

Compositional Analysis of Water-Soluble Materials in Corn Stover

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Corn stover is one of the leading feedstock candidates for commodity-scale biomass-to-ethanol processing. The composition of water-soluble materials in corn stover has been determined with greater than 90% mass closure in four of five representative samples. The mass percentage of water-soluble materials in tested stover samples varied from 14 to 27% on a dry weight basis. Over 30 previously unknown constituents of aqueous extracts were identified and quantified using a variety of chromatographic techniques. Monomeric sugars (primarily glucose and fructose) were found to be the predominant water-soluble components of corn stover, accounting for 30–46% of the dry weight of extractives (4–12% of the dry weight of feedstocks). Additional constituents contributing to the mass balance for extractives included various alditols (3–7%), aliphatic acids (7–21%), inorganic ions (10–18%), oligomeric sugars (4–12%), and a distribution of oligomers tentatively identified as being derived from phenolic glycosides (10–18%).

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

INTRODUCTION

Corn stover has been identified as a promising feedstock for biomass-to-ethanol conversions (1–4). The term corn stover refers to the above-ground portion of a corn plant (i.e., leaves, cobs, husks, and stalks) that remains after corn has been harvested for grain. It has been estimated that greater than 60 million tons of corn stover can be sustainably collected and used to produce over 3 billion gallons of ethanol transportation fuel on an annual basis (1). Additional attributes of corn stover for biomass-to-ethanol processing include its proximity to existing grain-to-ethanol production facilities (3) and its amenability to conventional harvesting practices (4).

Any technical or economic valuation of a feedstock is inherently dependent upon detailed knowledge of its chemical composition. Accepted analytical procedures for compositional analysis of biomass (5) enable near-quantitative mass closure on a dry weight basis. However, total water- and/or ethanol-soluble materials are typically quantified gravimetrically and identified only as extractives (6–9). Previous work has demonstrated that extractives can effect macrocomponent compositional determinations affiliated with analysis of herbaceous biomass (e.g., Klason lignin, total glycan, ash, and protein) (9). Most notably, it was demonstrated that hot water extraction prior

to analysis resulted in a significant reduction in the measurable glucan content of corn stover, fescue, and switchgrass. This result provides strong impetus to further investigate the composition of water extractives in herbaceous feedstocks, since the value of a feedstock for fermentation-based bioprocessing is directly related to its carbohydrate content.

The objective of the present study was to characterize unknown water-soluble constituents of corn stover. Five representative samples with diverse chemical compositions were extracted and investigated in a side-by-side comparison. Multiple analytical protocols were developed over the course of the study, collectively affording identification and quantitative assessment of over 30 chemical constituents that were 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 corn stover, and results are interpreted in terms of their potential impact on biomass processing, feedstock storage, and future analyses of feedstock composition.

MATERIALS AND METHODS

Corn Stover Feedstocks and Chemical Reagents. Dry corn stover (<10% moisture) was supplied by the National Renewable Energy Laboratory (NREL) (Golden, CO). Five samples (NREL sample IDs: Kramer 33A14, 2302-079, 2870-061, 2302-115, and 2893-026) were selected from a larger collection of feedstock at NREL that had been used previously to calibrate chemometric models based on near-infrared

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Table 1. Historical Data for Analyzed Corn Stover Feedstocks

NREL ID	seed company	location grown	harvest year
Kramer 33A14	Pioneer Hi-Bred International	Wray, CO	2002
2302-079	Monsanto	Oskaloosa, IA	1997
2870-061	Syngenta Seed Co.	Fond du Lac, WI	1997
2302-115	Monsanto	Perry, IA	1997
2893-026	Pioneer Hi-Bred International	Fond du Lac, WI	1997

spectra. A brief history of each stover is provided in **Table 1**. The only criteria employed in selecting feedstocks for the present study were diverse chemical compositions. All chemicals and reference standards were reagent grade or better, obtained from commercial vendors, and used as received. Distilled water was purified and deionized to 18 M Ω with a Barnstead Nanopure Diamond UV water purification system.

Sample Preparation. Each stover sample was "milled" for 90 s using a commercial coffee grinder. Milled stover was subsequently screened using a 40 mesh sieve, and material passing through the sieve was collected and utilized in compositional determinations. It was assumed that chemical fractionation does not occur during the sieving step.

