PAPER

Isolation and characterization of new lignin streams derived from extractive-ammonia (EA) pretreatment †

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One of the key challenges facing lignin conversion to fuels and chemicals is related to the level of carbohydrate and ash impurities found in extracted lignin. Structural modifications of lignin may also occur as a result of biomass pretreatment and harsh lignin extraction protocols. Extractive-Ammonia (EA) is a new pretreatment technology that uses liquid ammonia to cleave lignin–carbohydrate complexes, decrystallize cellulose, solubilize lignin, and selectively extract lignin from lignocellulosic biomass, enabling better utilization of both lignin and carbohydrate components in a biorefinery. The EA-based biorefinery produces two different lignin-rich streams, with different properties, that could potentially be upgraded to fuels and chemicals using green processes. In this work, a water/ethanol-based fractionation method was developed to enrich the ammonia-soluble extractives, resulting in a major product stream containing 92% lignin. Detailed characterization of the various streams resulting from EA treatment, including compositional analysis, structural characterization by nuclear magnetic resonance (NMR) spectrometry, elemental analysis, molecular weight analysis, and thermo-gravimetric analysis provides a broad evaluation of the EA-derived lignin product stream structures and properties, assessing their potential for commercial applications. In summary, EA-derived lignins preserve much of lignin's functionality, including the sensitive β-aryl ether units. Nitrogen incorporation was observed in the lignin-rich streams, notably due to the presence of hydroxycinnamoyl amides formed during ammonia pretreatment.

Introduction

Recent advances in ammonia-based pretreatment of lignocellulosic biomass have demonstrated the importance of transforming the naturally occurring crystalline allomorph of cellulose I (CI) into cellulose III$_{I}$ (CIII). [1–3] The accompanying rearrangement of hydrogen bonding networks can increase the cellulose conversion to glucose using fungal enzymes by 2 to 5 fold, contributing to significant enzyme savings for a biorefinery.[1] Efficient conversion to CIII is made possible by making anhydrous liquid ammonia come in contact with biomass at elevated liquid-to-solid ratios (>2 : 1, w/w) for relatively short residence times. [3] Such pretreatment conditions also enable partial solubilization of plant cell wall components, including lignin, monomeric and oligomeric carbohydrates, proteins, lipids, minerals, and other extractives. The presence of lignin has been considered a major barrier to achieving efficient cellulosic bioconversion because of its negative interactions (i.e., chemical inhibition and physical restriction) with cellulolytic enzymes and microorganisms during enzymatic hydrolysis and microbial fermentation. [4,5] Therefore, efficient lignin solubilization and its subsequent extraction from cell wall carbohydrates is desirable to enhance enzymatic hydrolysis and microbial fermentation performance. In this context, we developed a novel Extractive-Ammonia (EA) pretreatment process that is able to selectively extract lignin from corn stover under mild conditions, with minimal carbohydrate loss, and

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which can simultaneously convert cellulose I to the more digestible CIII. [3]

Lignin has received considerable attention as a possible bio-based precursor for aromatic building blocks that are currently obtained through petroleum refining. [6] Lignin represents about 10–40% of the lignocellulosic biomass content, depending on the species and variety of plant, as well as its maturity, and the developmental conditions under which it is grown. [7] Several methods have been developed to depolymerize and convert lignin to its constituent monomers (i.e., aromatic compounds), although none has yet achieved economic viability at scale. These methods involve catalytic cracking, hydrolysis, reductions, and oxidations, among other methods. [6,8] For most of these processes, preserving native functionalities such as β-aryl ether linkages in the extracted lignin is desirable for lignin's efficient conversion to value-added products. [6] Biorefining methods, such as dilute acid pretreatment, often result in condensation reactions that form more chemically resistant C–C bonds. The cleavage of such condensed units requires higher energy and less selective chemistry, thus making lignin de-polymerization less effective.[8–10] Therefore, lignin isolation methods that minimize lignin modification and degradation, providing lignin streams closer to "native lignin", are preferable to facilitate depolymerization chemistry with high selectivity and yield. In this work, we present a methodology based on sequential precipitation using green solvents, such as ethanol and water, to fractionate and isolate lignin resulting from EA pretreatment of corn stover. The different product streams generated by this fractionation process were structurally and thermochemically characterized to provide insights into their possible utilization.

