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ARTICLE

Evaluation of Process Severity on the Chemical Composition of Organosolv Switchgrass Lignins by Using Mass Spectrometry

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Organosolv treatment is an environmentally friendly fractionation method that affords a lignin stream with high purity. The severity of the treatment affects the chemical composition of the generated, very complex lignin degradation product mixtures. Understanding the effects of the process severity on the types and relative abundances of the individual compounds in the mixtures is of great importance for the optimization of the treatment and the development of downstream conversion processes. In this study, seven organosolv switchgrass lignin samples prepared at different reaction temperatures and using different acid concentrations and reaction times were initially analyzed by using high-resolution mass spectrometry with negative ion-mode electrospray ionization ((-)ESI HRMS). Fast pyrolysis coupled with negative ion-mode atmospheric pressure chemical ionization and HRMS (py/(-)APCI HRMS) was also used to characterize the lignin degradation products. This method generated similar data as (-)ESI HRMS. Lignin monomers and dimers were found to constitute the majority of compounds in the mixtures. High-performance liquid chromatography coupled with (-)ESI high-resolution multistage tandem mass spectrometry (HPLC/(-)ESI HRMS^n) based on collision-activated dissociation (CAD) was employed to obtain structural information for the most abundant compounds as well as a β-O-4 dimer with a relatively low abundance in the organosolv lignin samples. The relative abundances of lignin-carbohydrate complexes were found to be high under mild organosolv treatment conditions but become low under moderate and harsh treatment conditions. As lignin compounds with β-O-4 linkages are not stable under acidic conditions, the relative abundances of these compounds were found to be very low. The relative amounts of lignin monomers decreased as the treatment severity increased while the relative abundances of lignin dimers, trimers, and bigger oligomers increased, possibly due to repolymerization reactions under the harsher treatment conditions.

Introduction

The global increase in the consumption of non-renewable fossil fuels and the resultant undesirable environmental impacts have motivated the search for sustainable and renewable resources for energy and value-added chemicals. Lignocellulosic biomass is a promising source due to its wide availability and renewability.[1,2] Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin.[3] Lignin is the most abundant natural aromatic biopolymer and hence a promising source for aromatic compounds and biofuels. It is a macromolecule that is composed of three major monomeric subunits: syringyl (S-unit), guaiacyl (G-unit), and *p*-hydroxyphenyl (H-unit).[4-6] These monomeric units are connected through different types of linkages, including β-O-4 (most common), 5-5, β-5, β-1, β-β, 4-O-5, as well as others.[4-6]

A variety of processing technologies have been developed for the conversion of lignin into small molecules, including solvent fractionation, chemical treatment, biological treatment, and others.[7-9] The organosolv process is a solvent fractionation method that is environmentally friendly and affords a lignin stream with high purity.[7] Understanding the changes in the types and abundances of the individual compounds in the lignin degradation products obtained under different treatment severities is essential for the optimization of the organosolv lignin treatment and the development of downstream conversion processes.

In this study, high-resolution mass spectrometry equipped with negative-ion mode electrospray ionization ((-) ESI HRMS) and fast pyrolysis coupled with HRMS using negative-ion mode atmospheric pressure chemical ionization (py/(-)APCI HRMS) were used to obtain information for the chemical compositions of seven organosolv switchgrass lignin mixtures prepared under a variety of fractionation conditions (different reaction temperature, acid concentration, and reaction time).[10] High-performance liquid chromatography coupled with high-resolution multistage tandem mass spectrometry based on collision-activated dissociation (CAD) (HPLC/(-)ESI HRMS^n) was then employed to obtain structural information for the different components in the organosolv switchgrass lignin samples. The effects of the treatment severity on the chemical compositions were evaluated.

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Experimental

Materials

The seven organosolv switchgrass lignin samples were prepared at the University of Tennessee Center for Renewable Carbon using a previously published method.[10] The organosolv lignin samples were prepared at temperatures of 120, 140, or 160 °C, sulfuric acid concentrations of 0.05 or 0.1 M, and reaction times of 56 or 120 min. Klason analysis of the samples showed them to be typically >90-95% lignin. A detailed description of the preparation of the organosolv switchgrass lignin samples is given in supporting information. LC/MS grade methanol, acetonitrile, and water were purchased from Fisher Scientific (Pittsburgh, PA, USA) and used as received.

Instrumentation

All experiments were performed using a Thermo Scientific LTQ linear quadrupole ion trap (LQIT) mass spectrometer coupled with a high-resolution orbitrap mass analyzer with either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), both operated in the negative ion mode. The instrument was operated by using the LTQ Tune Plus interface of the Xcalibur 2.1 software. The linear quadrupole ion trap was filled with helium at a pressure of approximately 2 mTorr. In the orbitrap vacuum manifold, nominal pressure of approximately 1.5×10^{-11} Torr was maintained, as read by a cold ion gauge (IKR 270, Pfeiffer Vacuum, Asslar, Germany). A resolution of 60,000 (FWHM at *m/z* 200) was used for all of the high-resolution measurements. The LQIT mass spectrometer was coupled with a Thermo Surveyor MS Pump Plus HPLC system which was equipped with an autosampler, a quaternary pump, and a photodiode array (PDA) detector.

For direct-infusion (-)ESI HRMS experiments, each of the organosolv switchgrass lignin samples was dissolved in acetonitrile and water (1:1, v/v) at a concentration of 2 mg mL^{-1} . For the (-)ESI HRMS experiments performed with an internal standard, each of the lignin samples was dissolved in acetonitrile and water (1:1, v/v) at a concentration of 1.8 mg mL^{-1} and the solutions were spiked with an internal standard, $^{13}C_6$-vanillin, at a concentration of 6.0×10^{-3} mg mL^{-1}. The sample solutions were introduced into the mass spectrometer via a syringe drive at a flow rate of 10 µL min^{-1}. The sample solutions were diluted with a solution of acetonitrile and water (1:1, v/v) eluted at a flow rate of 100 µL min^{-1} from a Finnigan Surveyor MS Pump Plus via a tee connector to facilitate the formation of a stable ESI spray. The optimized mass spectrometer operating conditions were as follows: sheath gas (N_2) flow of 30 (arbitrary units), auxiliary gas (N_2) flow of 10 (arbitrary units), spray voltage of -3.5 kV, ion transfer capillary temperature of 275 °C, and capillary voltage of -10 V. Compounds in the samples were deprotonated by using (-)ESI, transferred into the ion trap and then into the orbitrap where they were analyzed and detected.