Water Extraction. In a typical extraction, 8 g of sieved stover was added to a Soxhlet thimble and extracted for 10 h as described in NREL Laboratory Analytical Procedure (LAP) Determination of Extractives in Biomass (6). Heating was adjusted to achieve a siphon rate of 4–5 cycles per hour. Aqueous extracts generated for compositional determinations were quantitatively transferred to volumetric flasks and diluted to 200 mL prior to analysis. For determination of percent extractives, aqueous extracts (prepared from independent water extractions) were evaporated under N₂ at 40 °C using a Zymark Turbovap LC concentration workstation (Zymark Corp., Hopkinton, MA), and residues were dried to constant weight in a vacuum oven at 40 °C. The moisture content of each feedstock was independently determined using the "convection oven method" described in NREL LAP Determination of Total Solids in Biomass (10). Approximately 1 g of each sieved stover was placed in a preweighed aluminum weighing dish and dried to constant weight at 105 °C. Percent total solids, defined as (dry weight of sample/sample weight as received) \times 100, was calculated for each tested feedstock and used to determine the oven dry weight for each extracted sample. Percent extractives were calculated as (dry weight of extractives/dry weight of extracted sample) \times 100.

Fractionation and Compositional Analysis of Aqueous Extracts. The analytical approach employed for identification and quantitation of water-soluble materials in corn stover involved fractional cleanup of aqueous extracts followed by one or more chromatographic analyses. Details of cleanup procedures and affiliated chromatographic methods developed during this study are described below with specific reference to the class of compounds assessed using each experimental protocol. Representative chromatograms resulting from analysis of samples isolated from a water extract are given in **Figure 1**.

Sugars and Related Alditols. A 2 mL aliquot of aqueous extract was loaded onto a Supelclean ENVI-Chrom P solid-phase extraction (SPE) cartridge that had been preconditioned with 15 mL of methanol followed by 15 mL of water. The cartridge was rinsed with slightly less than 23 mL of water, and the combined eluate was diluted to 25 mL in a volumetric flask. After a subsequent five-fold dilution, the sample was analyzed for monomeric sugars, sucrose, and related alditols via high-performance anion-exchange chromatography with pulsed amperometric detection at a disposable gold electrode (HPAE-PAD). All HPAE-PAD analyses were carried out on a Dionex DX-600 series liquid chromatograph equipped with a DG2410 degassing module, GP50 gradient pump, AS3500 autoinjector (10 μ L sample loop), LC30 chromatography oven, and ED40 electrochemical detector. Chromatographic separation (**Figure 1A**) was achieved at 27 °C using a 50 mm \times 4 mm CarboPac PA100 guard column and two 250 mm \times 4 mm CarboPac PA100 analytical columns connected in series and isocratic elution (mobile phase = aqueous 10 mM NaOH at 0.8 mL/min). A second aliquot

from the diluted eluate was also screened for sucrose, glucose, and fructose using a colorimetric enzyme assay available commercially from R-Biopharm. Spectroscopic monitoring affiliated with the enzyme assay was carried out on a Perkin-Elmer model Lambda 35 UV-visible spectrophotometer.

Assessment of oligosaccharides was conducted using a procedure similar to that described in NREL LAP Determination of Sugars, Byproducts, and Degradation products in Liquid Fraction Process Samples (11). Briefly, a 5 mL aliquot was taken from the 25 mL sample described above and hydrolyzed with 4% sulfuric acid at 121 °C for 1 h. A series of sugar recovery standards were treated in the same manner to correct for potential degradation. Once the samples cooled to room temperature, the pH was adjusted to 5–6 with calcium hydroxide, and the resulting mixtures were loaded onto preconditioned Supelclean ENVI-Chrom P SPE cartridges. Each cartridge was rinsed with water, and eluates were diluted to 25 mL. These samples were transferred directly to autosampler vials and analyzed for total sugar content (i.e., the sum total of monosaccharides present in hydrolysate resulting from treatment of aqueous extracts with sulfuric acid at elevated temperature and pressure) using the HPAE-PAD method described above. The amount of water-soluble oligomeric sugar present in analyzed stover samples was assessed by subtracting the summative monosaccharide content (excluding fructose) measured in native aqueous extracts from the total sugar content measured in the corresponding hydrolysate.

Aromatic Acids. The SPE cartridge used to isolate sugars and related alditols from the aqueous extract was subsequently rinsed with 10 mL of an aqueous 2% sodium bicarbonate solution. The eluate was collected and acidified to pH 1.8 with phosphoric acid. This sample was extracted with methyl-*tert*-butyl ether and screened for a variety of aromatic acids (and alternative lignocellulosic degradation products) using a previously reported reversed-phase liquid chromatography (RPLC) procedure (12). Chromatographic separation (**Figure 1B**) employed an RP 18 Opti-Guard column (Alltech, Deerfield, IL) and 250 mm \times 4.6 mm YMC Carotenoid S-3 analytical column (Waters Corp., Milford, MA) connected in series and a nonlinear gradient, consisting of 0.05% (v/v) aqueous H₃PO₄ and acetonitrile.

