Journal of Chromatography A, 1217 (2010) 2082–2087

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development and validation of a fast high pressure liquid chromatography method for the analysis of lignocellulosic biomass hydrolysis and fermentation products

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ARTICLE INFO

Article history:
Received 20 October 2009
Received in revised form 13 January 2010
Accepted 19 January 2010
Available online 4 February 2010

Keywords:
Biomass
Renewable energy
Cation-exchange HPLC
Acetic acid
Ethanol
Furfural
Fast acid method

ABSTRACT

A simple, precise, and accurate 10-min high pressure liquid chromatography (HPLC) method was developed and validated for the analysis of organic acids, alcohols, and furans from processing biomass into renewable fuels. The method uses an H^+ form cation-exchange resin stationary phase that has a five-fold shorter analysis time versus that in the traditional method. The new method was used for the analysis of acetic acid, ethanol, 5-hydroxymethyl furfural, and furfural. Results were compared with a legacy method that has historically has been used to analyze the same compounds but with a 55 min run time. Linearity was acceptable on the new method with $r^2 > 0.999$ for all compounds using refractive index detection. Limits of detection were between 0.003 and 0.03 g/L and limits of quantification were between 0.1 and 0.01 g/L. The relative standard deviations for precision were less than 0.4% and recoveries ranged from 92% to 114% for all compounds.

1. Introduction

The high cost of petroleum has led to a resurgence of interest in developing renewable fuels and chemicals. The US Department of Energy has developed a scenario for producing approximately 60 billion gallons per year of renewable fuel using domestic resources [1]. New, higher-throughput analytical methods will help accelerate the pace of research to reach the goals in these timetables.

Researchers routinely use HPLC methods to analyze ethanol, organic acids, and furans in biomass hydrolysate liquor, fermentation, and enzyme treated samples [2]. Such a method is often used for monitoring ethanol production during fermentation of biomass pretreatment hydrolysate; ethanol production during simultaneous saccharification and fermentation (SSF); and measuring acetic acid and furans formed during pretreatment [3,4].

It has been convenient to analyze organic acids, alcohols, furans using an "all-in-one" method based on cation-exchange resin HPLC. These resins are versatile and Pecina et al. [5] have published a paper on the behavior of 63 compounds on an H^+ form cation-exchange resin (Aminex HPX-87H, BioRad). Other benefits are that the method conditions are simple and convenient. Dilute sulfuric acid is used as an isocratic mobile phase and compounds are detected by refractive index. The acidic mobile phase continuously regenerates the stationary phase during chromatography [6].

While researchers have used the BioRad Aminex HPX-87H column for these purposes [4,7–14] analysis times are generally long because furans are strongly retained on this column. The aim of the present work is to compare the performance of the Aminex column to a Rezex RFQ column (Phenomenex) that has a five-fold shorter analysis time. The Rezex column, like the Aminex column, uses an H^+ form cation-exchange resin. Xu et al. [15] used the Rezex column for the fast analysis of dextrose and HMF in intravenous infusion fluids.

Compounds interact with these columns by multiple modes including ion-exchange, ion exclusion, size exclusion, ligand exchange, ion moderated partitioning, and interactions with the polymeric support matrix [16–19]. Temperature and flow rate have a strong effect on analysis times. The retention times of furans can be considerably reduced by running at high column temperatures [5]. The Phenomenex column tolerates both higher temperature and flow rates relative to the Aminex column enabling the shorter analysis time.

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doi:10.1016/j.chroma.2010.01.061

Table 1
Calibration curves for cellobiose, glucose, xylose, arabinose, xylitol, lactic acid, glycerol, acetic acid, ethanol, HMF, and furfural on the Rezex column.

Compound	Range (g/L)	R^2	Equation curve
Cellobiose	0.5–10	1.00000	$y = 54905x - 1337$
Glucose	0.5–40	1.00000	$y = 50488x - 1914$
Xylose	0.5–40	1.00000	$y = 50297x + 938$
Arabinose	0.5–30	1.00000	$y = 50297x - 1285$
Xylitol	0.5–10	0.99999	$y = 54549x - 1568$
Lactic acid	0.5–10	0.99999	$y = 34412x + 591$
Glycerol	0.5–10	1.00000	$y = 43991x - 1057$
Acetic acid	0.5–20	0.99999	$y = 22734x + 793$
Ethanol	4–80	0.99999	$y = 21262x + 9488$
HMF	0.35–7	0.99917	$y = 62525x + 333$
Furfural	0.25–5	0.99945	$y = 61430x - 5938$

x = concentration (g/L), y = peak area.