Organosolv lignin sample S4 was analyzed using HPLC/(-)ESI $HRMS^n$ to obtain structural information for some relatively abundant compounds that were found in all of the seven switchgrass lignin samples. Sample S4 was dissolved at a concentration of 10 mg/mL in acetonitrile and water (1:1, v/v), and 10 µL of the solution was loaded into a reversed-phase Zorbax SB Phenyl column (4.6 × 250 mm, particle size 5 µm, Agilent Technologies, Santa Clara, CA, USA) by using the partial loop injection mode. The mobile phase solvents used were water (A) and acetonitrile (B), and both solvents were doped with 0.1% (v/v) of formic acid to enhance HPLC separation. The flow rate used was 600 µL/min. The nonlinear gradient used was as follows: 0 min, 70% A, 30% B; 6.0 min, 70% A, 30% B; 36.0 min, 20% A, 80% B; 36.1 min, 5% A, 95% B; 45.0 min, 5% A, 95% B; 45.1 min, 70% A, 30% B; 50.0 min, 70% A, 30% B. The sample tray was refrigerated at 4 °C and the column temperature was kept at 40 °C. A volume of 400 µL of wash solvent (1:1 (v/v) water : methanol) was injected between each sample injection to prevent sample carryover between injections. An automated multistage tandem MS method was employed. The most abundant ions in the ion trap were isolated using an isolation window of 2 *m/z* units and a *q* value of 0.25. The isolated ions were fragmented by accelerating them and allowing them to undergo collisions with helium in the ion trap at a normalized collision energy of 35 (arbitrary units) for 30 ms at a *q* value of 0.25 (MS^2 experiment). The most abundant fragment ions were isolated and fragmented by repeating the above process (MS^3 experiment). This process was repeated one more time to reach an MS^4 experiment.

The experimental set-up for performing py/(-)APCI HRMS followed previously published reports.[11,12] Briefly, the solid organosolv lignin samples were placed onto a platinum ribbon pyroprobe (Pyroprobe 5200, CDS Analytical, Oxford, PA, USA) inside the ion max box and heated from room temperature to 600 °C at a rate of 1000 °C s^{-1}. The pyroprobe was held at 600 °C for 1s to enable sufficient evaporation of the compounds. A flush of acetonitrile and water (50/50, v/v) from a Finnigan Surveyor MS Pump Plus was introduced into the APCI source at a flow rate of 100 µL min^{-1} to enhance ionization. A sheath gas (N_2) flow rate of 30 (arbitrary units), an auxiliary gas (N_2) flow rate of 10 (arbitrary units), a discharge current of 5 µA, a vaporizer temperature of 300 °C, an ion transfer capillary temperature of 275 °C, and a capillary voltage of -10 V were employed.

Results and discussion

The organosolv treatment conditions used to generate the seven organosolv lignin degradation mixtures studied here differed in temperature, acid concentration, and reaction time. To directly compare the samples treated under different conditions, the combined severity factor (CSF) was used.[10,13-15] The CSF combines the effects of temperature, acid concentration, and reaction time into a single value and is defined as:

$$CSF = \log (R_0) - pH$$

where pH is the measure of acidity and R_0 is the reaction severity without considering acidity; $R_0 = t \times \exp [(T_r - T_b] / 14.75]$, where t is the reaction time (min), T_r is the reaction temperature (°C) and T_b is the base temperature, typically 100 °C. Based on the equation, mild treatment conditions with short reaction times, low acid

concentrations, and low reaction temperatures contribute to a low CSF. Harsh treatment conditions with long reaction times, high acid concentrations, and high reaction temperatures result in a high CSF.

In this study, organosolv treatments were performed at 120, 140, and 160 °C temperatures, 0.05 and 0.1 M sulfuric acid concentrations, and 56 and 120 min reaction times, to obtain seven samples. These conditions span a large range of severity (CSF from 1.04 to 2.85). Details about the organosolv lignin samples are given in

Table 1.

Table 1. Conditions of the organosolv lignin processes and the combined severity factors (CSF).

Sample	Temperature (°C)	Acid Concentration (M)	Time (min)	CSF
S1	120	0.05	56	1.04
S2	120	0.1	56	1.34
S3	140	0.05	56	1.62
S4	140	0.1	56	1.93
S5	160	0.05	56	2.21
S6	160	0.1	56	2.51
S7	160	0.1	120	2.85

Chemical composition information obtained by using (-)ESI HRMS

The organosolv lignin samples were initially analyzed by using (-)ESI HRMS to examine their chemical compositions. The HRMS spectra measured for samples S1, S4, and S7 prepared under mild, moderate, and severe conditions (CSF = 1.04, 1.93, and 2.85, respectively) are shown in Figure 1 and the mass spectra for the other samples (Samples S2, S3, S5, and S6) are shown in Figures S-1 to S-4 (supporting information). The most abundant ions (with their elemental compositions in parenthesis) observed in the mass spectrum measured for sample S1 prepared under mild conditions (CSF = 1.04) are those of *m/z* 323 ($C_{16}H_{19}O_7$) and m/z 353 ($C_{17}H_{21}O_8$). However, the mass spectrum measured for sample S4 prepared under harsher conditions (CSF = 1.93) shows major ions of *m/z* 329 ($C_{17}H_{13}O_7$). Finally, the ions of *m/z* 191 ($C_{11}H_{11}O_3$) were the most abundant ones for sample S7 prepared at the most severe conditions (CSF = 2.85). Ions of *m/z* 163 ($C_9H_7O_3$), 191 ($C_{11}H_{11}O_3$), 193 ($C_{10}H_9O_4$), 207 ($C_{11}H_{11}O_4$), 301 ($C_{16}H_{13}O_6$), 323 ($C_{16}H_{19}O_7$), 329 ($C_{17}H_{13}O_7$), 353 ($C_{17}H_{21}O_8$), and 415 ($C_{22}H_{23}O_8$) were observed under all treatment conditions but their relative abundances varied. Many additional ions were also observed (Figure 1). Therefore, it is obvious that these samples are very complex mixtures.