Red-Brown Fraction. Following successive rinses with water and 2% aqueous sodium bicarbonate, a narrow band of material that was reddish-brown in color remained at the head of the SPE cartridge used to isolate carbohydrate and aromatic acid fractions from the aqueous extract. The band was eluted with 5 mL of 1:1 water-acetonitrile, the solvent was evaporated under N₂ at 40 °C, and the quantity of remaining nonvolatile material was assessed gravimetrically. Qualitative analyses directed at characterization of material(s) in the red-brown fraction were also performed. The residue remaining after solvent evaporation was initially reconstituted in 5 mL of water, extracted with MTBE, and analyzed using the RPLC procedure noted above. An independent 5 mL sample was treated with 4% sulfuric acid at elevated temperature (as described above) and analyzed via HPAE-PAD. A third 5 mL sample was treated with 4% sulfuric acid and subsequently extracted with 95:5 *n*-butanol:hydrochloric acid using a previously reported procedure (13). Following phase separation, the colored *n*-butanol phase was back-extracted with 2% aqueous sodium bicarbonate, and the resulting aqueous extract was analyzed via RPLC.

Aliphatic Acids and Inorganic Anions. An independent 2 mL aliquot of aqueous extract was loaded onto a second preconditioned Supelclean ENVI-Chrom P SPE cartridge (see above). The cartridge was rinsed with slightly less than 3 mL of water, and the combined eluate was diluted to 5 mL in a volumetric flask. Polar organic acids and inorganic anions in this sample were analyzed via ion chromatography with suppressed conductivity detection. Chromatographic separation (**Figure 1C**) was carried out at 30 °C using a 50 mm \times 4 mm IonPac AS11-HC guard column and 250 mm \times 4 mm IonPac AS11-HC analytical column connected in series and gradient elution (1–200 mM aqueous KOH at 1.2 mL/min). Complementary screening of aliphatic acids in this sample was also conducted via independent ion-exclusion and reversed-phase separations with UV detection at 210 nm. Ion-exclusion separations were carried out at 55 °C using a 300 mm \times 7.6 mm IC-Pak column (Waters Corp., Milford, MA) and isocratic elution (0.01 N aqueous H₃PO₄). Reversed-phase separations were carried out using the procedure described above for analysis of aromatic acids. All three