2. Experimental

2.1. Reagents and standards

Ultrapure water, 18.2 megohm (Barnstead, NanoPure Diamond, Dubuque, IA, USA), 10N sulfuric acid (Mallinckrodt, Hazelwood, MO, USA). HPLC calibration standards were purchased from Absolute Standards, Inc. (Hamden, CT, USA) Standards contained cellobiose, glucose, xylose, arabinose, xylitol, lactic acid, glycerol, acetic acid, ethanol, 5-hydroxymethyl furfural (HMF), and furfural at 4 levels in the concentration ranges listed in Table 1. A concentration verification standard (CVS) was also purchased from Absolute Standards, Inc. containing the same compounds prepared from independent lots at final concentrations near the midpoint of the standards.

2.2. Samples

The hemicellulose hydrolysate [3] used in this work was produced from milled corn stover harvested in the fall of 2003 from a farm in northeastern Colorado. The stover was treated with dilute sulfuric acid in a 900 dry kg/day pilot-scale continuous reactor operated at a solids concentration of 30% (w/w), and 190 °C, 0.048 g acid/g dry biomass, and an approximate residence time of 1 min [20]. A portion of this pretreatment slurry was reserved for simultaneous saccharification and fermentation testing [21]. Hemicellulose hydrolysate was obtained through solid–liquid separation performed using a hydraulic press at 2000 psi that removed approximately 70% of the liquor from the pretreated slurry.

2.3. Instrument

The HPLC system used in this study was an Agilent 1100 (Santa Clara, CA, USA) equipped with an inline degasser, an isocratic pump, an autosampler with thermostatic cooling (samples held at 5 °C), and a refractive index detector. The system was controlled by Agilent Chemstation software running on a personal computer. An isocratic mobile phase of 0.01N sulfuric acid and a 6 μL sample injection volume was used for all experiments. The mobile phase was prepared by mixing 1 mL of 10N sulfuric acid with ultrapure water to a final volume of 1.0 L. The analytical column was either an Aminex HPX-87H 300 mm × 7.8 mm, 9 μm particle size ran as previously described (0.6 mL/min, 65 °C) [2], or a Rezex RFQ, 100 mm × 7.8 mm, 8 μm particle size (Phenomenex, Torrance, CA, USA). The same type of guard column, a Cation H from BioRad (Hercules, CA, USA), was used with either analytical column. The guard column was kept outside of the compartment to avoid heating it above the manufacturers recommended limit of 60 °C. The guard cartridge holder was wrapped with foam pipe insulation to keep the cartridge at a steady temperature (ca. 55 °C). Run conditions for the Rezex column were a flow rate of 1.0 mL/min and the column was heated to 85 °C. A macro (available from Agilent) was added to method run time checklist to set the column compartment thermostat to temperatures greater than 80 °C.

3. Results

Fig. 1 shows chromatograms of a standard solution from the traditional Aminex method versus those from the new Rezex Fast Acid method. The Rezex method is over five times faster than the