Samples prepared under mild treatment conditions yielded a smaller number of different ions upon ionization compared with those prepared under more severe treatment conditions (Figure 1). For example, sample S1 (CSF = 1.04) yielded only a small number of ions with m/z values greater than 500 while samples S4 (CSF = 1.93) and S7 (CSF = 2.85) yielded more ions with m/z values greater than 500, although these bigger ions had low relative abundances. Sample S7 (CSF = 2.85) generated more ions than sample S1 (CSF = 1.04). This can be rationalized based on lignin containing different types of linkages, some of which are easier to cleave than others. Therefore, it is likely that under mild treatment conditions, fewer linkages in the macromolecule lignin are cleaved and therefore fewer compounds are generated than under the harsh treatment conditions. The observation of larger ions under the harsher conditions suggests that repolymerization reactions are more common under these conditions, which also results in more compounds generated under the harsh treatment conditions.

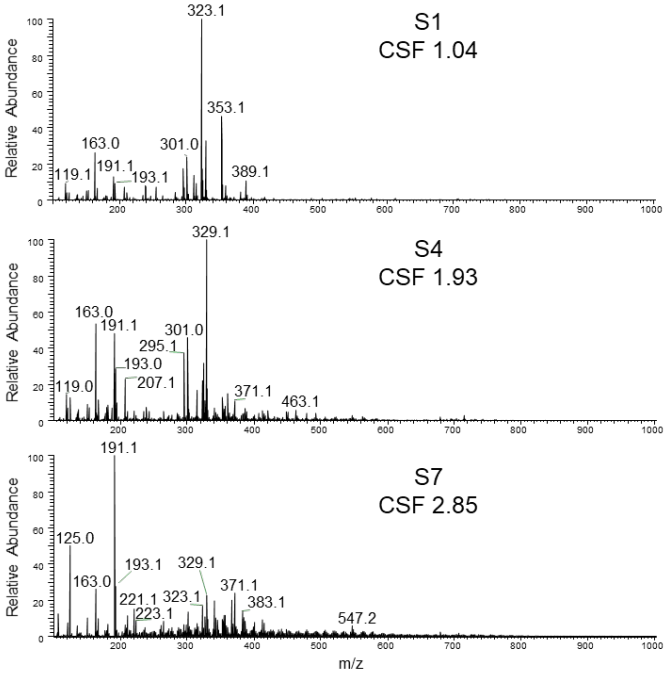

Figure 1. High resolution mass spectra measured for organosolv lignin samples S1, S4, and S7 prepared under mild (CSF = 1.04), moderate (CSF = 1.93), and high severity conditions (CSF = 2.85).

Chemical composition information obtained by using py/(-)APCI HRMS

Fast pyrolysis coupled with mass spectrometry (py/MS) is another analytical method that is widely used for biomass analysis.[16,17] The (-)ESI HRMS experiments discussed above require the sample to be dissolved in a solvent compatible with the MS method. However, some biomass samples do not dissolve in common solvents. In these cases, py/MS can be used to analyze them. To further investigate the chemical content of the above

organosolv lignin samples, py/(-)APCI HRMS was used to characterize some of the organosolv switchgrass samples. The solid samples were placed on a pyroprobe and degraded and evaporated by fast heating. The evaporated gaseous molecules were ionized by using (-)APCI. Acetonitrile and water (1:1, v/v) eluting from a Finnigan Surveyor MS Pump Plus at a flow rate of 100 μL min^{-1} were introduced into the APCI source to enhance ionization. The ions generated were introduced into the high-resolution mass spectrometer. Compared to ESI, the py/(-)APCI method is not nearly as soft evaporation/ionization method as ESI as fast pyrolysis degrades the organosolv lignin compounds further and APCI may do the same. The samples S1, S4, and S7 discussed above (obtained under mild, moderate, and harsh treatment severity conditions, respectively) were analyzed using this approach (Figures S-5 to S-11 in supporting information). The results were compared with those obtained for the same samples analyzed by using (-)ESI HRMS.

Comparison of the chemical composition information obtained by using (-)ESI HRMS and py/(-)APCI HRMS

Bubble charts were constructed from the (-)ESI HRMS and py/(-)APCI HRMS data to better visualize the distributions of the compounds generated under different organosolv treatment conditions and to ease comparison of the two analytical methods (Figure 2). The bubble charts show the ring and double bond equivalent (RDBE; RDBE = a + 1 - b/2 for ionized compounds containing no nitrogen or halogen atoms, wherein a = the total number of carbon atoms and b = the total number of hydrogen atoms) values as a function of the *m/z* values of the deprotonated compounds. RDBE values reflect the degree of unsaturation and/or number of rings in the ions. The size of each bubble represents the relative abundance of each ionized compound. Lignin monomers contain one benzene ring and have an RDBE value of equal or greater than 4. The RDBE values of deprotonated monomeric lignin-related compounds are greater than this by 0.5 due to the removal of a proton upon deprotonation. The RDBE values are greater if double bonds (C=C or C=O) and/or rings exist in the monomers. Hence, the minimum RDBE value for deprotonated monomers is 4.5. Lignin dimers contain two benzene rings and thus the minimum RDBE value for deprotonated dimers is 8.5. Similarly, lignin trimers and tetramers contain three and four benzene rings, respectively. The RDBE values for deprotonated trimers and tetramers are at least 12.5 and 16.5, respectively. Non lignin-related compounds, including fatty acids, lipids, and carbohydrates, were also detected in the lignin degradation mixtures. The RDBE values of these compounds are usually less than 4.5 since these compounds often do not contain a benzene ring. For all three samples S1, S4, and S7 (treated under different process severities), both (-)ESI HRMS and py/(-)APCI HRMS analyses indicate that the majority of the compounds are lignin monomers (RDBE 4.5-8) and dimers (RDBE 8.5-12). Lignin trimers (RDBE 12.5-16) and tetramers (RDBE 16.5-20) constitute a minor part of the samples. Overall, more different compounds were detected for samples obtained under harsher treatment conditions. As the treatment conditions became more severe, more lignin dimers and trimers (compared to monomers) were detected. The abundances of the tetramers and bigger oligomers (RDBE more than 20.5) detected was very low for both analysis methods. Generally, slightly more dimers (compared to monomers) were detected by using the py/(-)APCI HRMS method than the (-)ESI HRMS method (Figure 2). Overall, the (-)ESI HRMS and py/(-)APCI HRMS experiments gave similar results on the chemical compositions of the samples.

