column remodification requires a modest time commitment, it is comparable to the time spent washing/regenerating commercially available columns during routine analysis.

3.7. Mechanistic observations

While this study was primarily intended to demonstrate the preparation and stability of a carbonate-modified PA-20 column, there are some general observations that can be made regarding mechanisms that may be operable in affecting the observed separations. To the extent that added carbonate occupies anion-exchange sites on the column, a reduction in analyte retention would be expected based on reduced capacity. The PA-20 column is a microporous, pellicular anion-exchange resin employing a multifunctional quaternary-ammonium anion exchanger embedded in a latex coating and containing relatively few ion-exchange sites [~65 μeq/column] [33]. Thus, it is not unreasonable to suspect a significant reduction in anion-exchange capacity for carbohydrates on the modified stationary phase. Nevertheless, this simple argument does not readily explain the observation of a relatively larger shift to shorter retention times for sucrose than for other analytes listed in Table 2. Retention times appear to correlate directly with analyte pKa once the column is modified, which suggests that local pH at the surface of the stationary phase may also be important in understanding retention behavior. However, other factors may also be important. Further assessment of the mechanism(s) responsible for improvements in chromatographic performance reported in this study will likely require experimental stationary phase development, which is beyond the current capabilities of this laboratory.

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