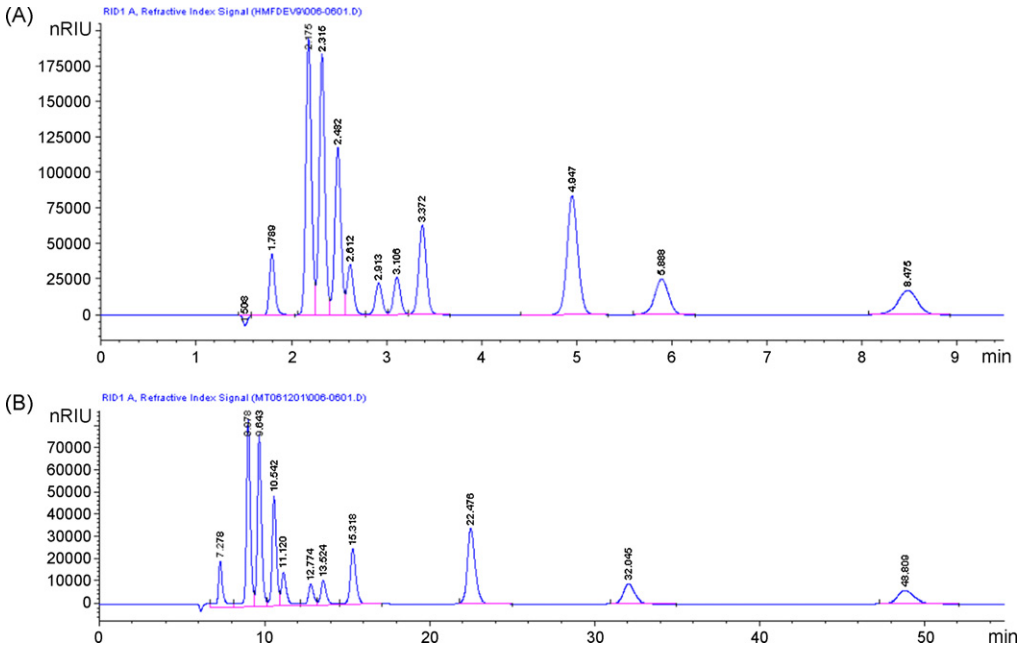

Fig. 1. (A) Chromatogram of a mixture of standards using the Rezex column; run time 9.5 min. (B) The same standard solution analyzed using the Aminex column; run time 55 min. Compounds in order of increasing retention time on either chromatogram are cellobiose, glucose, xylose, arabinose, xylitol, lactic acid, glycerol, acetic acid, ethanol, HMF, and furfural.

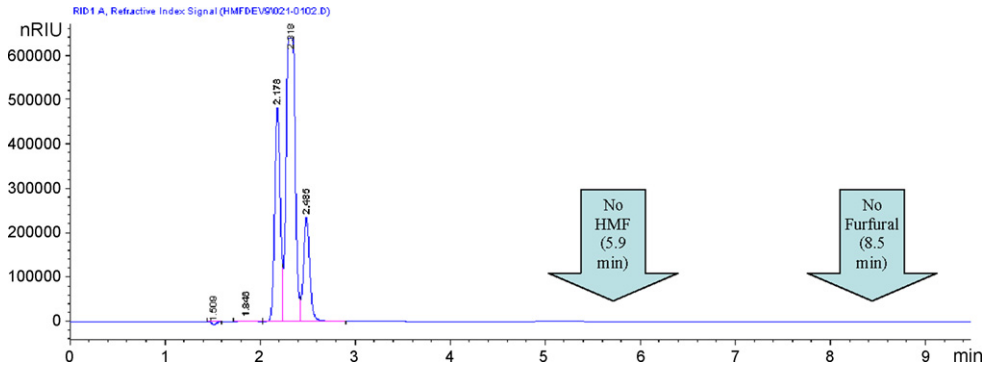

Fig. 2. No degradation of sugars to furans was detected when analyzing an aqueous sugar solution on the new method (glucose (40 g/L), xylose (100 g/L), galactose (20 g/L), arabinose (10 g/L), mannose (10 g/L)). Mixed sugars co-elute at 2.1–2.5 min.

traditional method. The same type of guard column (BioRad Cation H) was used with both analytical columns. Using the BioRad guard column improved the symmetry for all peaks and improved the resolution of the sugar peaks (data not shown). It is important to note that either column is not optimized for sugar analysis and some biomass derived sugars will co-elute or be poorly resolved (i.e., xylose, galactose, and fructose co-elute; glucose and mannose co-elute). These columns are best suited (i.e., peaks are baseline resolved) for the analysis of organic acids, alcohols, and furans.

The Rezex column used for the new method has some features that make this significant increase in analysis throughput possible. The column tolerates higher temperatures, up to 85 °C, enabling fast analysis of HMF and furfural. The column also tolerates a higher flow rate of 1 mL/min. A comparison of column efficiency shows that the Rezex column has a greater than 2-fold increase in theoretical plates per unit length when each method was ran as described above (the number of plates/cm for ethanol on the Rezex column = 850, Aminex = 370, calculated as $N = 2\pi((\text{retention time} \times \text{height})/\text{area})^2$). The high performance resin (i.e., higher efficiency, tolerance to higher temperature and flow) in the Rezex column enables the use of shorter column length, just 100 mm versus 300 mm for the Aminex column, decreasing analysis time.