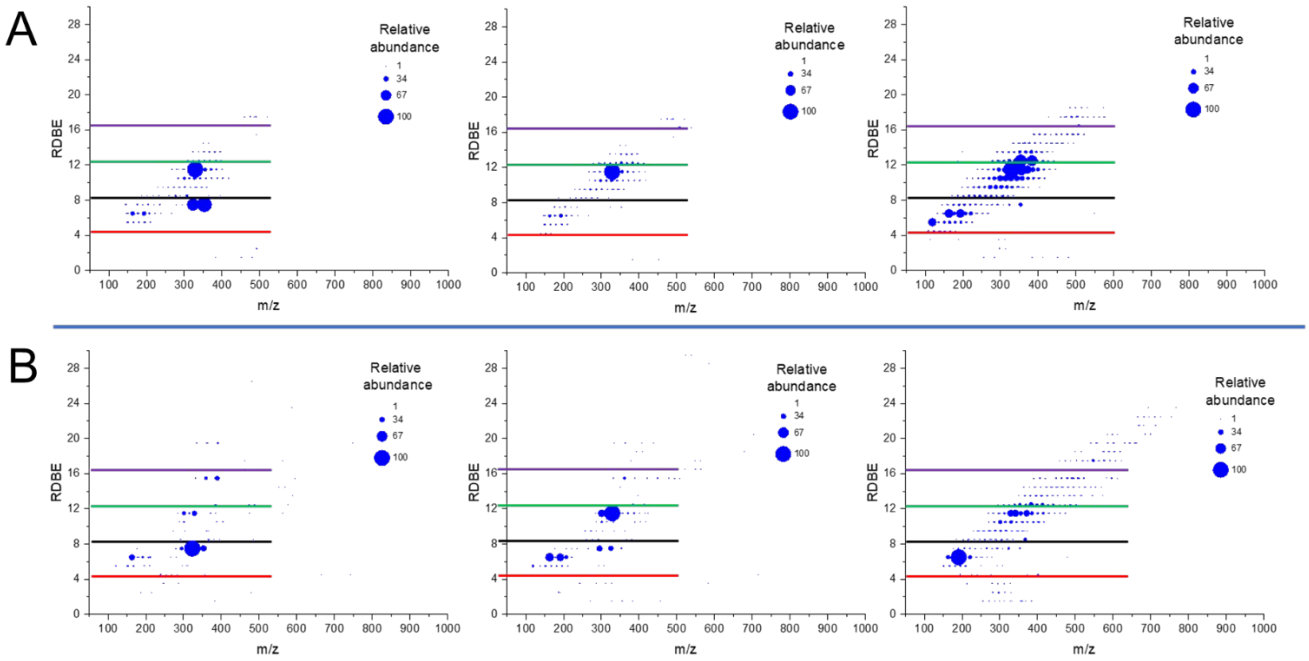

Figure 2. Chemical compositions of the organosolv switchgrass samples S1, S4, and S7 (prepared under mild (CSF = 1.04), moderate (CSF = 1.93), and high severity conditions (CSF = 2.85) based on py/(-)APCI HRMS (part A) and (-)ESI HRMS analyses (part B). Red, black, green, and

purple lines were drawn to separate compounds with RDBE values above 4.5, 8.5, 12.5, and 16.5, respectively. The size of each bubble represents the relative abundance (%) of each ionized analyte.

The relationship of the relative abundances and RDBE values of the ions measured using the py/(-)APCI HRMS and (-)ESI HRMS methods are also illustrated in a column graph (Figure 3). The py/(-)APCI HRMS and (-)ESI HRMS experiments again generated similar results. Deprotonated lignin monomers (RDBE 4.5-8) and dimers (RDBE around 8.5-12) are the most abundant detected compounds. More non lignin-related compounds (RDBE usually less than 4.5), lignin monomers (RDBE 4.5-8), and lignin oligomers larger than trimers (RDBE greater than 16.5) were detected using (-)ESI HRMS while more lignin dimers (RDBE around 8.5-12) were detected using py/(-)APCI HRMS analysis. The differences in compound relative abundances are likely due to degradation of the compounds in the organosolv lignin samples upon fast pyrolysis and the different ionization efficiencies of the different compounds when ionized using the two ionization methods.[18-20] In ESI, the analytes are commonly believed to be ionized in charged liquid droplets. Mechanisms such as solvent evaporation and droplet fission liberate the ionized analytes into the gas phase from the liquid phase.[21] In APCI, the solution containing the analytes is vaporized first, and then high-voltage corona discharge causes ionization of the analytes via a cascade of gas-phase ion-molecule reactions.[22] As the ionization mechanisms of ESI and APCI are different, different analytes can have very different ionization efficiencies upon (-)ESI and (-)APCI.

Lignin monomers (RDBE 4.5-8.5) and dimers (RDBE 8.5-12.5) constitute the majority of the compounds in all three samples obtained under different treatment conditions. The relative abundances of lignin monomers are highest under the mildest conditions (sample S1) but lignin dimers are more abundant as the treatment conditions became more severe. Also, more tetramers and bigger compounds (RDBE > 16.5) were observed as the treatment conditions became more severe. Finally, a larger number of compounds were detected under more severe conditions.

