JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 3251–3258 3251
DOI:10.1021/jf9033877

Compositional Analysis of Water-Soluble Materials in Switchgrass

Shou-Feng Chen,[†] Richard A. Mowery,[†] Richard S. Sevcik,[†] Christopher J. Scarlata,[§] and C. Kevin Chambliss*,[†]

[†]Department of Chemistry and Biochemistry, Baylor University, Waco, Texas 76798, and [§]National Renewable Energy Laboratory, Golden, Colorado 80401

Any valuation of a potential feedstock for bioprocessing is inherently dependent upon detailed knowledge of its chemical composition. Accepted analytical procedures for compositional analysis of biomass water-soluble extracts currently enable near-quantitative mass closure on a dry weight basis. Techniques developed in conjunction with a previous analytical assessment of corn stover have been applied to assess the composition of water-soluble materials in four representative switchgrass samples. To date, analytical characterization of water-soluble material in switchgrass has resulted in >78% mass closures for all four switchgrass samples, three of which have a mass closure of >85%. Over 30 previously unknown constituents in aqueous extracts of switchgrass were identified and quantified using a variety of chromatographic techniques. Carbohydrates (primarily sucrose, glucose, and fructose) were found to be the predominant water-soluble components of switchgrass, accounting for 18−27% of the dry weight of extractives. Total glycans (monomeric and oligomeric sugars) contributed 25−32% to the dry weight of extractives. Additional constituents contributing to the mass balance for extractives included various alditols (2−3%), organic acids (10−13%), inorganic ions (11−13%), and a distribution of oligomers presumed to represent a diverse mixture of lignin−carbohydrate complexes (30−35%). Switchgrass results are compared with previous analyses of corn stover extracts and presented in the context of their potential impact on biomass processing, feedstock storage, and future analyses of feedstock composition.

KEYWORDS: Switchgrass; compositional analysis; extractives; biomass; value-added products water-soluble materials; feedstock

INTRODUCTION

Switchgrass (*Panicum virgatum* L.) is a relatively drought-tolerant, warm-season C_4-species of tall prairie grass with a range that extends from Mexico to Canada. An assessment of herbaceous biomass by the U.S. Department of Energy (USDOE) was initiated in the late 1980s and led to the selection of switchgrass as one of the primary biofuel feedstocks (*1*−*3*). Switchgrass has great potential as an energy crop because it is a perennial crop that does not require annual reseeding. It also requires lower agricultural inputs (fertilizer and pesticides) than other annual crops and can often be grown on marginal cropland (*4*). Overall, up to 34.6 Mg ha^{-1} $year^{-1}$ of switchgrass can be sustainably collected (*1*). Net energy and economic studies recently reported by several authors indicated that switchgrass-derived ethanol could generate 540% more energy than needed to produce it (*2*, *3*). These data suggest that production of cellulosic ethanol from switchgrass may be economically feasible and significantly more energy efficient than other feedstocks (*4*).

*Address correspondence to this author at the Department of Chemistry and Biochemistry, One Bear Place, Box 97348, Waco, TX 76798 [telephone (254) 710-6849; fax (254) 710-4272; e-mail kevin_chambliss@baylor.edu].

Accepted analytical procedures for compositional analysis of biomass provide near-quantitative mass closure on a dry weight basis (*5*). Total water- and/or ethanol-soluble materials are typically quantified gravimetrically and identified only as extractives (i.e., qualitative and quantitative assessments of extracts are not performed) (*6*−*10*). As a result, any valuable materials contained in the extractive fraction are omitted when a feedstock's potential is assessed. Previous work has shown that extractives can affect macrocomponent compositional determinations (*9*). More significantly, a detailed study of aqueous corn stover extracts recently demonstrated that corn stover feedstocks contain as much as 12% fermentable sugar (on a dry weight basis) in water-soluble form (*11*). These findings have potentially significant implications for technical and economic valuations of biomass-to-biofuel conversion processes, as well as feedstock storage practices, providing strong impetus to further investigate the composition of water-soluble materials native to herbaceous biomass.

The objective of the present study was to characterize water-soluble constituents of switchgrass. Four opportunistic samples, grown in different locations and harvested in different years, were extracted and investigated in a side-by-side comparison. Analytical protocols developed in conjunction with our previous

© 2010 American Chemical Society
Published on Web 02/19/2010

evaluation of corn stover (*11*) were utilized with minimal modification in most cases and enabled identification and quantitative assessment of more than 30 chemical constituents common to each sample. Compositional data for carbohydrates, alditols, organic acids, inorganic ions, and a tentatively identified oligomeric fraction of aqueous extracts are reported as a percentage of total water-soluble materials in switchgrass, and results are compared with similar data obtained previously in our laboratory for corn stover materials.

MATERIALS AND METHODS

Feedstock and Reagents. All chemicals and reference standards were of reagent grade or better, obtained from commercial vendors, and used as received. Distilled water was purified and deionized to 18.2 MΩ with a Barnstead Nanopure Diamond UV water purification system. The four dried switchgrass samples analyzed in this study were provided by the National Renewable Energy Laboratory (NREL), Golden, CO. These were opportunistic samples, representing multiple cultivars grown in different locations and harvested in different years (**Table 1**). Although the NREL ID given in **Table 1** for three of the four samples designates the cultivar, it is important to point out that the first entry was also believed to be a Forestburg variety that was processed in St. Anthony and mislabeled prior to delivery at NREL.

Water Extraction. Each switchgrass sample was "milled" for 90 s using a commercial coffee grinder. Milled switchgrass was subsequently screened using stackable sieves to achieve a particle size distribution ranging from 20 to 80 mesh. Two extraction protocols, Soxhlet extraction and accelerated solvent extraction (ASE), were employed in initial work. Extraction protocols followed procedures described in NREL Laboratory Analytical Procedure (LAP) "Determination of Extractives in Biomass" (*6*), and details of the Soxhlet approach have been described previously (*11*). Methodology employed for the ASE approach was as follows. In a typical extraction, 1.5 g of sieved switchgrass was added to an 11 mL extraction cell and extracted in triplicate by pressurized liquid extraction using an ASE-200 accelerated solvent extractor (Dionex Corp.). ASE parameters were as follows: pressure, N_2 at 1500 psi; temperature, 100 °C; preheat time, 0 min; heat time, 5 min; static time, 7 min; flush volume, 150%; purge time, 120 s; static cycles, 3. Following extraction, aqueous extracts generated for compositional determinations were quantitatively transferred into a 50 mL volumetric flask and diluted to volume with water. The amount of water-soluble material present in all extracts (i.e., those generated by either Soxhlet or ASE) was assessed gravimetrically and used to calculate percent extractives, as described previously (*11*).