Researchers routinely have the need to run samples with high sugar concentrations (e.g., >15%, w/v, total sugars) through this type of analysis. One concern with using the higher operating temperature in the Rezex column in the presence of an acidic mobile phase is the potential to degrade sugars. A test solution of monosaccharides simulating corn stover hemicellulose hydrolysate (40 g/L glucose, 100 g/L xylose, 20 g/L galactose, 10 g/L arabinose, 10 g/L mannose in water) was ran on the Rezex column at 85 °C. No evidence of the sugar degradation products HMF and furfural was detected (Fig. 2).

3.1. Validation of the proposed method

3.1.1. Linearity

A linear relationship (Table 1) was found for all compounds tested (cellobiose, glucose, xylose, arabinose, xylitol, lactic acid, glycerol, acetic acid, ethanol, HMF, and furfural) with an $r^2 > 0.999$.

3.1.2. Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) for this method are displayed in Table 2. The LOD was defined as the lowest concentration of each compound that gave an average signal to noise ratio greater than 3 over five replicate injections based on peak area. The LOQ was defined as the lowest concentration of each compound that gave peak area counts with a relative standard deviation (RSD) less than 2.5% over five replicate injections. Samples were prepared from serial dilutions of the lowest concentration calibration standard.

3.1.3. Precision

Data from multiple injections ($n = 14$), collected on the same day, of a calibration verification standard were used to estimate the precision of the method (Table 3). The percent RSD was <1% in all cases.

3.1.4. Recovery

Cellobiose, glucose, xylose, arabinose, xylitol, lactic acid, glycerol, acetic acid, ethanol, HMF, and furfural were added to a biomass corn stover hydrolysate at two levels (Table 4). Each sample was analyzed in triplicate. Recovery amounts were slightly high for most compounds. Refractive index (RI) detection is a universal type of detection where any compound with a RI different from the mobile phase will be observed. Therefore there may be sample matrix dependent effects on recovery.

Table 2
Limit of detection (LOD) and limit of quantification (LOQ).

	Cellobiose	Glucose	Xylose	Arabinose	Xylitol	Lactic acid	Glycerol	Acetic acid	Ethanol	HMF	Furfural
Cal level 1 (gravimetric)	0.50	2.0	2.0	1.5	0.50	0.50	0.50	1.0	4.0	0.35	0.25
LOQ (RSD < 2.5%)	0.01	0.01	0.02	0.02	0.05	0.1	0.1	0.04	0.04	0.04	0.05
LOD (S/N > 3)	0.005	0.004	0.004	0.004	0.003	0.005	0.005	0.01	0.02	0.02	0.03

All values are in g/L; $n = 5$ for each test. The values for LOQ and LOD are from serial dilutions of Cal level 1.

Table 3
Precision of the method.

	Cellobiose	Glucose	Xylose	Arabinose	Xylitol	Lactic acid	Glycerol	Acetic acid	Ethanol	HMF	Furfural
Average CVS values ($n = 14$)	3.0	15.0	15.1	10.1	3.0	3.0	3.0	14.5	29.6	4.0	3.9
Std Dev	0.01	0.02	0.02	0.01	0.01	0.003	0.003	0.02	0.05	0.004	0.02
% RSD; ((SD/average) × 100)	0.28	0.15	0.12	0.09	0.33	0.12	0.11	0.11	0.18	0.10	0.42

Table 4
Results of recovery tests.