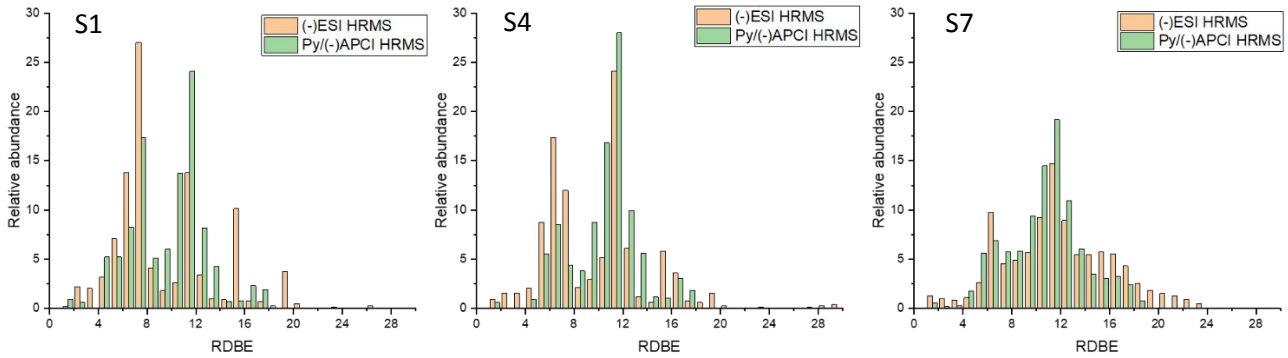

Figure 3. Relative abundances of deprotonated compounds as a function of their RDBE values derived from the organosolv switchgrass samples S1, S4, and S7 (prepared under mild (CSF = 1.04), moderate (CSF = 1.93), and high severity conditions (CSF = 2.85)) and analyzed by using the (-)ESI HRMS and py/(-)APCI HRMS methods.

Structural elucidation of compounds in the organosolv lignin samples by using HPLC/(-)ESI HRMS^n

Ionized lignin monomers of *m/z* 163 ($C_9H_7O_3$), 191 ($C_{11}H_{11}O_3$), 193 ($C_{10}H_9O_4$), and 207 ($C_{11}H_{11}O_4$), ionized lignin dimers of m/z 301 ($C_{16}H_{13}O_6$), 329 ($C_{17}H_{13}O_7$), and 415 ($C_{22}H_{23}O_8$), and ionized lignin-carbohydrate compounds of m/z 323 ($C_{16}H_{19}O_7$) and 353 ($C_{17}H_{21}O_8$) were relatively abundant in the mass spectra and were observed for all treatment conditions. To obtain structural information for these compounds that were found in all of the seven switchgrass lignin samples, an HPLC/(-)ESI HRMS^n method was employed on sample S4 to separate the compounds in this sample and to obtain structural information on the compounds eluting from the HPLC by deprotonating them via (-)ESI, transferring them into the linear quadrupole ion trap, isolating them by ejecting all other ions from the trap, and fragmenting them by subjecting them to collision-activated dissociation (CAD), followed by high-resolution detection using the orbitrap. The total ion chromatogram is shown in Figure 4. The fragmentation patterns of the abundant ions and the proposed structures for their neutral forms are shown in Table S1 (in supporting information (SI).

The elemental compositions determined for the ionized compounds and their fragment ions as well as previously published dissociation patterns were used to elucidate the structures of the aforementioned compounds derived from the organosolv lignin samples.[23-26] Among the compounds that were detected in all of the samples, five were determined to be monomeric lignin-related compounds (correspond to ions of m/z 163, 191, 193 (two isomers), and 221), three are lignin dimers (correspond to ions of m/z 301, 329, and 415), and two are lignin-carbohydrate complexes (correspond to ions of m/z 323 and 353). Among the three lignin dimers, two contain a 5-5 linkage and the third contains a β-O-4 linkage. A detailed discussion on the fragmentation patterns and structural elucidation of these abundant ions is provided in the supporting information. The fragmentation patterns of the deprotonated compounds of *m/z*

163 match those of deprotonated *p*-coumaric acid (lost CO_2 upon CAD),[26] which suggests that their structures are the same. The same applies to ions of *m/z* 193 and deprotonated ferulic acid (lost CO_2 and CH_3 upon CAD),[26] thus confirming the structure of the unknown ions of 193. An isomeric ion of *m/z* 193 was also examined by using the HPLC/(-)ESI HRMS^n method. Based on the elemental composition and the fragmentation pattern (see the supporting information for details), the isomeric ions of *m/z* 193 are identified as deprotonated 3-(4-hydroxy-3-methoxyphenyl)-3-oxopropanal (Figure 4).

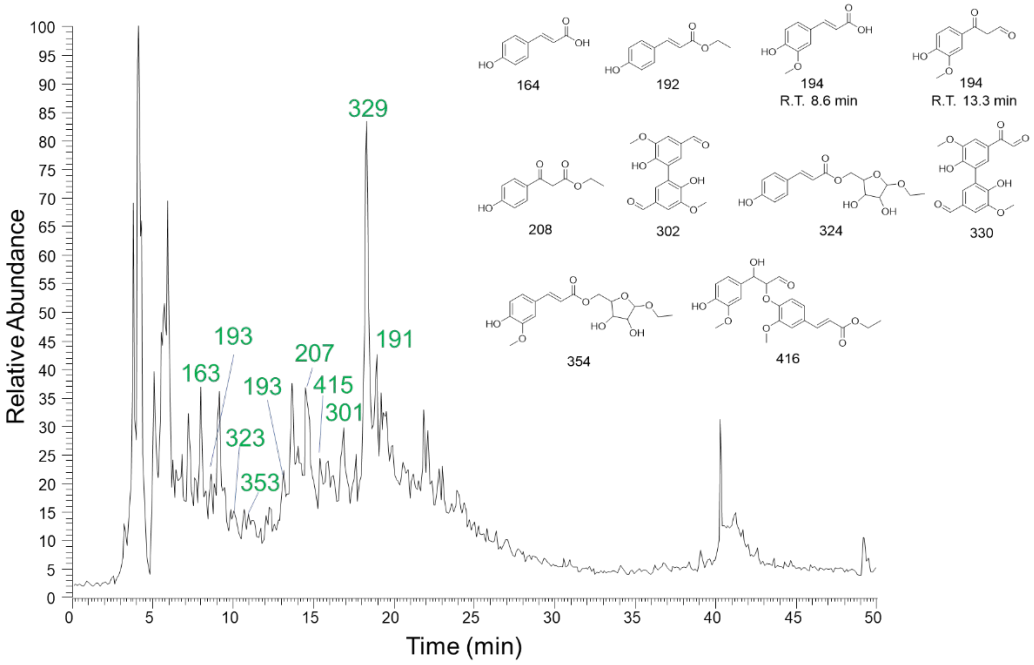

Figure 4. HPLC total ion chromatogram measured by using HPLC/(-)ESI HRMS^n for the organosolv switchgrass sample S4. The *m/z* values for some deprotonated compounds and structures proposed for the corresponding neutral compounds and their molecular weight (in Da) are shown.