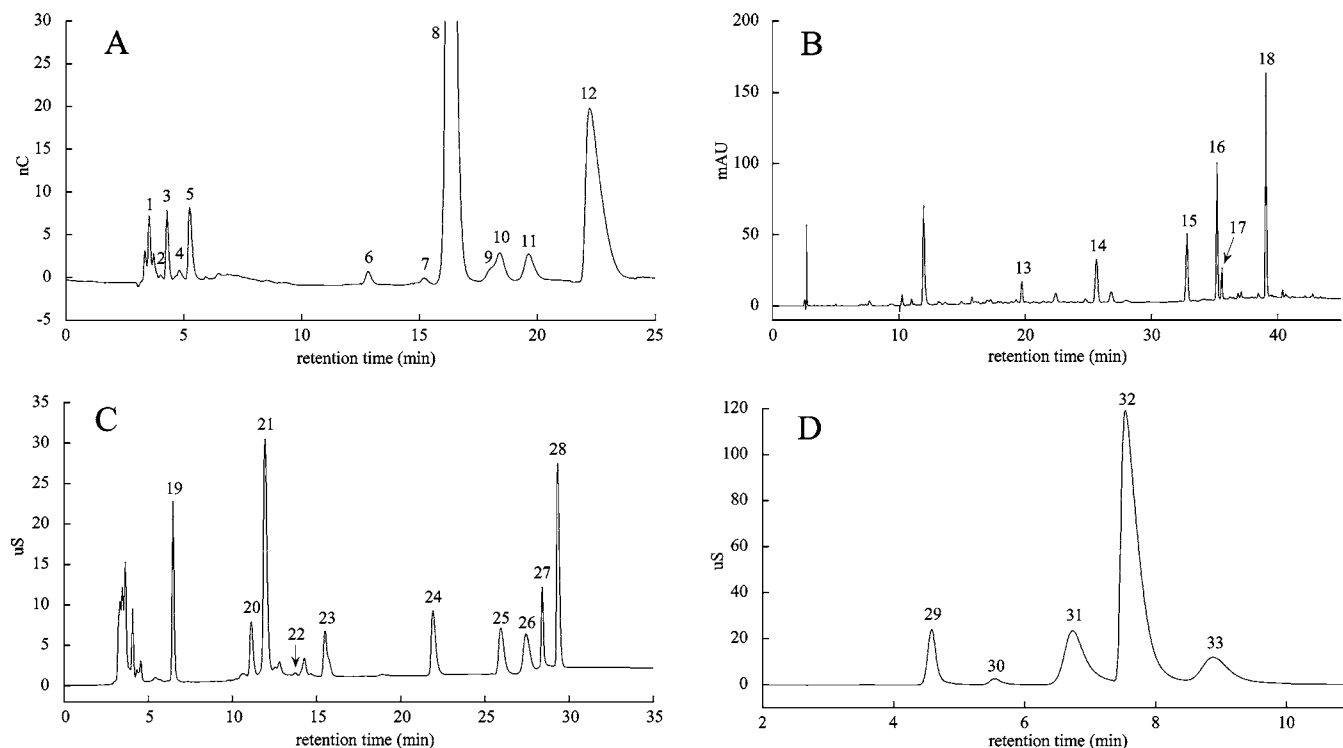


Figure 1. Representative chromatograms resulting from analysis of (A) sugars and related alditols, (B) aromatic acids, (C) aliphatic acids and inorganic anions, and (D) inorganic cations in sample fractions derived from an aqueous extract of corn stover feedstock 2302-079. See the text for details. Peak identifications are as follows: 1, glycerol; 2, mannitol; 3, arabinitol; 4, sorbitol; 5, xylitol; 6, arabinose; 7, galactose; 8, glucose; 9, mannose; 10, xylose; 11, sucrose; 12, fructose; 13, 3,4-dihydroxybenzoic acid; 14, 4-hydroxybenzoic acid; 15, vanillic acid; 16, caffeic acid; 17, syringic acid; 18, *para*-coumaric acid; 19, chloride; 20, nitrate; 21, malic acid; 22, maleic acid; 23, fumaric acid; 24, phosphate; 25, citric acid; 26, isocitric acid; 27, *cis*-aconitic acid; 28, *trans*-aconitic acid; 29, sodium; 30, ammonium; 31, magnesium; 32, potassium; and 33, calcium.

chromatographic analyses were carried out on a Dionex DX-600 series liquid chromatograph equipped with a DG2410 degassing module, GP50 gradient pump, AS50 autoinjector (10 μ L sample loop), LC30 chromatography oven, UVD170U multiwavelength ultraviolet detector, and ED40 electrochemical detector.

Inorganic Cations. An independent 1 mL aliquot of aqueous extract was combined with an equal volume of 100 mM aqueous methanesulfonic acid and extracted with 1 mL of *n*-butanol. The clear, colorless aqueous phase was analyzed directly for cations via ion chromatography with suppressed conductivity detection. Chromatographic separation (**Figure 1D**) was achieved at 40 °C using a 50 mm \times 5 mm IonPac CS16 guard column and 250 mm \times 5 mm IonPac CS16 analytical column connected in series and isocratic elution (mobile phase = 48 mM aqueous methanesulfonic acid at 1 mL/min).

Identification and Quantitation of Analytes. The criteria employed for analyte identification varied with compound class. Monomeric sugars, alditols, and inorganic ions were assigned based on retention time and an expected doubling of analyte concentration ($\pm 20\%$) in subsequent analyses of spiked samples. Although retention time is not a unique qualifier of identity, this approach was deemed acceptable due to the unique nature of the analytical response expected for individual compounds when pulsed amperometry or conductivity is employed for analyte detection. In contrast, assignment of aliphatic acids required confirmation of a retention time match with a reference standard in three complementary separations (i.e., anion exchange, ion exclusion, and reversed phase). Identification of aromatic acids was based on a comparison of retention time and UV absorbance data with reference standards as described previously (12). As evidenced by the chromatograms in **Figure 1**, this approach resulted in assignment of nearly all major peaks in sample fractions derived from water extracts.

Quantitation methods also varied with compound class. Sugars and related alditols were determined via HPAE-PAD using multipoint, external standard calibration curves. External standard calibration curves were also employed in the determination of aliphatic acids and inorganic ions via ion chromatography. In contrast, aromatic acids were deter-

Table 2. Mass Percent of Water-Soluble Materials (Extractives) in Oven-Dried Corn Stover^a

sample ID	mean ($n = 3$) (% dry weight)	RSD (%)
Kramer 33A14	14.2	0.90
2302-079	20.3	0.15
2870-061	16.4	0.56
2302-115	26.7	0.61
2893-026	14.3	0.94

^a Values based on 10 h Soxhlet extractions. See the text for details. RSD, relative standard deviation.

mined using an internal standard calibration approach (12). Constituents in the strongly retained red-brown fraction were quantified gravimetrically. Mean values reported in **Tables 2–5** were based on triplicate extractions of corn stover, and except for aromatic acids, assume quantitative recovery of analytes in all sample preparation steps.