	Recovery (%)										
	Cellobiose	Glucose	Xylose	Arabinose	Xylitol	Lactic acid	Glycerol	Acetic acid	Ethanol	HMF	Furfural
Level 1	111 ± 9	104 ± 0.5	100 ± 0.8	101 ± 0.2	112 ± 4	114 ± 5	100 ± 7	109 ± 8	103 ± 1	104 ± 5	105 ± 3
Level 2	112 ± 5	103 ± 0.3	92 ± 1	101 ± 0.3	107 ± 0.7	110 ± 0.7	102 ± 0.6	105 ± 0.3	103 ± 0.7	101 ± 0.3	104 ± 6

Level 1 = corn stover hemicellulose hydrolysate spiked with 2.5 g/L cellobiose, 10 g/L glucose, 10 g/L xylose, 7.5 g/L arabinose, 2.5 g/L xylitol, 2.5 g/L lactic acid, 2.5 g/L glycerol, 5 g/L acetic acid, 20 g/L ethanol, 1.75 g/L HMF, and 1.25 g/L furfural. Level 2 = corn stover hemicellulose hydrolysate spiked with 5 g/L cellobiose, 20 g/L glucose, 20 g/L xylose, 15 g/L arabinose, 5 g/L xylitol, 5 g/L lactic acid, 5 g/L glycerol, 10 g/L acetic acid, 40 g/L ethanol, 3.5 g/L HMF, and 2.5 g/L furfural. Results are reported as the mean of triplicate injection ± standard deviation.

3.2. Interferences

The potential for compounds to co-elute when using the BioRad Aminex column has been described [5]. That research showed the potential for biomass derived sugars, organic acids, and other compounds to have identical or overlapping capacity factors. Likewise, some compounds can co-elute on the Phenomenex RFQ column. Researchers interested in using this new method should be aware of interferences that can affect the quality of their data. Levulinic and formic acids are degradation products of sugars via furans [22,23]. They are observed most commonly in samples where aggressive conditions were used to hydrolyze cellulose (i.e., total acid hydrolysis of biomass). Levulinic acid will co-elute with acetic acid and formic acid will co-elute with glycerol when using the Fast Acid method (data not shown). Certain biomass-derived sugars co-elute on the Fast Acid column (i.e., xylose, galactose, and fructose co-elute (Fig. 3); glucose and mannose co-elute (not shown)). Therefore the Fast Acid method is best suited (i.e., peaks are baseline resolved) for the analysis of organic acids, alcohols, and furans.

3.3. Applications and transferring methods

The Fast Acid method is suitable for the analysis of hemicellulose hydrolysate produced during dilute acid treatment of biomass

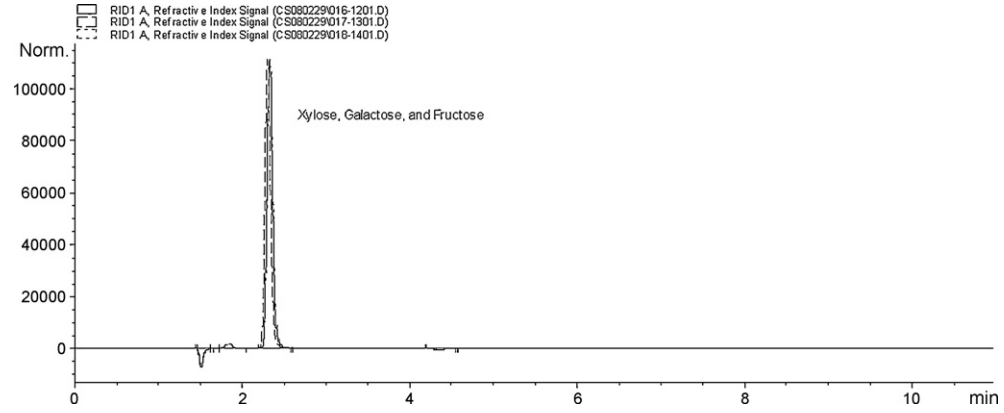

Fig. 3. Overlay of 3 chromatograms showing the co-elution of xylose, galactose, and fructose on the Fast Acid column.