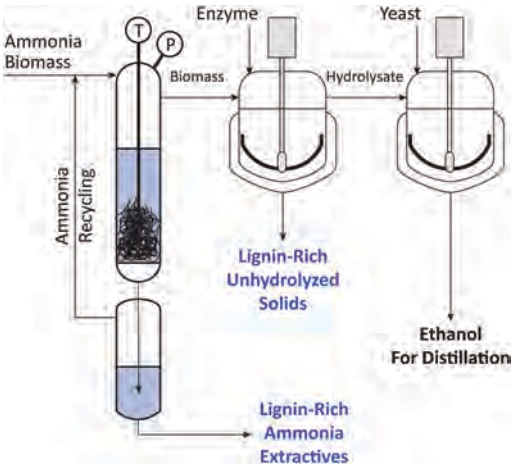
Results and discussion

Extractive-ammonia (EA) pretreatment process and lignin mass balance

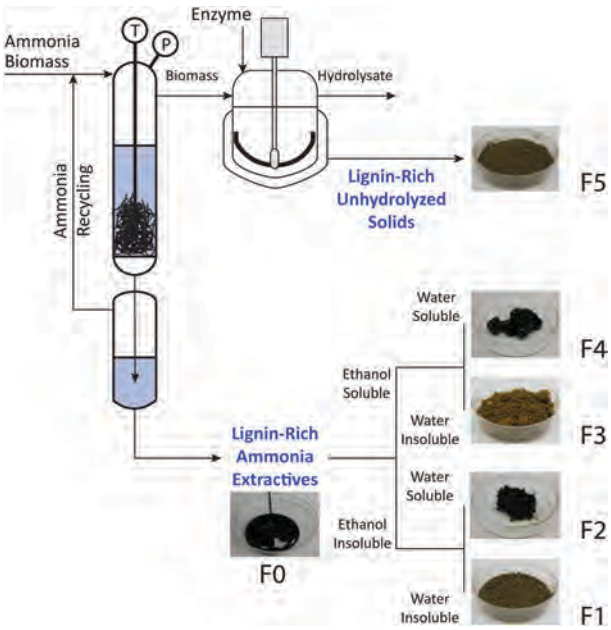
Our previous work has demonstrated that several structural modifications occurring in the plant cell wall during EA pretreatment promoted reduced biomass recalcitrance toward commercial fungal enzymes. [3] An important feature of EA pretreatment is that it uses liquid ammonia at elevated temperatures to extract lignin from lignocellulosic biomass. Lignin is known to inhibit both enzymes and microorganisms during cellulosic bioconversion to fuels and chemicals (Scheme 1). We have demonstrated that most of the native lignin functionalities are still present in the crude EA-extracted lignin from corn stover (Fraction 0 or F0), indicating that EA-derived lignin offers potential for chemical conversion to fuels and chemicals. [3]

In this study, we performed EA pretreatment on corn stover using an ammonia-to-biomass (NH_3:biomass) ratio of 6:1, a residence time of 30 min, and 10% (w/w) biomass moisture. These conditions enable high lignin removal, while simultaneously converting CI to CIII. [3] The ammonia-soluble EA product that was recovered after extraction and evaporation of ammonia (F0 in Scheme 2) is a black viscous fluid with pH of 6.5 and contains 44% of the total lignin from the untreated corn stover. This initial F0 material was further fractionated by sequential precipitation using ethanol and water at room temperature, as shown in Scheme 2. In pursuit of an economical and sustainable biomass fractionation process, the choice of solvents was based on their cost and availability in an ethanol biorefinery. In this context, ethanol (produced using bio-based feedstocks) is one of the cheapest and most readily available green solvents.

Using this methodology, it was possible to fractionate the ammonia-soluble EA product into four different streams or fractions (F1–F4 in Scheme 2). The ethanol-insoluble/water-



Scheme 1 Major steps in the biorefinery based on Extractive-Ammonia (EA) pretreatment.



Scheme 2 Biomass fractionation process based on ammonia extraction of corn stover, followed by sequential ethanol and water precipitation of lignin-rich ammonia extractives. Mass yields oflignin for each fraction are given based on 100 g lignin input.