RESULTS AND DISCUSSION

Mass Balance for Water-Soluble Materials. Water-soluble materials accounted for as much as 27% of the dry weight of corn stover feedstocks utilized in this study, clearly justifying the importance of understanding the composition of extractives. However, the mass percent of extractives varied by almost a factor of 2 between samples (**Table 2**). While a detailed explanation of compositional variation among stover samples is beyond the scope of this work, previous studies have demonstrated differing chemical composition in various stovers derived from maize hybrids (14) and between different anatomical fractions (i.e., leaves, cobs, husks, and stalks) of corn plants (3) and corn stover (15). Crop maturity (3) and storage method

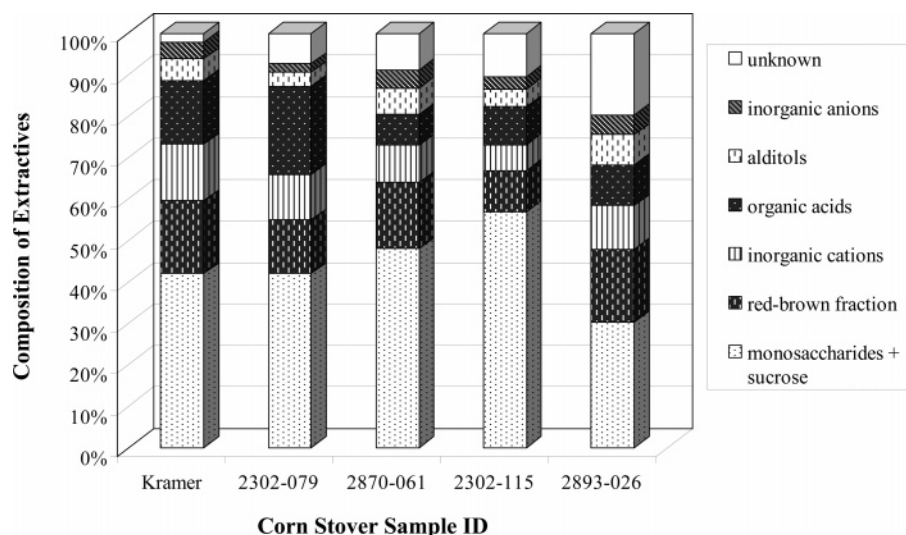


Figure 2. Composition of extractives in corn stover (expressed as a percentage of oven-dried water-soluble material recovered from the native feedstock). See the text for details.

(15) have also been shown to influence composition. Stover feedstocks utilized in this study were composite samples, collected from multiple locations in different years. Thus, some variation in chemical composition is to be expected. All values reported in **Table 2** are consistent with previous analyses of extractives in herbaceous biomass (9).

Compositional analysis of aqueous extracts revealed that monosaccharides were the largest contributor to the overall mass balance for extractives (**Figure 2**). Additional compounds identified in aqueous extracts and assessed in this work include various alditols, organic acids, and inorganic ions. A visible band of material that was reddish-brown in color and could not be eluted with water, aqueous sodium bicarbonate, or acetonitrile was consistently retained near the top of SPE cartridges used to cleanup aqueous extracts prior to analysis. It was later discovered that the colored band could be eluted with a 1:1 water–acetonitrile mixture, and gravimetric analysis of the residue remaining after solvent evaporation demonstrated that this fraction of the aqueous extract (i.e., the red-brown fraction) also contributed significantly to the mass balance for water-soluble materials (10–18%). The mass percentages of individual constituents in each compound class identified in **Figure 2** are reported in **Tables 3–5**, and notable features of these data are discussed below.

Carbohydrates. Monosaccharides, primarily glucose and fructose, represented 30–46% of the dry weight of water-soluble materials in tested feedstocks (**Table 3**). Note that the glucose quantities reported here are more than sufficient to explain the apparent reduction in glucon content reported previously for analysis of structural carbohydrates in water-extracted corn stover (9). Additionally, it is noteworthy that approximately equal amounts of glucose and fructose were detected in all samples, suggesting that the presence of these sugars may be derived from a common sucrose origin. This observation led to divergent hypotheses that one or more sample preparation/analysis steps may promote hydrolysis of extracted sucrose or that latent enzyme activity may be present in harvested feedstocks.

A simple experiment was subsequently conducted to evaluate the potential of procedural factors to affect sucrose hydrolysis. An aqueous solution of sucrose at pH 5.4 (the typical pH of