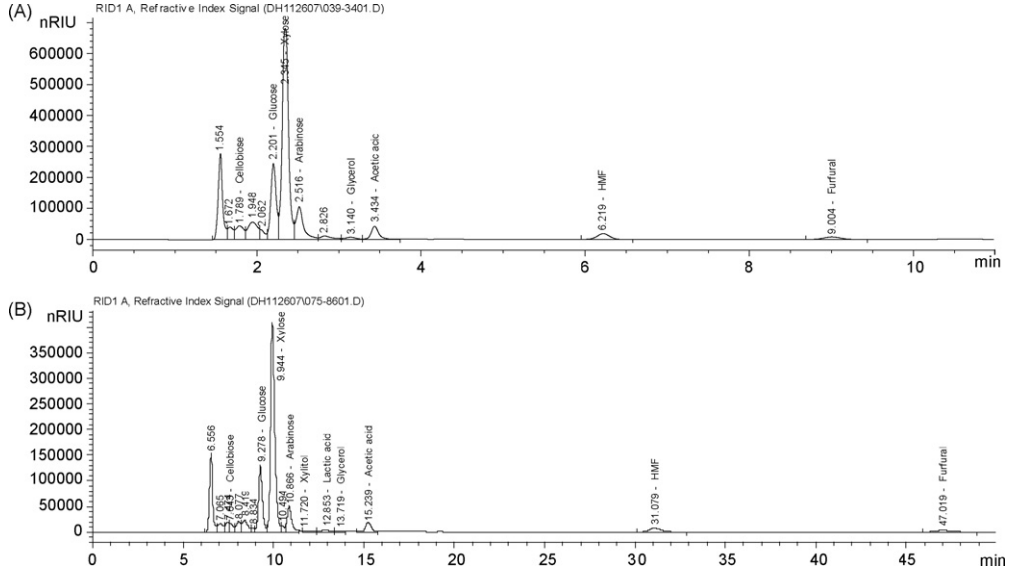

Fig. 4. Chromatograms of corn stover hemicellulose hydrolysate analyzed using (A) the Phenomenex RFP column and (B) the Aminex HPX-87H column. The scatter plot for acetic acid had a slope of less than one. This was due to the co-elution of levulinic acid on the Fast Acid column as measured by LC–MS (see text).

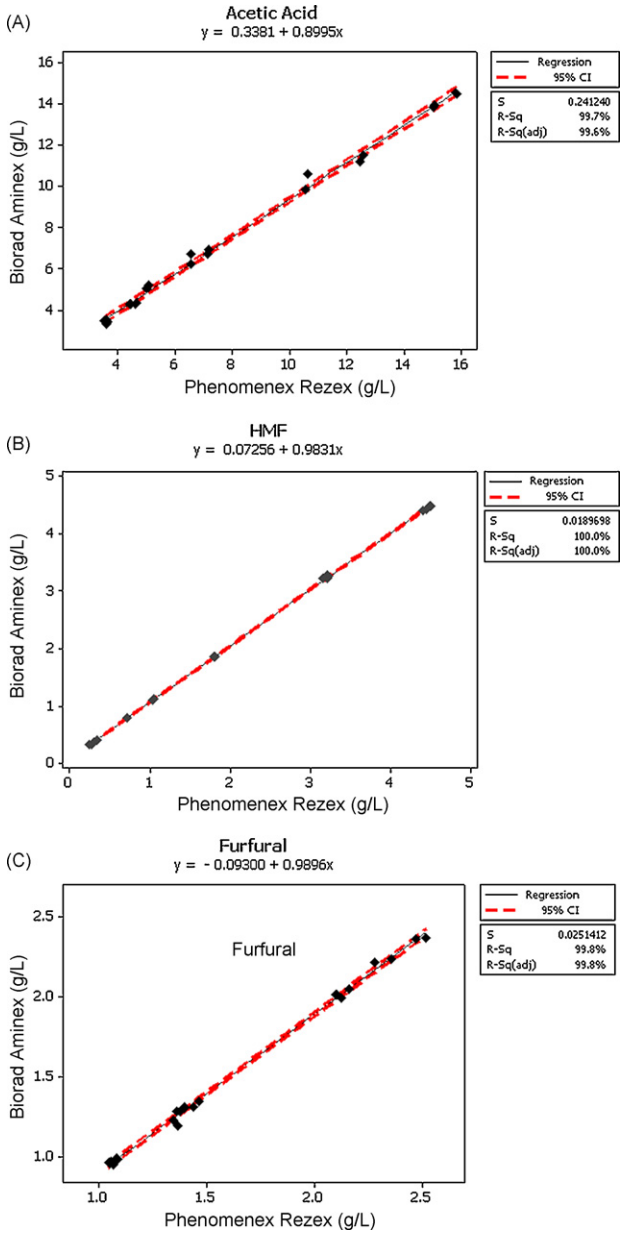

Fig. 5. Regression line plots with the 95% confidence interval bands of the prediction comparing the performance of the Phenomenex Rezex RFQ and the BioRad Aminex HPX-87H columns for the analysis of acetic acid (A), HMF (B), and furfural (C) in hemicellulose hydrolysate liquor.