Table 1 Composition offractions F1 to F5 generated from EA pretreatment of corn stover

	Composition (%)				
	F1	F2	F3	F4	F5
Glucan	0.5 ± 0.0	13.3 ± 0.3	0.0 ± 0.0	4.4 ± 0.1	19.0 ± 0.2
Xylan	0.2 ± 0.0	11.7 ± 0.0	0.0 ± 0.0	1.3 ± 0.0	5.5 ± 0.1
Arabinan	0.0 ± 0.0	3.2 ± 0.1	0.0 ± 0.0	0.5 ± 0.0	1.3 ± 0.0
Acetyl	0.4 ± 0.1	1.2 ± 0.1	0.4 ± 0.1	27.3 ± 0.7	0.4 ± 0.0
Lignin	69.1 ± 1.0	14.4 ± 0.4	92.4 ± 0.9	13.5 ± 0.3	42.9 ± 0.8
Ash	1.4 ± 0.0	4.0 ± 0.1	0.2 ± 0.0	2.4 ± 0.0	4.1 ± 0.0

insoluble fraction, Fraction 1 (F1), represents approximately 1.5% (dry weight) of the untreated corn stover and contains approximately 1% ofits lignin. This fraction is 69 wt% lignin and contains relatively low levels of carbohydrates and ash, as demonstrated in Table 1. Fraction 2 (F2) is ethanol-insoluble and water-soluble, and contains approximately 3 wt% of the lignin initially present in untreated corn stover. Compositional analysis shows that this stream is abundant in water-soluble carbohydrates (28 wt%), ash (4 wt%), and water-soluble lignin (14 wt%). The ethanol-soluble fractions (Fraction 3 or F3 and Fraction 4 or F4) contain most of the F0 lignin extracted during EA pretreatment, and represent approximately 40 wt% of the lignin present in the untreated corn stover. Upon adding water to the ethanol-soluble extracts, light brown aggregates were formed, whereas the darker brown component of the ethanol-soluble extract was soluble in water. Mass balance and compositional analysis showed that about 32% of the lignin present in untreated corn stover is recovered in the water-insoluble F3, whereas the remaining 8% is water soluble (recovered in F4). More importantly, this green-solvent-based fractionation approach isolated a product stream, F3, which contained 92 wt% lignin.

A residual solid stream (Fraction 5 or F5) and a liquid hydrolysate stream were obtained following enzymatic hydrolysis of the EA pretreated corn stover (EA-CS). The hydrolysate mostly contains sugars that are destined for fermentation to ethanol, whereas F5 is mostly composed oflignin (43 wt%) and recalcitrant carbohydrates (26 wt%) that are di fficult to hydrolyze during enzymatic hydrolysis.

F1, F2, and F4 do not show great potential for lignin utilization due to either low yield or low lignin content. These fractions contain about 1, 3, and 8% of the total lignin mass in untreated corn stover, respectively. In contrast, F3 and F5 are highly enriched in lignin and may provide a good source of aromatic precursors for producing fuels and chemicals in an EA-based biorefinery. To evaluate the potential of these EA product streams, a detailed characterization was performed.

NMR analysis

F0, F3, and F5 as well as the native corn stover feedstock were subjected to whole-cell-wall-gel 2D-heteronuclear single quantum coherence (HSQC) NMR analysis. Fig. 1 shows the NMR spectra and respective peak assignments for these fractions in comparison with the native corn stover (control). As expected, the NMR spectrum for the crude ammonia-extracted fraction F0 (Fig. 1B) shows many unassigned peaks that represent polysaccharides and other unknown ammonia-soluble compounds, but clearly shows that the lignin is essentially intact and that the various cell wall esters (p-coumarates and ferulates) extract into this ammonia-soluble fraction, with significant conversion to their amide analogs. Fraction F3, originally generated from F0 by sequential water and ethanol precipitation, is immediately seen to be a fraction highly enriched in lignin and phenolics, with consequently many fewer unassigned peaks than F0. The compositional analysis data presented in Table 1 confirm the much lower presence of carbohydrates in fraction F3 than in F0. Comparing the NMR spectra of native corn stover (Fig. 1A) with those from the remaining extracted lignin samples (Fig. 1B and C) indicates not only that the aromatics have some additional complexity, suggesting some slight modification of the lignin, but also that the major, and in fact the most sensitive functionalities, the β-ethers, are well preserved during EA pretreatment.