Table 1. Historical Data for Analyzed Swtichgrass Feedstocks

NREL ID	location grown	harvest year
St. Anthony	South Dakota	2004
Forestburg	South Dakota	2006
Trailblazer 2003	Nebraska	2003
Trailblazer 2004	Nebraska	2004

Fractionation and Compositional Analysis of Aqueous Extracts. Details of extract fractionation procedures and most chromatographic protocols employed for identification and quantitation of sample constituents in this study have been reported elsewhere (*11*). Unless noted below, experimental protocols were identical to the previous paper. Representative chromatograms resulting from analysis of isolated fractions of an aqueous switchgrass extract that were used for quantitation of sample constituents are provided in **Figure 1**.

Monomeric Sugars and Related Alditols. A 2 mL aliquot of aqueous extract was loaded onto a Supelclean ENVI-Chrom P solid phase extraction (SPE) cartridge, which was preconditioned, loaded, and eluted as described previously (*11*). A 250 μL aliquot of the resulting eluate was then loaded onto an Alltech Extract-Clean SAX SPE cartridge, which had been preconditioned with 9 mL of methanol followed by 9 mL of water. A sample was collected after the second SPE cartridge had been rinsed with slightly less than 10 mL of water, and the eluate was diluted to 10 mL in a volumetric flask. The sample was analyzed for monomeric sugars and

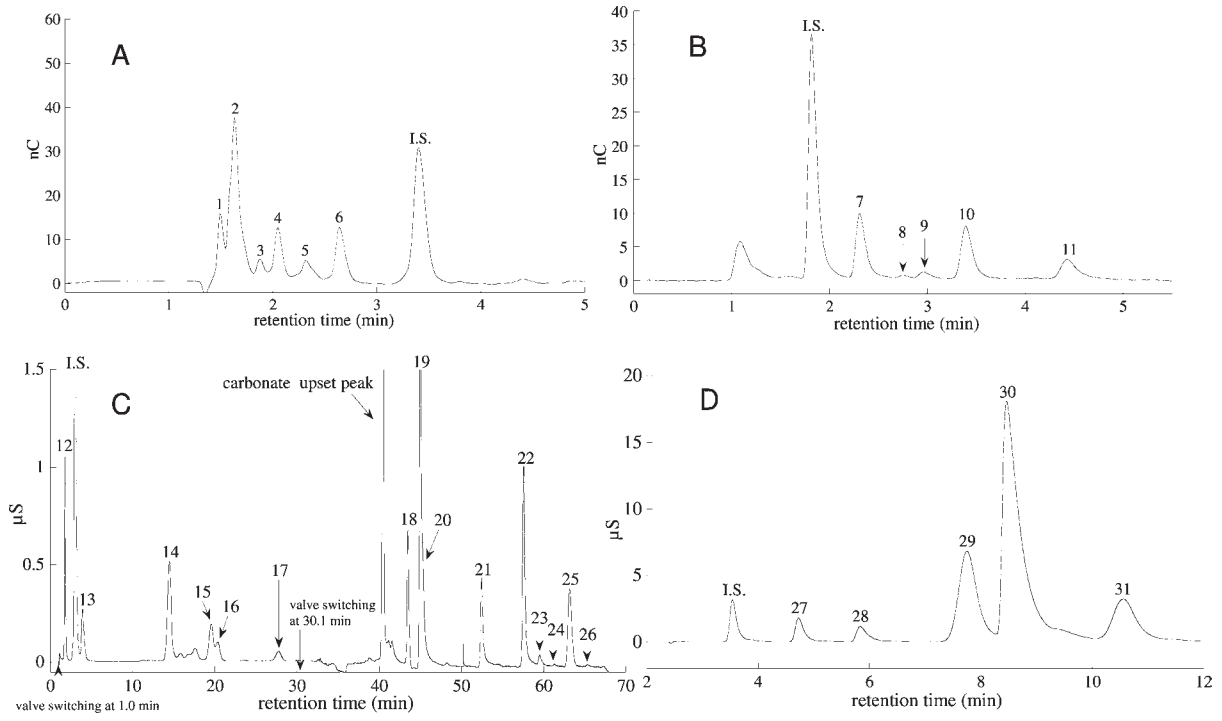

Figure 1. Representative chromatograms resulting from analysis of (**A**) alditols, (**B**) sugars, (**C**) organic acids and inorganic anions, and (**D**) inorganic cations in sample fractions derived from an aqueous extract of switchgrass feedstock St. Anthony. See text for details. Peaks: (1) glycerol, (2) inositol, (3) xylitol, (4) arabitol, (5) sorbitol, (6) mannitol, (7) sucrose, (8) arabinose, (9) galactose, (10) glucose, (11) fructose, (12) chloride, (13) nitrate, (14) dihydroxypentanoic acid, (15) lactic acid, (16) acetic acid, (17) formic acid, (18) sulfate, (19) oxalic acid, (20) fumaric acid, (21) phosphate, (22) citric acid, (23) isocitric acid, (24) *cis*-aconitic acid, (25) chelidonic acid, (26) *trans*-aconitic acid, (27) sodium, (28) ammonium, (29) magnesium, (30) potassium, (31) calcium. Internal standards (I.S.) in each chromatogram: (**A**) fucose, (**B**) fucose, (**C**) trifluoroacetate (TFA), (**D**) lithium.

related alditols via high-performance anion-exchange chromatography with pulsed amperometric detection at a disposable gold electrode (HPAEC-PAD).

All HPAEC-PAD analyses were carried out on a Dionex ICS-3000 series liquid chromatography system equipped with an EG eluent generator, DP gradient pump, AS autoinjector (10 μL sample loop), DC chromatography oven, and DC electrochemical detector. Chromatographic separation of alditols (**Figure 1A**) was achieved at 40 °C using a 50 mm × 4 mm CarboPac PA100 guard column and a 250 mm × 4 mm CarboPac PA100 analytical column connected in series and isocratic elution (mobile phase = aqueous 50 mM NaOH at 1 mL/min). Chromatographic separation of monomeric sugars (**Figure 1B**) was achieved at 40 °C using carbonate-modified 30 mm × 3 mm CarboPac PA20 guard and 150 mm × 3 mm CarboPac PA20 analytical columns connected in series with isocratic elution (mobile phase = aqueous 1.0 mM NaOH at 0.5 mL/min). Details of the procedure used to modify the PA20 columns are reported elsewhere (*12*).