In addition to lignin monomers with a carboxylic acid, aldehyde, or keto functionality, esters were also detected in the lignin degradation mixtures. Based on their elemental compositions and fragmentation pattern (see SI), ions of *m/z* 191 were identified as deprotonated ethyl *p*-coumarate[26], and ions of *m/z* 207 were proposed to correspond to a deprotonated β-ketoester (Figure 4).

In plants, ferulic acid, and to some extent, *p*-coumaric acid, form esters with polysaccharides (primarily arabinoxylans in grasses).[6,27,28] Based on literature, the ester bond is formed between the carboxylic acid functionality of ferulic acid (or *p*-coumaric acid) and the C5 hydroxy-functionality of arabinose. Arabinose is attached to xylan, which is the major hemicellulosic polysaccharide in grasses.[6,27,28] The arabinose esters of ferulic acid and *p*-coumaric acid belong to a family of compounds called lignin-carbohydrate complexes.[6,27,28] Based on their elemental compositions and fragmentation patterns (see SI), two major ions of *m/z* 323 and 353 were identified as deprotonated *p*-coumarate and ferulate carbohydrate esters (Figure 4), respectively.

The β-O-4 linkage is the most abundant linkage type in lignin. The CAD patterns of deprotonated oligomeric lignin β-O-4 model compounds have been identified to involve three major pathways (Scheme 1A):[23] elimination of the charge-remote unit(s) B; elimination of the charged end unit A; and elimination of water and formaldehyde from the side chain. Cleavage of the β-O-4 linkage is involved in both the elimination of the charge-remote unit(s) and the charged end unit A upon CAD.[23-25] Based on their fragmentation patterns (see SI), the unknown ions of m/z 415 were identified as containing a β-O-4 linkage. The structure proposed for the ions of *m/z* 415 and the pathways proposed for the formation of their major fragment ions of m/z 193 and 221 are shown in Scheme 1B.

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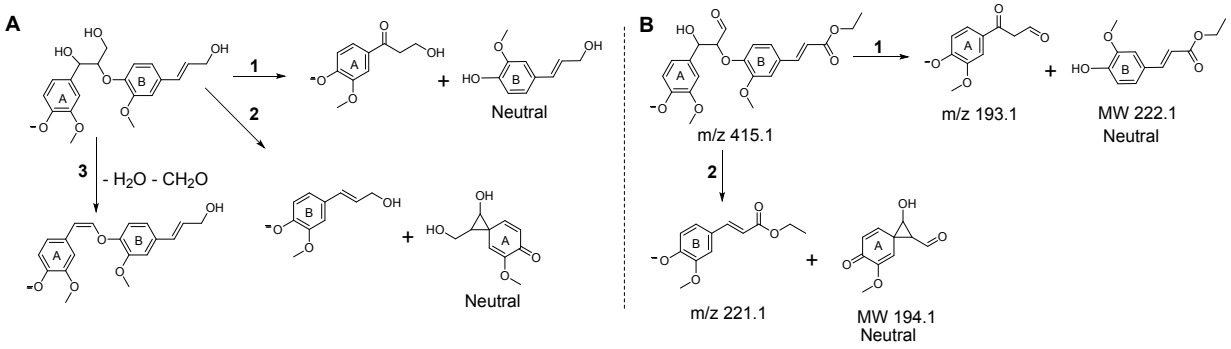

Scheme 1. Fragmentation pathways reported previously[23] for deprotonated lignin model compounds containing a β-O-4 linkage (part A) and proposed fragmentation pathways for the formation of the two major fragment ions upon CAD of the deprotonated compounds of *m/z* 415 in this study (part B).

Two abundant compounds with a 5-5 linkage were detected in the organosolv lignin mixture studied. The 5-5 linkage is another type of linkage that is commonly found in lignin. The recalcitrant 5-5 linkage stays intact during organosolv pretreatment. When a deprotonated lignin model compound is subjected to CAD, the C-C bond of the 5-5 linkage stays intact.[24,29] Only losses of molecules smaller than an aromatic ring were observed upon CAD.[24,29] Upon CAD of the ions of *m/z* 301 and 329, no losses of neutral molecules bigger than a benzene ring were observed throughout the MS^2 to MS^4 experiments (Table S1), indicating that all these ions contain a 5-5 linkage. The fragmentation pattern of ions of *m/z* 301 matches that reported for deprotonated 5-5' bisvanillin,[29] thus confirming its structure. Based on their elemental composition and fragmentation pattern (see SI), a structure was proposed for the deprotonated compounds of *m/z* 329 with a 5-5 linkage (Figure 4).

Changes in the relative abundances of compounds in organosolv lignin samples as a function of process severity

Detection and rationalization of changes in the relative abundances of the individual compounds in organosolv lignin samples obtained under different organosolv treatment conditions may facilitate the understanding of the decomposition and formation of compounds in organosolv lignin processes. The compounds with relatively high abundances that were found in all of the samples obtained at different treatment conditions are mainly small monomeric lignin-related compounds, lignin dimers with 5-5 linkages, and lignin-carbohydrate complexes. Lignin monomeric ions of *m/z* 163 ($C_9H_7O_3$), 191 ($C_{11}H_{11}O_3$), 193 ($C_{10}H_9O_4$), and 207 ($C_{11}H_{11}O_4$), lignin dimeric ions of m/z 301 ($C_{16}H_{13}O_6$), 329 ($C_{17}H_{13}O_7$), and 415 ($C_{22}H_{23}O_8$), and lignin-carbohydrate complex ions of m/z 323 ($C_{16}H_{19}O_7$) and 353 ($C_{17}H_{21}O_8$) were relatively abundant in the mass spectra and were observed for samples generated under all treatment conditions. Therefore, changes in their relative abundances were investigated.