The most significant evidence of chemical interactions between ammonia and the corn cell wall is the presence of products of ammonolysis reactions of esters, such as feruloyl amide and p-coumaroyl amide in the NMR spectra of F0, F3, and F5. These are chemical species that derive from their ester counterparts in the cell wall, and are themselves not present in native corn stover lignin (Fig. 1A) but are quite abundant in ammonia pretreated biomass, as previously demonstrated for the case of Ammonia Fiber Expansion (AFEX ™ – a trademark of MBI International) pretreated corn stover, [11,12] or in the aromatic components derived from other ammonia-based pretreatment processes. [13] Ferulates in F0, F3, and F5 were mostly present as feruloyl amide; even though ferulates are associated primarily with the polysaccharides, their e fficient cleavage by ammonia ensures that they are well represented in these product streams. p-Coumarate, which acylates both polysaccharides and lignins in corn stover, [14] also features heavily in these fractions. Although the amide is again the major component, the modest level retained as either the ester or the acid suggests that its amidation is incomplete, possibly because ofits original association with the lignin. Fig. 1 shows the relative abundance of p-coumarate, feruloyl amide, and p-coumaroyl amide, with respect to the total syringyl and guaiacyl NMR peak volumes for all fractions analyzed in this study. The high levels of some nitrogenous compounds show