Oligomeric Sugars. Acid hydrolysis for determination of oligomeric sugar content was detailed in an earlier paper (*11*). Once hydrolyzed samples had cooled to room temperature, a 250 μL aliquot of the hydrolysate was loaded onto an Alltech Extract-Clean SAX SPE cartridge, which had been preconditioned with 9 mL of methanol followed by 9 mL of water. The SPE cartridge was rinsed with slightly less than 10 mL of water, and the collected eluate was diluted to 10 mL in a volumetric flask. An aliquot of each sample was transferred directly to an autosampler vial and analyzed for total sugar content (i.e., total monosaccharides present in hydrolysate after acid hydrolysis at elevated temperature and pressure) using the HPAEC-PAD method described above. The amount of water-soluble oligomeric sugar present was assessed by subtracting the free sugar content measured in native aqueous extracts from the total sugar content measured in the corresponding hydrolysate. Note that acid hydrolysis of sucrose results in complete degradation of the fructose unit (i.e., half the weight percent of sucrose) and is therefore unaccounted for when total sugar content is assessed.

Organic Acids and Inorganic Anions. Preparation of switchgrass extracts for analysis of aliphatic acids and inorganic anions was performed using a Supelclean LC-18 SPE cartridge without further modification of the protocol described in Chen et al. (*11*, *13*). Chromatographic separation (**Figure 1C**) was carried out using a slightly modified procedure. Analytes were separated at 30 °C using two 50 mm × 4 mm IonPac AS11-HC guard columns and a 250 mm × 4 mm IonPac AS11-HC analytical column connected in series with gradient elution (1−40 mM aqueous NaOH at 1.5 mL/min). The columns were configured with a valve arrangement (i.e., a forward column step, as described in ref *14*) to optimize resolution between compounds. The valve arrangement also helps to reduce the large carbonate upset peak that is often seen in ion chromatography when dilute aqueous NaOH is used as the mobile phase and is not prepared by an eluent generator. This modified procedure enabled monitoring of formic, lactic, acetic, and oxalic acids along with other aliphatic acids and inorganic anions in a single run. Consistent with our previous corn stover study (*11*), aromatic acids were also monitored in these samples using a previously reported protocol (*15*).

ESI-MS Identification of Chelodonic and Dihydroxypentanoic Acids. Chelodonic and dihydroxypentanoic acids were identified using high performance liquid chromatography coupled with electrospray ionization−mass spectrometry in negative-ion detection mode (HPLC-ESI-MS). Briefly, a Varian ProStar model 210 binary pump, equipped with a model 410 autosampler, was used to drive analytical separations. Sample constituents were separated on a 150 mm × 4.6 mm YMC Carotentoid S-3 analytical column with nonlinear binary gradient elution at a flow rate of 0.75 mL/min (0.05% formic acid/H_2O and 90% acetonitrile/H_2O). Both compounds were monitored by tandem mass spectrometry (MS/MS) using a Varian model 1200 L triple-quadrupole mass analyzer. Instrumental settings of the mass spectrometer held constant in all HPLC-ESI-MS experiments were as follows: nebulizing gas, N_2 at 60 psi; drying gas, N_2 at 22 psi; temperature, 400 °C; needle voltage, 4500 V ESI^-; declustering potential, 40 V; collision gas, argon at 2.0 mTorr.

Once identified, quantitation of chelodonic acid and dihydroxypentanoic acid was performed via internal standard calibration in the same chromatographic run that was utilized to monitor additional aliphatic acids and inorganic ions (see above). However, reference standards were not available for dihydroxypentanoic acid (in any form); thus, quantitation of dihydroxypentanoic was estimated using calibration curves constructed for quinic acid, which eluted at the same retention time as the tentatively identified dihydroxypentanoic acid. The rationale for use of quinic acid was that, in suppressed conductivity detection, the hydrogen ion provides >90% of total conductance measured at the detector, and the balance of the analytical signal is related to the pK_a of the organic acid comprising the counterion (*16*). Because the pK_a for quinic acid falls within the range of expected pK_a values for dihydroxypentanoic acids (3.8−4.5), essentially identical analytical responses may be expected for these acids when suppressed conductivity detection is employed.

Table 2. Mass Percentage of Water-Soluble Materials (Extractives) in Switchgrass[a]

	accelerated solvent extractor		Soxhlet	
sample ID	mean (n = 3)	RSD (%)	mean (n = 3)	RSD (%)
St. Anthony	13.4	1.17		
Forestburg	11.8	1.25		
Trailblazer 2003	12.8	2.39	12.66	2.39
Trailblazer 2004	14.9	0.33	13.21	5.80

[a] Values reported as a percentage of oven-dried sample weight. RSD, relative standard deviation.

RESULTS AND DISCUSSION

Comparison of Extraction Techniques. ASE and Soxhlet techniques are typically assumed to be interchangeable methods for extracting soluble constituents from solid sample matrices. Scientists at the National Renewable Energy Laboratory have previously confirmed that the two techniques are interchangeable for generating aqueous extracts from corn stover (*6*). However, alternative sources of biomass were not investigated in that work. Data in the last two rows of **Table 2** clearly suggest that application of the two techniques resulted in similar removal of water-soluble materials from the two switchgrass samples extracted by both methods in this study. However, ASE offered a 7-fold reduction in the amount of time required to prepare triplicate samples as compared to the Soxhlet approach (1.5 h for ASE versus 10 h for Soxhlet). Accordingly, extract preparation via ASE was favored in all subsequent analyses affiliated with this study.

Mass Balance for Water-Soluble Materials. Water-soluble material accounted for as much as 15% of the dry weight of switchgrass feedstocks utilized in this study (**Table 2**). All values reported in **Table 2** are slightly lower but in reasonable agreement with the mass percentage of water-soluble material (16.42%) observed in a previous study of extracted switchgrass (*9*). They also fall near the range of mass percentages (14−27%) previously observed for five water-extracted corn stover samples (*11*). In contrast to the relatively narrow range of values reported in **Table 2**, the U.S. Department of Energy's Biomass Feedstock Composition and Property Database (*17*) documents 5−25% variability in the percentage of water-soluble materials that may be expected for switchgrass. Although the origin of this discrepancy is presently unknown, it is important to emphasize that the intent of our study was to evaluate the chemical composition of water-soluble materials in four opportunistic samples, not necessarily to provide a comprehensive comparison of variability that may be expected for all switchgrass materials.