It is noteworthy that the reported relative abundances of ions may vary over time and for different MS measurements due to slight fluctuations in the experimental conditions. Therefore, the samples were spiked with an internal standard, $^{13}C_6$-vanillin, in order to report the abundances of the lignin-derived ions relative to the abundance of the ionized internal standard. The concentration of the organosolv lignin sample in acetonitrile and water (1:1,v/v) was 1.8 mg mL^{-1} and the concentration of the internal standard was 6.0 × 10^{-3} mg mL^{-1}. The solution was analyzed using the direct-infusion (-)ESI HRMS experiments described above. The ratio of the abundances of the ions of interest (ions of m/z 163, 191, 193, 207, 301, 323, 329, and 353) to the abundance of the ionized internal standard (m/z 157) was used to represent the relative abundances of the ions of interest. The mass spectra measured for samples S1 to S7 spiked with the internal standard are shown in Figures S-12 to S18 (supporting information). The internal standard experiment is very robust as several repeats revealed closely similar trends. The observed trends are discussed below.

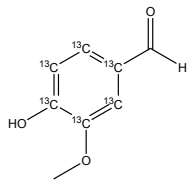

Figure 5. $^{13}C_6$-Vanillin was used as an internal standard.

The abundance of the deprotonated phenolic monomeric compound *p*-coumaric acid (ions of *m/z* 163) was found to be smaller in the mass spectra measured for samples treated under more severe conditions (Figure 6A). On the other hand, the abundance of

the deprotonated ethyl *p*-coumarate (ions of *m/z* 191) fluctuated under different treatment conditions, although the overall trend was a slight increase in abundance. The combined abundance of the isomeric ions corresponding to deprotonated phenolic monomeric ferulic acid and 3-(4-hydroxy-3-methoxyphenyl)-3-oxopropanal (ions of *m/z* 193) first increased as the treatment conditions became more severe and then decreased at the harsh treatment conditions.

The abundance of the deprotonated β-ketoester of *m/z* 207 became slightly smaller as the process severity increased. The formation of the β-ketoester may involve oxidation of a hydroxyl group in a side chain into a keto- functionality. The oxidation reactions may compete with other reactions during the organosolv treatment process, such as dehydration that affords α,β-unsaturated esters rather than β-ketoesters. The overall outcome is a slightly smaller abundance of the deprotonated β-ketoester of *m/z* 207.

The abundances of deprotonated lignin-carbohydrate complexes (ions of *m/z* 323 and 353) were large in the mass spectra measured for the samples treated using mild conditions while their abundances became significantly smaller as the treatment conditions became more severe (Figure 6B). This finding indicates that the *p*-coumaroylated- and feruloylated-carbohydrate complexes are relatively stable under the mild conditions but degrade under moderate and harsh conditions.

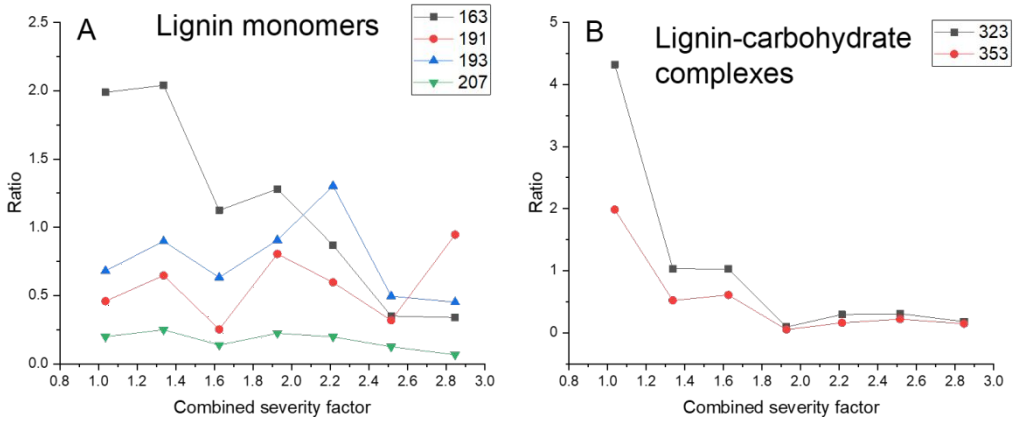

Figure 6. Changes in the relative abundances of the deprotonated monomeric compounds (part A) and deprotonated lignin-carbohydrate complexes (part B) in the mass spectra as a function of process severity for the differently treated organosolv lignin samples.

The relative abundance of one compound with a 5-5 linkage (ionized compound of *m/z* 301, **Error! Reference source not found.**) only slightly changed as the treatment conditions became more severe (Figure 7). Based on its fragmentation behavior, the ionized compound (*m/z* 301) corresponds to deprotonated 5,5'-bisvanillin (**Error! Reference source not found.**).[29]

On the other hand, the abundance of another compound with a 5-5 linkage (ionized compound of *m/z* 329, **Error! Reference source not found.**) was quite high in the mass spectra measured for samples treated using mild conditions but significantly smaller for samples treated under more severe conditions (Figure 7). The unknown ions of *m/z* 329 were identified to contain three carbonyl functionalities (**Error! Reference source not found.**). These functionalities may have been generated upon cleavage of two side chains and oxidation of a hydroxyl-group in another side chain during the organosolv process. Under harsher treatment conditions, the competition from other pathways may become more dominant.

The β-O-4 linkage that is the most abundant linkage in intact lignin is readily cleaved upon acidic and thermal treatments.[30-33] Therefore, it is not surprising that in the organosolv lignin samples studied, only one compound with a β-O-4 linkage (corresponding to ions of m/z 415) was identified (with low relative abundance: ca. 1% of all detected compounds) (Figure 7), indicating that the majority of the labile β-O-4 linkages were cleaved under the acidic organosolv treatment conditions.