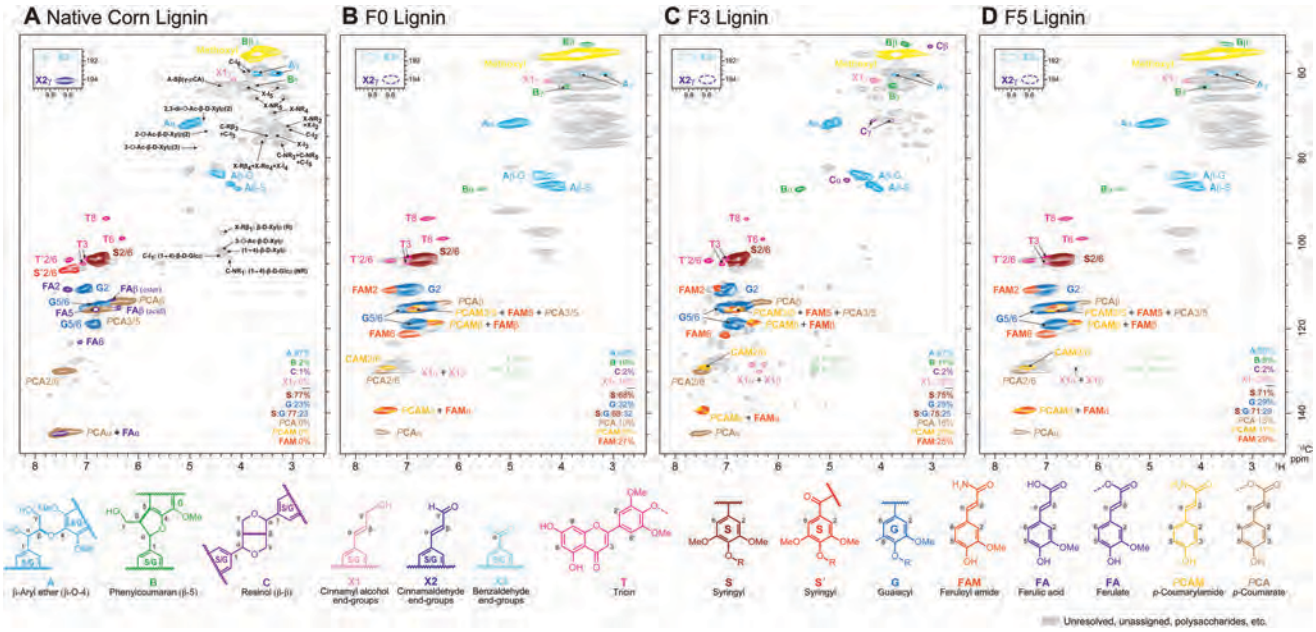


Fig. 1 2D-HSQC NMR of (A) native corn lignin; (B) F0; (C) F3, and (D) F5. Legend: C – I, cellulose internal unit; C-NR, cellulose non-reducing end unit; C-R α, cellulose reducing end unit, α-anomer; C-R β, cellulose reducing end unit, β-anomer; X-I, xylose internal unit; X-NR, xylan non-reducing end unit; X-R α, xylan reducing end unit, α-anomer; X-R β, xylan reducing end unit, β-anomer; R, reducing end; NR, non-reducing end. [44] NMR spectra have correlation contours color-coded to match those of the aromatics and lignin structures shown here. Percentages are integrals of key component correlation contours and are not absolute quanti fications; they are for comparative purposes only. [40] The aliphatics are based on A + B + C = 100%; similarly, aromatics are based on S + G = 100%. Note that some correlations are not evident at the contour level plotted, but are visible at lower contour levels and can still be integrated.

that, as expected, chemical modifications are occurring in the hydroxycinnamates that acylate both cell wall polysaccharides and lignin during EA pretreatment. Ferulates are responsible for important polysaccharide –polysaccharide and lignin –polysaccharide cross-linking in the corn stover plant cell wall; [14] the cleavage reactions evidenced in ammonia (and base in general) pretreatments go a long way to explaining the improved access to the cell wall following such pretreatments of grasses. Other reactions involving nitrogen incorporation into the lignin and into the more complex diferulates from corn stover may occur; [15] however, the characterization of such reaction products requires much more detailed work and is beyond the scope of this study.

An important property of F0, F3, and F5 lignins is that they preserve the β-aryl ether linkages, functionalities that many catalytic processes selectively target for lignin de-polymerization and eventual chemical upgrading to value-added products. [6,8,16] This feature was already highlighted for fraction F0 in a previous report that described in detail the important aspects of the EA pretreatment process. [3] However, these results show that EA and the ethanol –water fractionation scheme shown here, well preserve the β-aryl ether units in both the lignin-rich F3 and F5 fractions. β-Aryl ether bonds account for the majority of the inter-unit linkages between lignin monomer-derived units in F0, F3, and F5. These results show that the fractionated lignins have a comparable abundance of β-aryl ether units as in the native corn stover lignin, according to semi-quantitative 2D NMR analysis (Fig. 1). The presence of phenylcoumaran and resinol linkages in native corn lignin is relatively small, accounting for a minor percentage of the total inter-unit linkages between native lignin monomers. Such units are, however, concentrated in the various EA fractions presumably because they are associated with shorter lignin chains (as they are favored in lignin-initiating dimerization reactions). The high relative abundance of β-aryl ether units suggests that the lignin streams derived from EA-based processing have good potential for catalytic processes that target such bonds for chemical upgrading. [6,8] Not all pretreatment technologies preserve β-aryl ether units intact. For example, pretreatments operating under acidic conditions depolymerize lignin by cleaving β-aryl ether bonds. The resulting lignin is somewhat unstable under acidic conditions, with repolymerization reactions forming new C –C bonds between lignin units, [17] although mild acidolytic methods, including the recent γ-valerolactone [18,19] and ionic liquid [20] methods as well as organosolv/acetolysis, [8] can retain high β-ether contents, as can the lignins from other ammonia-based methods. [13] Depolymerization of highly condensed lignins, like those resulting from severe acid pretreatment conditions, [8] becomes challenging, as it requires higher energy inputs that a ffect the selectivity of the process. Therefore, pretreatment methods that preserve the native lignin structure and properties o ffer greater potential for lignin upgrading in a biorefinery to provide a more valuable supplement to the burning oflignin for producing heat and power.

The semi-quantitative 2D NMR analysis of the spectra in Fig. 1 was used to estimate syringyl-to-guaiacyl ratios (S : G) for

each fraction derived from EA-pretreatment in comparison with the native corn lignin control. Native corn lignin has an S : G ratio of 77 : 23, whereas F0, F3, and F5 show S : G values of 68 : 32, 75 : 25, and 71 : 29. Such results suggest that EA pretreatment generates lignins that are quite representative of the lignin in the native corn stover.