Compositional analysis of water-soluble materials in switchgrass resulted in >85% mass closure for extractives in three of four analyzed feedstocks, and mass closure was >78% in all cases (**Figure 2**). Data in **Figure 2** are qualitatively similar to results of compositional determinations conducted previously on aqueous extracts of corn stover (*11*) in that identical compound classes represent the majority of the mass balance in each case. In our

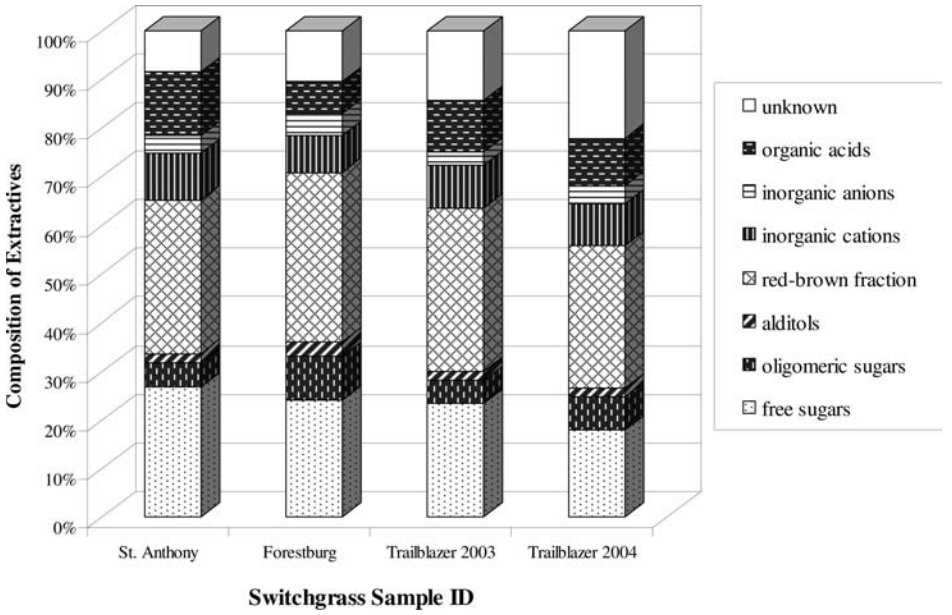

Figure 2. Composition of extractives in switchgrass (expressed as a percentage of oven-dried water-soluble material recovered from the native feedstock). Mass closure for the four samples is as follows: St. Anthony, 91.73%; Forestburg, 89.68%; Trailblazer 2003, 85.73%; Trailblazer 2004, 77.95%.

previous study, a band of material that was reddish brown in color was found to be strongly retained on solid phase extraction cartridges used to fractionate aqueous extracts. This band could not be eluted with either water or neat acetonitrile but was easily recovered using a 50:50 water/acetonitrile mixture. Subsequent analysis of material present in the recovered band revealed a complex mixture, characterized by two broad distributions in UV absorbance stretching from 30 to 60 min in reversed-phase HPLC chromatograms (*11*). When the band was hydrolyzed prior to HPLC analysis, the resulting chromatogram was relatively clean and showed major peaks at retention times corresponding to *p*-coumaric, ferulic, and sinapic acids (*11*). The hydrolyzed sample also contained significant amounts of arabaniose, galactose, glucose, and xylose (*11*), leading us to tentatively postulate that the red-brown fraction of corn stover extracts represented a diverse mixture of lignin−carbohydrate complexes. The red-brown fraction identified in **Figure 2** is also presumed to represent a mixture of lignin−carbohydrate complexes, because this fraction of switchgrass extracts was similar in color and exhibited identical retention behavior on SPE cartridges to the red-brown fraction derived from corn stover.

In most cases, the relative contribution of a given compound class to the overall mass balance for water-soluble materials in switchgrass was similar to that observed previously for corn stover (*11*). However, the relative contribution of free sugars was found to be lower for switchgrass extracts than for corn stover. In contrast, the red-brown fraction represented 30−35% of the mass balance for extractives in switchgrass, yet contributed only 10−18% to the mass balance for extractives in corn stover. Collectively, these observations suggest that an appreciable amount of fermentable sugar present in aqueous extracts of switchgrass may be conjugated to aromatic constituents, whereas the majority of fermentable sugar present in corn stover extracts is available as free sugar. The mass percentages of individual constituents of each compound class identified in **Figure 2** (excluding the red-brown fraction that was assessed only quantitatively by gravimetric means) are reported in **Tables 3**−**5**, and notable features of these data are discussed in more detail below.

Carbohydrates. Free sugars (i.e., monomeric sugars plus sucrose) represented 18−27% of the dry weight of water-soluble material in switchgrass (**Table 3**). As noted above, the observed contribution of free sugars to the overall mass balance for extractives in this study was lower than that observed for corn stover (30−57%) in previous work (*11*). In that study, the free-sugar content of extracts was composed almost entirely of glucose and fructose, which were present in approximately equal amounts. Statistically equivalent levels of glucose and fructose were also observed in the present study. However, sucrose dominated free-sugar composition in two instances and was

Table 3. Mass Percent of Monomeric Sugars, Oligomeric Sugars, Sucrose, and Related Alditols in Water Extracts of Switchgrass[a]

	sample ID			
analyte	St. Anthony	Forestburg	Trailblazer 2003	Trailblazer 2004
free sugars	26.79	24.01	23.44	17.90
glucose	6.3(3)	4.1(2)	7.2(1)	5.3(3)
fructose	6.4(6)	4.96(9)	6.3(3)	6.2(4)
sucrose	12.7(1)	14.5(4)	9.1(2)	5.6(2)
xylose	nd[b]	nd	nd	nd
arabinose	0.29(1)	nd	nd	nd
galactose	1.1(2)	0.45(4)	0.84(7)	0.80(1)
mannose	nd	nd	nd	nd
oligomeric sugars[c]	4.99	8.97	4.75	6.97
glucan	0.97(3)	5.04(6)	0.28(1)	2.73(3)
xylans	0.70(5)	0.44(1)	0.64(1)	0.50(1)
arabinans	0.04(1)	0.38(4)	0.32(2)	0.28(1)
galactans	1.02(9)	1.55(3)	0.98(4)	1.16(3)
mannans	2.26(2)	1.56(6)	2.53(7)	2.30(7)
alditols	1.93	2.97	1.79	1.75
glycerol	0.21(1)	nd	0.21(2)	0.21(1)
inositol	0.74(1)	1.24(5)	0.63(3)	0.59(3)
xylitol	0.16(1)	0.23(1)	0.12(1)	0.14(2)
arabinitol	0.44(1)	0.61(1)	0.34(3)	0.29(2)
sorbitol	0.10(1)	0.14(1)	0.08(1)	0.18(2)
mannitol	0.28(1)	0.75(6)	0.41(3)	0.344(4)

[a] Mass percentages represent the average of triplicate determinations. Values in parentheses represent one standard deviation in the least significant digit. [b] nd, not detected. [c] Oligomeric sugars are calculated differences between total and free sugars. See Materials and Methods for details.