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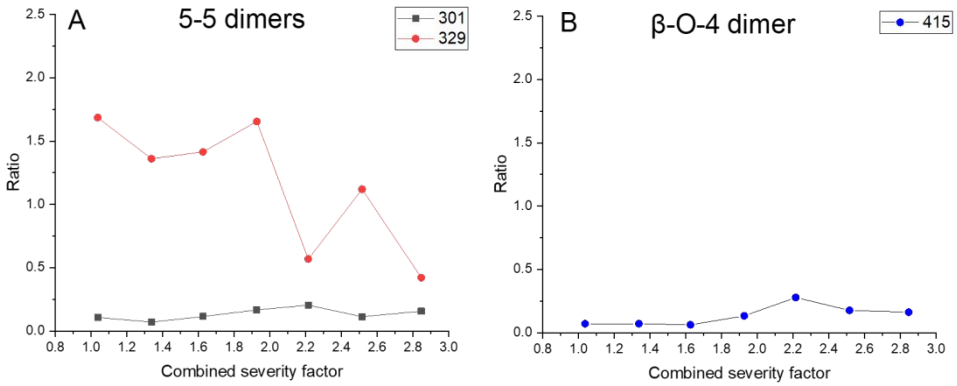

Figure 7. Changes in the relative abundances of the deprotonated dimeric lignin compounds with (A) a 5-5 linkage and (B) a β-O-4 linkage as a function of process severity for the studied organosolv lignin samples.

It is noteworthy that different analytes can have different ionization efficiencies upon (-)ESI. A previous (-)ESI HRMS study using an equimolar mixture of 11 lignin model compounds consisting of lignin-related monomers, dimers, and a tetramer demonstrated that the majority of the compounds studied (7 out of 11 compounds) had a similar ionization efficiency.[29] Moreover, the largest compound, a tetramer, showed a higher ionization efficiency than monomers.[29] This result demonstrates that the (-)ESI HRMS method does not discriminate against the large lignin-related oligomers in the mixture.

Changes in the relative abundances of different compound classes in the organosolv lignin samples as a function of process severity

The sum of the relative abundances of deprotonated compounds in each of the categories (monomers, dimers, trimers, tetramers and bigger oligomers, and not lignin-related compounds) obtained from the (-)ESI HRMS experiments using the internal standard was plotted as a function of process severity for each sample (Figure 8). As the treatment conditions became more severe, the relative abundances of lignin monomers became smaller, possibly due to acid-catalyzed repolymerization reactions of the monomeric lignin compounds.[15,34] The overall abundances of lignin dimers, trimers, and tetramers and bigger oligomers became greater as the treatment conditions became more severe, partially due to repolymerization reactions. The relative abundances of not lignin-related compounds were relatively low and slightly changed as the treatment conditions varied.

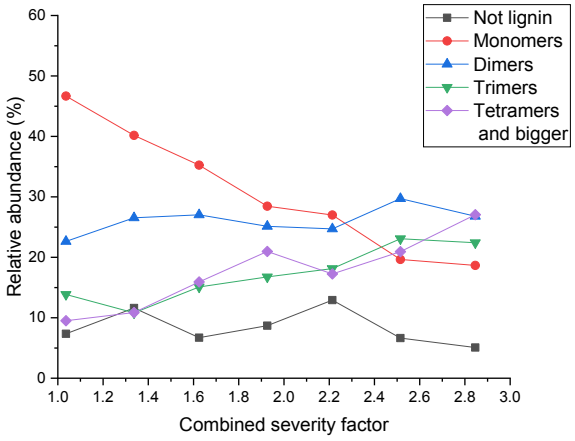

Figure 8. Changes in the relative abundances of compounds in the different compound classes obtained from the (-)ESI HRMS experiments using an internal standard were plotted as a function of process severity.

Conclusions

Organosolv switchgrass lignin samples prepared at different temperatures and using different acid concentrations and reaction times were analyzed by using three analytical approaches. (-)ESI HRMS and py/(-)APCI HRMS measurements revealed similar although not identical data for the overall chemical compositions of the samples. Lignin monomers and dimers constitute the majority of the compounds in the samples. In general, slightly more dimers were detected by using the py/(-)APCI HRMS method than the (-)ESI HRMS method. More not lignin-related compounds, lignin monomers, and

lignin oligomers larger than trimers were detected using (-)ESI HRMS while more lignin dimers were detected using py/(-)APCI HRMS analysis. Some lignin monomers, lignin-carbohydrate complexes, and lignin 5-5 dimers were relatively abundant and were found in all of the samples obtained under different treatment conditions. A lignin β-O-4 dimer was also found in all the samples, although with a relatively low abundance. The relative abundances of all of these compounds changed as the treatment conditions varied.

An HPLC/(-)ESI HRMS^n method based on CAD was employed to separate and structurally characterize the relatively abundant compounds in the samples. Structural information for the above compounds was obtained via examination of the elemental compositions and fragmentation behavior of their deprotonated forms. Among the detected compounds, lignin monomers, including ferulic acid, *p*-coumaric acid, 3-(4-hydroxy-3-methoxyphenyl)-3-oxopropanal, ethyl *p*-coumarate, β-ketoester, lignin-carbohydrate complexes, and lignin dimers with 5-5 linkages or a β-O-4 linkage were observed.

The changes in the relative abundances of the individual compounds as a function of treatment severity were investigated. Lignin-carbohydrate complexes are not stable under moderate and harsh conditions and their abundances were significantly smaller as the treatment conditions became more severe. A lignin monomer, *p*-coumaric acid, might undergo esterification reactions with ethanol in the system to afford esters, as suggested by the smaller abundance of *p*-coumaric acid and greater abundances of the *p*-coumarate esters as treatment severities increased. Lignin β-O-4 type compounds are labile under the acidic conditions of organosolv treatment. Thus, only one such compound was identified and it had a low abundance. The abundance of one of the two lignin 5-5 type compounds detected was only slightly changed while the other one became significantly smaller as the treatment conditions became more severe. As the treatment conditions became more severe, the abundances of the lignin monomers became smaller while the abundances of lignin dimers, trimers, tetramers and bigger oligomers were greater. These results suggest that monomers repolymerized to dimers, trimers, or bigger oligomers under harsher treatment conditions.

Conflicts of interest

There are no conflicts to declare.

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Supporting information

High-resolution mass spectra measured for the organosolv lignin samples.

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