Tricin is also an important corn stover lignin component (Fig. 1A).[21] Similar to that in the native corn lignin, tricin has been detected in the lignin of F0, F3 and F5, suggesting that this substructure is conserved throughout EA pretreatment and enzymatic hydrolysis of corn stover.

In summary, the 2D-NMR results showed that the major F3 material, derived from EA pretreatment extraction, shares various characteristics with native lignin from corn stover, except for the nitrogenous compounds that result from ammonolysis ofits ferulate and p-coumarate esters. F5 also exhibits the same functionalities as F3, but in di fferent relative amounts. A major feature of the F5 composition is that it contains levels of unhydrolyzed carbohydrates that could be detrimental to some applications for lignin. Therefore, lignin isolation techniques for the F5 fraction should be investigated in the future to increase the range of potential applications. Ideally, commercial enzyme cocktails should allow near-complete conversion of complex carbohydrates to soluble sugars, which would result in a low-carbohydrate F5 fraction. To achieve such a goal, it is important to understand the recalcitrant polysaccharide fraction at a structural level, so that key enzyme activities can be supplemented into the current commercial cocktails for improving carbohydrate solubilization.

To evaluate the relative distribution of the hydroxyl groups in the various EA product streams, quantitative ^{31}P NMR was performed. This technique uses a phosphitylating agent that reacts with hydroxyl groups present, allowing their analysis by ^{31}P NMR. Table 2 summarizes the hydroxyl group distribution contained within EA product streams F1 –F5. These results show that aliphatic hydroxyl groups are the most abundant in all the fractions analyzed. Similar results have been reported by different researchers for other biomass materials including wheat straw,[22] switchgrass,[23] and poplar.[24] Also, the most hydrophilic fractions (i.e., water-soluble F2 and F4) have more hydroxyl groups, with a total of 9.4 and 11.6 mmol of OH per g of solid residue, respectively. The total hydroxyl contents of the water-insoluble F1 and F3 are 4.9 and 5.1 mmol of OH per g of solid residue. The relative level of carboxylic acids is higher for ethanol-soluble F3 and F4, with values of 0.95 (19% of the total 5.0) and 4.3 (37% of the total 11.6) mmol of COOH per g of solid residue.

The di fferences in the hydroxyl content and in the percentage of the various types of hydroxyl groups can provide insights into the chemical diversity among the di fferent product streams generated from corn stover using EA pretreatment and sequential solvent fractionation. These data, combined with 2D-NMR analysis, provide valuable information about the chemical attributes of these fractions (e.g., solubility) and their potential for further high-value applications.

Molecular weight analysis

The weight average molecular weights (M_w) of the acetylated solid residues isolated from the various fractions (F1 –F5), were measured by gel-permeation chromatography (GPC) (Table 3). These values were calculated with reference to polystyrene standards. Therefore, the M_w values presented in this work are not absolute, but can help in comparing the average sizes of the macromolecules present in the various fractions and also with other literature values based on this method.[25]

The chromatograms (shown in ESI Fig. S1 †) of acetylated solid residues isolated from F1, F2, F3 and F4 all show a broad multi-modal peak with its major intensity shifting towards longer retention times from F1 to F4. This indicates that the molecular weights for the acetylated solid residues decrease from F1 to F4, as demonstrated by the weight average molecular weight (M_w) values dropping from 3310 g mol $^{-1}$ to 420 g mol^{-1} (Table 3). The ethanol-insoluble fractions contain higher molecular weight polymers compared to the ethanol-soluble fractions. F2, though having a smaller polydispersity index, has a significant series of convoluted peaks at lower molecular weights than the more mono-modal appearance from F1, as shown in ESI Fig. S1. † This suggests that the lower molecular weight components present in F2 and absent in F1 are water soluble. The ^{31}P NMR results support the above claim, and clearly show that F2 and F4 are rich in hydroxyl groups, and therefore highly hydrophilic.