Table 4. Mass Percent of Organic Acids in Water Extracts of Switchgrass[a]

	sample ID			
analyte	St. Anthony	Forestburg	Trailblazer 2003	Trailblazer 2004
total organic acids	13.38	6.60	10.59	9.80
dihydroxypentanoic acid	2.34(4)	1.03(6)	1.91(1)	1.26(6)
lactic acid	0.35(2)	0.41(5)	0.30(2)	0.36(6)
acetic acid	0.42(2)	0.33(3)	0.73(2)	0.95(6)
formic acid	0.134(4)	0.13(1)	0.11(1)	0.09(1)
oxalic acid	2.7(1)	0.68(1)	1.80(9)	1.88(7)
fumaric acid	0.46(5)	0.26(1)	0.73(2)	0.38(3)
citric acid	2.36(6)	1.72(7)	1.24(5)	1.34(2)
isocitric acid	0.39(1)	0.37(6)	0.41(3)	0.35(3)
cis-aconitic acid	nd[b]	0.12(1)	nd	0.31(5)
chelidonic acid	4.2(3)	1.42(9)	3.21(1)	2.34(1)
trans-aconitic acid	0.121(3)	0.13(1)	0.15(1)	0.54(8)

[a] Mass percentages represent the average of triplicate determinations. Values in parentheses represent one standard deviation in the least significant digit. [b] nd, not detected.

Table 5. Mass Percent of Inorganic Ions in Water Extracts of Switchgrass[a]

	sample ID			
analyte	St. Anthony	Forestburg	Trailblazer 2003	Trailblazer 2004
total cations	9.68	7.56	8.73	8.62
K^+	7.3(2)	5.3(2)	7.2(2)	7.1(2)
Ca^{2+}	0.13(2)	0.70(4)	0.19(2)	0.15(1)
Na^+	0.09(1)	0.085(3)	0.07(1)	0.06(1)
Mg^{2+}	1.93(5)	1.33(3)	1.10(3)	1.03(5)
NH_4^+	0.23(2)	0.14(1)	0.17(1)	0.28(2)
total anions	3.34	4.67	2.90	3.68
Cl^-	0.29(2)	1.36(3)	0.41(1)	0.68(4)
PO_4^{3-}	2.5(3)	2.25(8)	1.7(1)	1.90(1)
NO_3^-	0.181(3)	0.08(1)	0.17(1)	0.55(4)
SO_4^{2-}	0.37(2)	0.98(9)	0.62(3)	0.55(8)

[a] Mass percentages represent the average of triplicate determinations. Values in parentheses represent one standard deviation in the least significant digit.

present in all four aqueous extracts derived from switchgrass (**Table 3**). These results further confirm the previous hypothesis (*11*) that the presence of glucose and fructose in aqueous extracts of herbaceous biomass is derived from a common sucrose origin.

In our previous study of corn stover, it was discovered that a portion of polysaccharides (4−12% of the overall mass balance for water-soluble materials) was also released into aqueous extracts (*11*). Therefore, samples used to determine the free sugar content of switchgrass extracts were further hydrolyzed with acid at elevated temperature, and total sugar content was determined via HPAEC-PAD. These analyses revealed that oligomeric sugars, calculated as the observed difference between total and free sugars, contributed an additional 5−9% to the overall mass balance for extractives in switchgrass (**Table 3**). Note that this determination does not account for sugar present in the red-brown fraction of switchgrass extracts. However, it is important to recall that the red-brown fraction of analyzed samples dominated the overall mass balance for extractives (**Figure 2**), and the representative chromatogram shown in **Figure 3** clearly demonstrates that the red-brown fraction of aqueous extracts represents an additional source of fermentable sugars.

Alditols. Alditols identified in aqueous extracts of switchgrass are contained in **Tables 3**. The relative contribution of alditols to the overall mass balance for extractives in switchgrass is somewhat lower than that observed previously for corn stover (*11*);

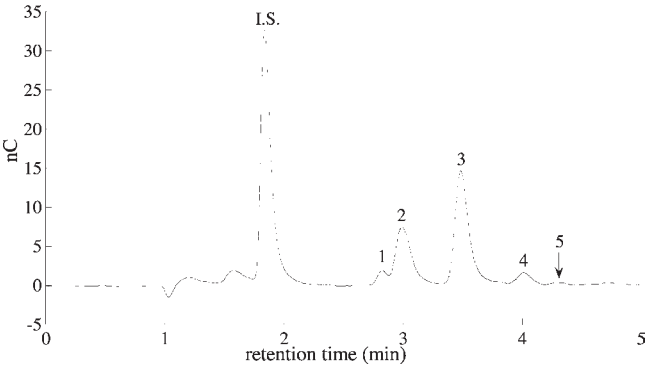

Figure 3. Chromatogram resulting from HPAEC-PAD analysis of a red-brown fraction with acid hydrolysis at elevated temperature. See text for details. Peaks: (1) arabinose, (2) galactose, (3) glucose, (4) xylose, (5) mannose. Internal standard (I.S.): fucose.

summative contributions ranged from 2 to 3% and from 3 to 7% in extracts of switchgrass and corn stover, respectively. This difference is almost entirely due to significantly reduced levels of glycerol in extracts of switchgrass (≤0.2%) relative to corn stover (1.7−3.6%). Other alditols in **Table 3** that were found to be common to both sample types were present at comparably low levels. However, it is important to note that the presence of inositol was unique to switchgrass extracts.