[3]. This method may be substituted for the traditional method previously described by Sluiter et al. [2]. See Fig. 4 for chromatograms of hemicellulose hydrolysate analyzed by each method. The chromatograms demonstrate the ability to do five-fold faster analysis on the Phenomenex column.

Selectivity is the same on each column. Resolution between glucose, xylose, and arabinose is better (i.e., closer to baseline) on the Aminex column. However each column has the potential for co-elution from matrix derived compounds including other biomass derived sugars as mentioned above. Resolution is best on both columns for acetic acid, HMF, and furfural. Regression line plots are shown in Fig. 5 with the 95% confidence interval bands of the prediction for the analysis of hemicellulose hydrolysate liquor. The performance of the Phenomenex and the Aminex column show the best correlation (i.e., slope close to 1) for HMF and furfural. The plot for acetic acid had a slope of less than one. This was due to

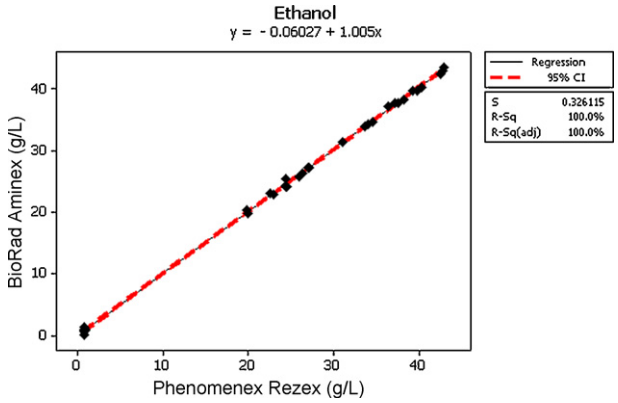

Fig. 6. Regression line plot with the 95% confidence interval bands of the prediction comparing the performance of the Phenomenex Rezex RFQ and the BioRad Aminex HPX-87H columns for the analysis of ethanol in samples from a simultaneous saccharification and fermentation experiment.

the co-elution of levulinic acid on the Fast Acid column as measured by LC–MS (data not shown) [24]. Acetic acid was present at levels of 16–300 fold higher concentration than that of levulinic acid. Therefore levulininc acid had a small but measureable effect on acetic acid measurement.

There was also good correlation (Fig. 6) between the two columns for the analysis of ethanol produced by simultaneous saccharification and fermentation (SSF) of biomass [21]. Comparisons for the analysis of cellobiose, glucose, xylose, arabinose, xylitol, lactic acid, and glycerol between columns were less well correlated. These compounds can suffer from co-elution with interfering matrix derived compounds than the ones mentioned above (many of the compounds have been described by Chen et al. [25]). Therefore the data quality from either column may be dependent on the sample matrix.

4. Conclusion

We developed a fast and simple method for the precise and accurate analysis of certain compounds in biomass hydrolysate liquor and fermentation samples. The new method correlated well with the traditional method for the analysis of acetic acid, HMF, furfural, and ethanol. Other compounds, including carbohydrates, can be detected using this method. The data quality for those compounds will be impacted by co-elution compounds from the sample matrix. Flow rate and column temperature have strongly influenced analysis times. The conditions described enable a five-fold shorter run time than that in the traditional method. This is a significant increase in throughput and productivity for this type of analysis.

Acknowledgements

This project was funded by the US Department of Energy, Office of Biomass Programs. HPLC data for ethanol produced by simultaneous saccharification and fermentation was provided by Gary McMillen. LC–MS data for the co-elution of levulinic and acetic acid was provided by Richard Mowery and Kevin Chambliss. Pretreated corn stover hemicellulose hydrolysate was prepared in the NREL Process Development Unit by Bob Lyons, Wes Hjelm, and Jody Farmer.

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