Table 3. Mass Percent of Monosaccharides, Sucrose, and Related Alditols in Water Extracts of Corn Stover^a

analyte	sample ID				
	Kramer 33A14	2302-079	2870-061	2302-115	2893-026
total sugars	42	42	48	57	30
glucose	18(1)	20(1)	24(2)	21(1)	15.0(4)
fructose	22(2)	19(2)	22(2)	24(2)	11.6(7)
sucrose	0.9(1)	1.53(3)	0.36(6)	11(1)	ND
xylose	0.53(5)	0.36(2)	0.69(8)	0.115(8)	2.2(2)
arabinose	0.27(2)	0.150(3)	0.49(4)	0.057(5)	0.8(2)
galactose	0.21(2)	0.097(4)	0.34(2)	0.13(2)	0.36(5)
mannose	0.43(5)	0.54(2)	0.27(4)	0.23(2)	0.3(1)
total alditols	5.2	3.4	6.4	4.3	7.3
glycerol	3.4(3)	1.7(1)	3.6(4)	3.2(3)	3.1(5)
xylitol	0.43(3)	0.74(2)	1.26(7)	0.472(8)	1.7(2)
arabinitol	0.56(4)	0.63(2)	1.2(1)	0.42(4)	2.0(2)
sorbitol	0.69(4)	0.196(8)	0.18(2)	0.15(2)	0.26(3)
mannitol	0.11(1)	0.097(3)	0.13(1)	0.08(1)	0.22(4)

^a Mass percentages represent the average of triplicate determinations. Values in parentheses represent one standard deviation in the least significant digit; ND, not detected.

corn stover extracts) was refluxed for 10 h in a Soxhlet apparatus. Once the solution cooled to room temperature, an aliquot was analyzed for sucrose, glucose, and fructose via HPAE-PAD and an independent colorimetric enzyme assay. Results for both analyses were consistent and indicated essentially negligible quantities of glucose and fructose (less than 5% of sucrose originally added to the aqueous solution). These results suggest that the extraction and analysis protocols utilized in this study do not contribute significantly to sucrose hydrolysis.

The confirmed presence of water-soluble sugar in corn stover has potentially significant implications for technical and economic valuations of bioconversion processes as well as feedstock storage practices. The data in **Tables 2** and **3** collectively demonstrate that fermentable sugars represent as much as 12% of the dry weight of corn stover feedstocks. Other sources of water-soluble sugar include oligomeric sugar and sugars derived from the red-brown fraction of aqueous extracts (see below). However, the recommended analytical procedure for determination of total carbohydrates in biomass requires that extractives be removed from feedstocks prior to analysis (16). As a result, the contribution of water-soluble sugars to total carbohydrates

Table 4. Mass Percent of Organic Acids in Water Extracts of Corn Stover^a

analyte	sample ID				
	Kramer 33A14	2302-079	2870-061	2302-115	2893-026
total aliphatic acids	15.34	21.20	7.43	9.14	9.75
malic acid	5.70(2)	5.40(3)	1.59(2)	3.26(1)	2.37(4)
isocitric acid	3.52(1)	5.48(1)	2.09(1)	2.81(1)	2.12(5)
citric acid	1.45(1)	4.06(3)	1.81(2)	1.54(1)	2.36(4)
<i>trans</i> -aconitic acid	2.097(4)	3.622(9)	0.514(2)	0.672(4)	0.67(1)
fumaric acid	0.98(4)	1.01(4)	1.18(1)	0.536(7)	1.29(1)
<i>cis</i> -aconitic acid	1.01(1)	1.58(1)	0.231(4)	0.301(5)	0.291(7)
maleic acid	0.58(2)	0.046(1)	0.013(1)	0.020(1)	0.650(7)
total aromatic acids	<0.06	<0.08	<0.06	<0.06	<0.1

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

is not typically considered in technical and economic models of biomass-to-ethanol processes.

In practice, implications of this oversight could have both positive and negative consequences. For example, the presence of water-soluble glucose in feedstocks would be expected to result in increased ethanol yields relative to those predicted by current models. In contrast, water-soluble fructose would likely have a negative impact on ethanol yield in processing schemes involving dilute acid. Under these conditions, fructose is rapidly degraded to 5-hydroxymethylfurfural (17), which is a known fermentation inhibitor (18). Last, it is important to point out that the confirmed presence of water-soluble sugars in corn stover raises an interesting question related to biomass storage practices, namely, whether outdoor storage of feedstocks in rainy climates has an impact on feedstock composition.

Alditols and Aliphatic Acids. Various alditols and aliphatic acids identified in aqueous extracts (Tables 3 and 4, respectively) have been cited among the top 30 value-added chemicals that can be derived from biomass (19). Glycerol, sorbitol, xylitol, arabinitol, malic acid, and fumaric acid are identified in the first tier of potential synthetic starting materials (i.e., the top 12), while citric and aconitic acid fall into the second tier. To our knowledge, the present study represents the first demonstration that these chemicals are among the primary constituents of purely aqueous extracts of corn stover. While commercial syntheses of many of these compounds are relatively straightforward, the same is not true of aconitic acid (1-propene-1,2,3-tricarboxylic acid). The primary commercial source of this compound has been its recovery as a byproduct of sugar cane processing since the 1950s (20).