Organic Acids. The summative contribution of organic acids to the overall mass balance for switchgrass extracts ranged from near 7 to > 13% (**Table 4**). These values are in general agreement with comparable data reported previously for corn stover (*11*). The contribution of citric acid was also found to be similar in extracts of both switchgrass and corn stover. However, the relative contribution of acids common to both sample types was typically different between studies. For example, contributions of isocitric, *trans*-aconitic, and *cis*-aconitic acids reported in **Table 4** are approximately an order of magnitude lower than their respective contributions in extracts of corn stover. Individual acids contributing to the overall mass balance were also different between studies. For example, malic acid was typically the largest contributor in extracts of corn stover, yet absent in extracts of switchgrass. Similarly, chelidonic and dihydroxypentanoic acids, two of the more dominant contributors to the mass balance in switchgrass extracts, were not present in samples derived from corn stover. The confirmed presence of lactic, acetic, formic, and oxalic acids in switchgrass extracts may also be unique. However, the chromatographic conditions employed for monitoring organic acids in the corn stover study did not accommodate these analytes (i.e., if present, these acids would likely have eluted with the solvent front). Thus, their presence in corn stover extracts cannot be ruled out definitively.

Many of the compounds reported in **Table 4** were observed previously in water extracts of corn stover. Thus, their presence in switchgrass extracts was readily confirmed via analysis of reference standards using prior knowledge of analyte retention times. However, chelidonic acid and dihydroxypentanoic acid have not been previously identified in aqueous extracts of herbaceous biomass. Their presence in switchgrass extracts was initially observed as two sizable peaks in reversed-phase chromatograms with UV detection (unknown peaks 1 and 2 in **Figure 4A**), and subsequent LC-MS analysis was performed to further characterize these unknown sample constituents as detailed below.

Identification of chelidonic acid was supported by comparison of ESI mass spectra observed for unknown peak 1 in **Figure 4A** with spectra observed for a pure standard. The full-scan LC-MS mass spectrum observed for unknown compound **1** is given in

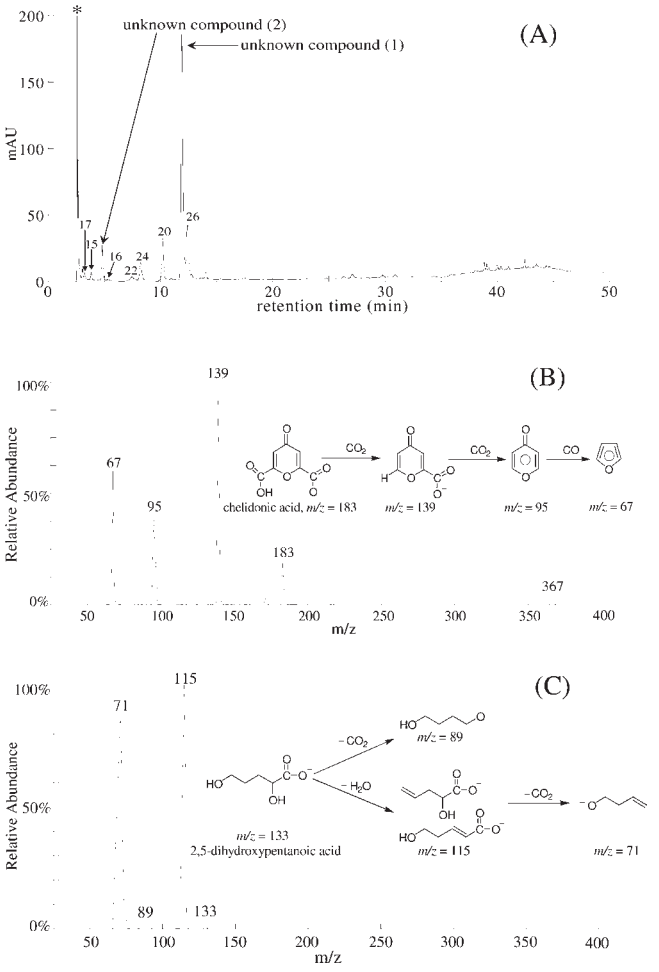

Figure 4. Unknown compound identification of switchgrass extractives. (**A**) HPLC-UV chromatogram of switchgrass extractives. Asterisk above the unretained peak indicates that the solvent front peak may potentially contain sugar acids. Labeled peak numbers correspond to **Figure 1C**. See text for details. (**B**) LC-MS mass spectrum of chelidonic acid in switchgrass sample and postulated structures of major mass fragments. (**C**) LC-MS/MS mass spectrum of dihydroxypentanoic acid in switchgrass sample and postulated structures of mass fragments.

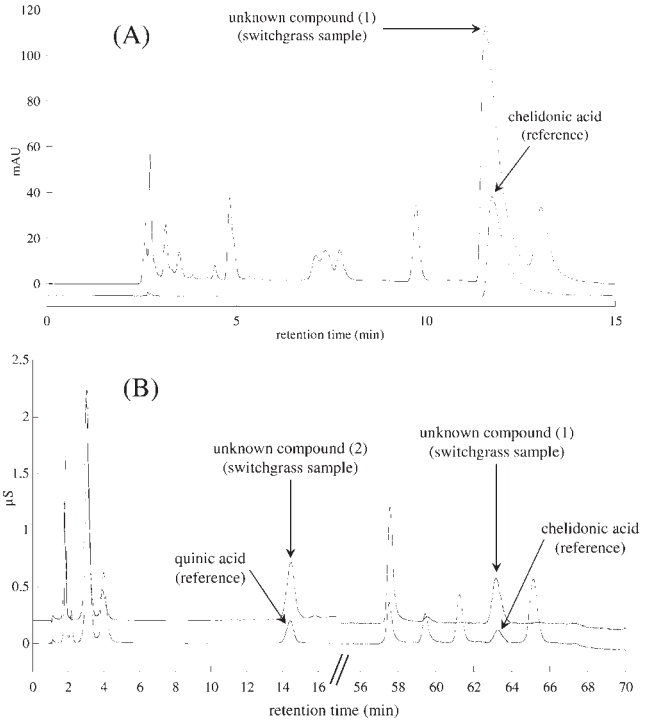

Figure 5. Two chromatographic methods for complementary screening of unknown compound in switchgrass extractives: (**A**) chromatogram of reversed-phase separation; (**B**) chromatogram of anion-exchange separation. See text for details.

Figure 4B. Subsequent MS/MS fragmentation of *m*/*z* 367 produced a base peak at *m*/*z* 139 and less abundant fragments at *m*/*z* 183, 95, and 67. Fragmentation of *m*/*z* 183 also produced ions at *m*/*z* 95 and 67. These data suggest that all ions were derived from a single component that was fragmented upon electrospray ionization and led to the tentative structural assignments given in **Figure 4B**. Although not specified in the figure, it was postulated that *m*/*z* 367 represented a singly charged dimer of chelidonic acid that formed potentially via a Diels−Alder reaction in the gas phase, as the dimer would not be expected to elute at the retention time observed for unknown compound **1**. LC-MS analysis of a pure standard produced comparable mass spectra (i.e., peaks were observed for the deprotonated molecule (*m*/*z* 183), its dimer (*m*/*z* 367), and fragments at *m*/*z* 95 and 67. It was subsequently discovered in direct infusion studies that the relative intensity of the ion at *m*/*z* 367 increased with increasing temperature of the electrospray, further confirming the hypothesis of dimer formation via chemical reaction. An abundant peak at *m*/*z* 91, presumed to be the doubly deprotonated ion $[M - 2H]^{2-}$, was also observed in infusion studies of the pure standard. Careful inspection of mass spectra resulting from LC-MS analysis of switchgrass extracts also revealed the presence of *m*/*z* 91, but at significantly reduced intensity.

The identity of unknown compound **1** was further confirmed by comparing retention times observed in three different HPLC separations with those observed for chelidonic acid. Comparative data for reversed-phase and anion-exchange separations are shown in panels **A** and **B** of **Figure 5**, respectively. A third comparative separation based on ion exclusion also confirmed equivalent retention times for unknown compound **1** and chelidonic acid (4.4 min). Absorbance ratios calculated at the retention time of chelidonic acid in reversed-phase HPLC separations of switchgrass extracts were also in agreement (> 98%) with those observed for the reference standard (data not shown); ratios were calculated using four wavelengths (210, 254, 275, and 320 nm) and normalized to 210 nm. The weight of evidence presented here supports definitive identification of chelidonic acid in aqueous extracts of switchgrass.

Tentative identification of unknown compound **2** in **Figure 4A** was based primarily on ESI mass spectral characterization. The full-scan mass spectrum of unknown compound **2** exhibited a single abundant ion at *m*/*z* 133, presumably the deprotonated ion $[M - H]^-$. Subsequent MS/MS fragmentation of *m*/*z* 133 produced the mass spectrum given in **Figure 4C** and led to the postulation that unknown compound **2** may be one of several structural isomers of dihydroxypentanoic acid. Comparative studies with a pure standard were not possible in this case, as a commercially available source of dihydroxypentanoic acid (in any form) could not be located. However, it was serendipitously discovered that unknown compound **2** eluted at the retention time of quinic acid in anion-exchange separations (**Figure 5B**). This finding was significant for two reasons. Not only did it provide for an analytical standard that could be used for quantitation of unknown compound **2** (see Materials and Methods for details), but it also further supports its tentative

identification. Retention of polar organic acids in anion-exchange chromatography is largely governed by pK_a, because only deprotonated ions are strongly retained. Thus, unknown compound **2** would be expected to have a pK_a similar to that of quinic acid [predicted $pK_a = 4.3 \pm 0.5$ (*18*)]. This decreases the likelihood that unknown compound **2** is the 2,2-isomer, as the predicted pK_a for this compound is 2.9 (*18*). In contrast, the predicted pK_a values for alternative dihydroxypentanoic acid isomers range from 3.8 to 4.5 (*18*), in agreement with the value predicted for quinic acid. Plausible structures of observed ions (i.e., m/z) are given in **Figure 4C** for the 2,5-isomer, which is a known degradation product of mannose (*19*) and several other sugars (*20*). However, it is important to point out that ions of equivalent m/z would also be expected for alternative forms.

Aromatic acids did not contribute significantly to the overall mass balance of switchgrass extracts. However, reversed-phase HPLC analysis confirmed that numerous compounds were in fact present at trace levels (a representative chromatogram is given in **Figure 4A**), consistent with previous analyses of corn stover extracts (*11*). As noted in that earlier investigation, the negligible contribution of these components (as well as acetic and formic acids in the present study) to the overall mass balance demonstrates that preparation of water extracts via ASE, according to the protocol reported here, does not result in significant hydrolysis of structural plant biopolymers (i.e., cellulose, hemicellulose, and lignin). Thus, the primary constituents of aqueous extracts identified in this work likely represent nonstructural plant components.

Inorganic Ions. The mass percentages of inorganic ions observed in aqueous extracts are given in **Table 5**. The total contributions of cations and anions to the overall mass balance for extractives in switchgrass are in general agreement with those reported previously for corn stover extracts (*11*). Cation composition was dominated by potassium in both studies, but magnesium replaced calcium as the second most abundant cation present in switchgrass extracts. Relative levels of other detected cations in this work were low. Anion composition was dominated by phosphate in the present study. Although phosphate was previously observed in corn stover extracts, its relative contribution to the mass balance for extractives was typically lower than that reported here. Relative levels of other anions observed in switchgrass extracts were more balanced but typically decreased in the order chloride $\geq$ sulfate $>$ nitrate. The confirmed presence of sulfate was a unique feature of switchgrass extracts relative to corn stover. Nitrite was also monitored in this work but not detected.

Contributors to the Unknown Fraction of Water Extracts. As demonstrated in **Figure 2**, compositional analysis did not result in quantitative mass closure for water-soluble materials. The additional presence of ash, protein, or both may be inferred from the previous work of Thammasouk et al. (*9*). The observation that UV absorbance for the solvent-front peak in reversed-phase analyses (i.e., the peak marked with an asterisk in **Figure 4A**) was almost 2 times greater for the switchgrass sample with 78% mass closure as compared to the three switchgrass samples for which mass closure exceeded 85% suggests that unretained components in this separation that were not detectable in other analyses may also contribute to mass closure. This peak essentially disappears from the chromatogram once extracts are passed through an anion-exchange SPE cartridge, suggesting the presence of highly polar organic acids (e.g., glucuronic acid, gluconic acid). It is not unreasonable to expect these to be ubiquitous constituents of herbaceous biomass. Alternatively, they could potentially form via sugar oxidation during the ASE process. A final contributor to the absence of complete mass closure may occur during the hydrolysis step utilized to assess oligomeric sugars. Once hydrolyzed, it is possible that monomeric sugars recondense or undergo additional side reactions and thus be unaccounted for in subsequent analyses. Thammasouk et al. has expressed similar concerns (*9*). Although each of these potential explanations for incomplete mass closure is worth noting, it is important to point out that the unknown fraction is most likely composed of many additional constituents present at low concentration rather than one or a few constituents representing the remaining mass balance for water-soluble materials.