The presence of water-soluble aconitic acid may indicate a novel opportunity to lower overall processing cost for biomass-to-ethanol conversion via its recovery from aqueous process streams. In living systems, aconitic acid is an ubiquitous intermediate of the Krebs cycle (this statement also applies to most other acids in Table 4), and *trans*-aconitic acid is synthesized in maize (*Zea mays* L.) via enzyme-catalyzed dehydration of citric acid (21). Therefore, it is likely that the occurrence of *cis*-aconitic acid in corn stover extracts is due primarily to geometric isomerization at elevated temperature (22). Data in Tables 2 and 4 collectively demonstrate that the dry weight concentration of aconitic acid (i.e., the sum of *cis*- and *trans*-isomers) in tested feedstocks varied between 0.1 and 1%. These percentages suggest that a significant amount of aconitic acid could be recovered if biomass-to-ethanol processing reaches its projected potential (e.g., consuming 60–80 million tons of corn stover on an annual basis). It is also likely

Table 5. Mass Percent of Inorganic Ions in Water Extracts of Corn Stover^a

analyte	sample ID				
	Kramer 33A14	2302-079	2870-061	2302-115	2893-026
total cations	13.62	11.01	8.98	6.31	10.55
K ⁺	10.93	8.18	6.10	3.98	5.70
Ca ²⁺	1.16	1.40	1.50	1.21	1.73
Na ⁺	0.76	0.57	0.75	0.43	0.83
Mg ²⁺	0.56	0.71	0.46	0.57	1.67
NH ₄ ⁺	0.21	0.15	0.17	0.12	0.62
total anions	4.07	2.14	4.40	3.03	4.81
Cl ⁻	1.78	0.43	3.24	1.72	3.00
PO ₄ ³⁻	0.83	1.31	1.05	1.01	1.50
NO ₃ ⁻	1.46	0.40	0.11	0.30	0.31

^a Mass percentages represent the average of triplicate determinations. The standard deviation of reported means was <0.001 in all cases.

that this strategy for reducing cost is not limited to corn stover, as previous work (23) has demonstrated that dry weight concentrations of aconitic acid in early season range grasses typically vary between 1 and 2.5% and were as high as 12.2% in the leaves of western larkspur (*Delphinium hesperium*).

Aromatic Acids. As demonstrated in Table 4, aromatic acids do not contribute significantly to the mass balance for organic acids. Aromatic monomers assessed in this study (Figure 1B) are lignin-derived constituents of hydrolysates resulting from pretreatment of corn stover with dilute acid (12, 24). Significant hydrolysis of corn stover constituents upon Soxhlet extraction would also produce a variety of additional degradation products that are easily detected using the RPLC procedure employed for analysis of aqueous extracts (12). Although careful inspection of the chromatogram shown in Figure 1B demonstrates that numerous compounds are present in these samples at trace levels, the negligible contribution of these components to the overall mass balance suggests that hydrolysis of lignocellulosic materials is not a significant contributor to the composition of water-soluble materials in corn stover. This is to be expected, as hot water pretreatments designed to initiate hydrolysis of corn stover (25, 26) are typically conducted at elevated temperatures (e.g., 170–220 °C) and pressures relative to the conditions employed here.

Inorganic Ions. The mass percentages of inorganic ions in aqueous extracts are given in Table 5. The cation composition was dominated by potassium and to a lesser extent calcium (and magnesium in one case). Relative levels of detected anions were more balanced but typically decreased in the order chloride > phosphate > nitrate. Note that nitrite was also monitored but not detected in aqueous extracts. Charge–balance relationships were calculated for each aqueous extract and revealed a bias in favor of cations by as much as a factor of 3 (data not shown). However, this is easily rationalized considering that di- and trifunctional aliphatic acids identified in aqueous extracts (i.e., citric, malic, and aconitic acids) are widely recognized as chelating agents for cations, such as Ca²⁺ and Mg²⁺, in aqueous solution.

Tentative Identification of Constituents in the Red-Brown Fraction. Qualitative analysis of the red-brown fraction of water extracts suggested the presence of a complex oligomeric mixture. A near continuum of peaks, characterized by two broad distributions stretching from roughly 30 to 60 min, was observed in the chromatogram resulting from RPLC analysis of the crude red-brown residue (Figure 3A). The intense color of material in this fraction initially led us to postulate the presence of