Potential Implications for Biomass Processing. The findings reported in this work have the potential to influence future decisions regarding preferred biomass processing schemes, as well as feedstock storage practices. In this context, the most significant observation from this (and our earlier) study on extractives in biomass is that fermentable sugars were identified as significant components of water-soluble materials. As noted previously (*11*), these sugars are not typically considered in theoretical yield calculations for biobased products. However, the net effect their presence would have on overall process efficiency is difficult to speculate. The presence of water-soluble glucose would be expected to increase product yields in most biofuel processing schemes under consideration for production of ethanol. Fructose, on the other hand, is rapidly degraded at low pH to 5-hydroxymethylfurfural, a known fermentation inhibitor (*21*, *22*). Thus, the presence of fructose may be expected to counteract potential efficiency gains due to the presence of other fermentable sugars in processing schemes utilizing dilute acid. Whether positive or negative, the influence exerted by extractives may be expected to be less for switchgrass than for corn stover, due to the relatively smaller contribution of free sugars to the overall mass balance for extractives. In contrast, the opposite would be true if oligomeric sugars could also be utilized as a fermentation substrate in future processing paradigms (i.e., the influence of extractives in switchgrass would be expected to have a greater effect relative to corn stover). Finally, that fermentable sugars are present in herbaceous biomass in water-soluble form raises an interesting question that could influence feedstock storage practices. Namely, what percentage of total sugar is lost if feedstocks are stored outdoors and subjected to prolonged rain events? Neither this nor our earlier study was designed to answer this question. However, the question may become increasingly interesting if biomass materials containing a higher percentage of extractives with significant levels of free sugars are identified.

ACKNOWLEDGMENT

We gratefully acknowledge Dr. Christopher Becker (Baylor University) for valuable insight and assistance with manuscript preparation and Lekh N. Sharma (Baylor University) for experimental assistance with LC-MS analyses.

LITERATURE CITED

(1) McLaughlin, S. B.; Kszos, L. A. Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. *Biomass Bioenergy* **2005**, *28*, 515–535.

(2) Schmer, M. R.; Vogel, K. P.; Mitchell, R. B.; Perrin, R. K. Net energy of cellulosic ethanol from switchgrass. *Proc. Natl. Acad. Ssci. U.S.A.* **2008**, *105*, 464–469.

(3) Farrell, A. E.; Plevin, R. J.; Turner, B. T.; Jones, A. D; O'Hare, M.; Kammen, D. M. Ethanol can contribute to energy and environmental goals. *Science* **2006**, *311*, 506–508.

(4) Varvel, G. E.; Vogel, K. P.; Mitchell, R. B.; Follett, R. F.; Kimble, J. M. Comparison of corn and switchgrass on marginal soils for bioenergy. *Biomass Bioenergy* **2008**, *32*, 18–21.

(5) National Renewable Energy Laboratory. *Standard Biomass Analytical Procedures*; http://www1.eere.energy.gov/biomass/analytical_procedures.html (accessed August **2008**).
(6) Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of Extractives in Biomass*; National Renewable Energy Laboratory, Golden, CO, July 17, **2005**; http://devafdc.nrel.gov/pdfs/42618.pdf (accessed August 2008).
(7) Hames, B. R.; Thomas, S. R.; Sluiter, A. D.; Roth, C. J.; Templeton, D. W. Rapid biomass analysis. New tools for compositional analysis of corn stover feedstocks and process intermediates. *Appl. Biochem. Biotechnol.* **2003**, *105*−*108*, 5–16.
(8) Sanderson, M. A.; Agblevor, F.; Collins, M.; Johnson, D. K. Compositional analysis of biomass feedstocks by near infrared reflectance spectroscopy. *Biomass Bioenergy* **1996**, *11*, 365–370.
(9) Thammasouk, K.; Tandjo, D.; Penner, M. H. Influence of extractives on the analysis of herbaceous biomass. *J. Agric. Food Chem.* **1997**, *45*, 437–443.
(10) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of Total Solids in Biomass and Total Dissolbed Solids in Liquid Process Samples; National Renewable Energy Laboratory, Golden, CO, July 17, **2005**; http://devafdc.nrel.gov/pdfs/42621.pdf (accessed August 2008).
(11) Chen, S.-F.; Mowery, R. A.; Scarlata, C. J.; Chambliss, C. K. Compositional analysis of water-soluble materials in corn stover. *J. Agric. Food Chem.* **2007**, *55*, 5912–5918.
(12) Sevcik, R. S.; Mowery, R. A.; Chambliss, C. K. Rapid analysis of sugars in aqueous extracts and hydrolysates of biomass using a carbonate-modified anion-exchange column. *Anal. Chem.* **2010**, submitted for publication.
(13) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples; National Renewable Energy Laboratory, Golden, CO, December 8, **2006**; http://devafdc.nrel.gov/pdfs/42623.pdf (accessed August 2008).
(14) Mowery, R. A. *Automated Stream Analysis for Process Control*; Manka, D. P., Ed.; Academic Press: New York, 1982; Vol. 1, p154.
(15) Chen, S.-F.; Mowery, R. A.; van Walsum, G. P.; Chambliss, C. K. High-performance liquid chromatography method for simultaneous determination of aliphatic acid, aromatic acid and neutral degradation products in biomass pretreatment hydrolysates. *J. Chromatogr., A* **2006**, *1104*, 54–61.
(16) Wightman, E. P. A study of the conductivity and dissociation of organic acids in aqueous solutions between zero and thirty-five degrees. Ph.D. Dissertation, Johns Hopkins University, Baltimore, MD, **1911**; pp 15−20.
(17) U.S. Department of Energy. *Biomass Feedstock Composition and Property Database*; http://www1.eere.energy.gov/biomass/printable_versions/feedstock_database.html.
(18) Calculated values obtained from the SciFinder Database (Copyright 2007 American Chemical Society).
(19) Niemela, K.; Sjostrom, E. Alkalin degradation of mannan. *Holzforschung* **1986**, *40*, 9–14.
(20) Sjostrom, E. Carbohydrate degradation products from alkaline treatment of biomass. *Biomass Bioenergy* **1991**, *1*, 61–64.
(21) Newth, F. H. The formation of furan compounds from hexoses. In *Advances in Carbohydrate Chemistry*; Academic Press: New York, 1951; Vol. 6, pp 83−106.
(22) Klinke, H. B.; Thomsen, A. B.; Ahring, B. K. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl. Microbiol. Biotecnol.* **2004**, *66*, 10–26.

Received for review September 25, 2009. Revised manuscript received January 24, 2010. Accepted January 26, 2010. This work was supported by the U.S. Department of Energy, National Renewable Energy Laboratory (Subcontract ZCO-7-77397-01; Prime DE-AC36-99GO10337).