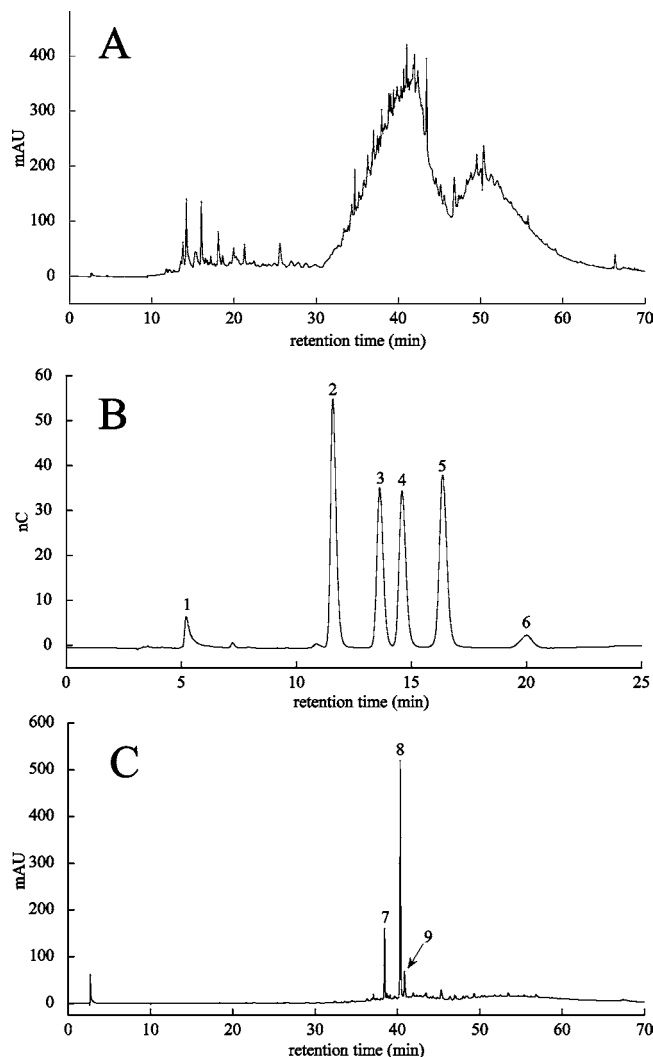


Figure 3. Chromatograms resulting from (A) RPLC analysis (detection at 210 nm) of an eluted red-brown fraction, (B) HPAE-PAD analysis of a red-brown fraction following acid hydrolysis at elevated temperature, and (C) RPLC analysis (detection at 320 nm) of an aqueous sample resulting from extraction of a red-brown fraction with hot *n*-butanol and back-extraction of the butanol phase with 2% aqueous sodium bicarbonate. See the text for details. Peak identifications are as follows: 1, xylitol; 2, arabinose; 3, galactose; 4, glucose; 5, xylose; 6, fructose; 7, *para*-coumaric acid; 8, ferulic acid; and 9, sinapic acid.

anthocyanidins and/or proanthocyanidins (i.e., condensed tannins). However, two confirmatory assays for these materials (13, 27) yielded negative results. The chromatogram resulting from HPAE-PAD analysis of a hydrolyzed residue (Figure 3B) was relatively clean and indicated significant quantities of hemicellulosic sugars (i.e., arabinose, galactose, glucose, and xylose). RPLC analysis of this sample, following successive *n*-butanol and aqueous bicarbonate extractions, also resulted in a surprisingly clean chromatogram (Figure 3C) and confirmed the presence of the oxidized form of three lignin monomers (i.e., coniferyl, coumaryl, and sinapyl alcohols). These results strongly suggest that the red-brown fraction is in part (if not primarily) composed of a diverse mixture of phenolic-glycosides. It is likely that this fraction of extractives is responsible for previously observed positive bias in Klason lignin determinations performed on native corn stover (9). The presence of conjugated sugars in this fraction may also influence glycan composition of corn stover. However, it is unclear at this point how these

constituents should be categorized or assessed in future compositional analyses of herbaceous feedstocks.

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. Samples used to determine the carbohydrate content of extracts were also hydrolyzed with 4% sulfuric acid at elevated temperature, and the total sugar content (i.e., monomeric plus oligomeric sugar) was determined via HPAE-PAD. Marginal increases (0–28%) were observed in mass percentages for total glucan relative to the monomeric glucose values reported in Table 3. In contrast, mass percentages of total xylose, arabinose, galactose, and mannose typically differed from monomeric assessments by a factor of 2–3. Calculated differences in observed total and free sugars for each stover sample (data not shown) suggest that oligomeric sugars contribute an additional 4–12% to the overall mass balance for extractives in corn stover.

Additional insight into the composition of the unknown fraction of water extracts may be inferred from literature. The study conducted by Thammasouk et al. (9) provides an indirect assessment of water-soluble materials and implies that ash accounts for up to 30% of the mass balance for extractives in corn stover. Inorganic ions monitored in the present study represented roughly 10–18% of the mass balance. Thus, alternative inorganic materials may also be present in water extracts. Data reported in the same paper also imply that protein represents 6–15% of the mass balance for water-soluble materials in herbaceous feedstocks. Protein was not monitored in the present study; however, this observation suggests that inclusion of protein analysis in future compositional assessments of water extracts may improve mass closure.

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