The ethanol-soluble F3 and F4 materials exhibit relatively narrower molecular weight distributions compared to that of

Table 2 Abundance of assigned hydroxyl groups in the EA fractions of corn stover, determined by phosphitylation and ^{31}P NMR[23]

	Percentage of total hydroxyl				
Hydroxyl assignments	F1	F2	F3	F4	F5
Aliphatic	62%	84%	47%	52%	74%
Syringyl and condensed (C5 + C3 substituted)	7%	3%	8%	1%	7%
Guaiacyl (C3 substituted)	22%	4%	26%	11%	12%
Carboxylic acid	9%	8%	19%	37%	8%
Total hydroxyl (mmol per g oflignin)	5.1	9.4	5.0	11.6	4.9

Table 3 Number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity index (PDI) of the solid residues isolated from the various fractions (F1 – F5). These values were calculated based on gel-permeation chromatography (GPC) analysis, using polystyrene standards

	M_n (g mol^{-1})	M_w (g mol^{-1})	PDI[a]
F1	130	3310	25.5
F2	690	2880	4.2
F3	740	1470	2.0
F4	230	420	1.8
F5	950	4140	4.4

[a] PDI – polydispersity index, PDI = M_w/M_n.

F2. However, F3 has an M_w value of 1470 g mol^{-1}, which is relatively lower compared to F1 and F2 (Table 3) and significantly lower than the M_w value from dioxane-extracted lignin from corn stover (3400 –3900 g mol^{-1}).[26] The lowest M_w found was for F4. However, a significant proportion of the chromatographic intensity for some of the fractions was outside the range where the molecular weight can be accurately resolved by the GPC column and/or calibration curve. Therefore, some of the values presented in Table 3 may be overestimated.

The highest molecular weight fraction obtained in this study was F5. The lignin contained was not solubilized by ammonia during EA pretreatment and remained water-insoluble after enzymatic hydrolysis. Due to its abundance, this stream would ideally be used in chemical conversion or the production of polymer materials (e.g., carbon fiber and polymer composites),[27] especially in applications that favor higher molecular weight feedstock or material. The M_w value obtained by GPC for lignin isolated from F5 was approximately 4140 g mol^{-1} and is slightly higher than literature values from lignin isolated directly from corn stover (3400 –3900 g mol^{-1}) by dioxane or acidic dioxane extraction.[26] This result is expected, as the fractionation process extracted a large part of the lower molecular weight components. There may be carbohydrates attached to the remaining lignin, resulting in a higher M_w value for the F5 fraction. The polydispersity index of the lignin from F5 was also calculated to be approximately 4.38, which is considerably higher than the values (2.2 –2.5)[26] usually observed from dioxane-extracted lignin. One downside offraction F5 is the relatively high levels of carbohydrate contamination. Utilization of the F5 lignin fraction for materials applications requires economically viable methods of removing these carbohydrates while preserving native lignin functionality. This may be possible via further enzymatic treatment,[28] or mild acidolytic methods,[9] both of which can result in lignins with low polysaccharide contamination.

Table 4 Mineral inorganic elements and CHNO analysis offractions derived from the EA process

	Composition (mg per kg offraction)				
Element	F1	F2	F3	F4	F5
Al	12.5	11.7	3.0	2.1	390.1
B	0.4	11.4	<0.3	<0.3	3.2
Ba	2.5	3.3	1.3	1.0	29.0
Be	<0.0	<0.0	<0.0	<0.0	<0.0
Ca	361.7	288.6	27.6	24.5	2714.0
Cd	<0.5	<0.5	<0.5	<0.4	<0.5
Co	<0.7	<0.7	0.7	1.0	<0.7
Cr	4.8	2.7	0.9	4.6	14.6
Cu	134.0	36.6	33.2	51.7	59.2
Fe	248.5	63.6	11.5	17.5	750.3
K	3830.0	26 643.4	131.2	75.6	845.9
Mg	1120.0	496.8	11.2	7.5	345.3
Mn	34.6	20.9	2.1	1.0	28.7
Mo	<1.6	4.3	1.6	3.2	3.0
Na	40.3	388.5	39.6	12.3	187.9
Ni	3.7	2.5	1.5	10.2	9.8
P	898.9	2573.3	209.7	1033.1	754.9
S	1340.0	2305.0	1310.0	911.6	1860.0
Sb	<5.1	<5.1	<5.1	<4.6	<5.1
Se	<11.3	<11.3	<11.3	<10.1	<11.4
Si	100.9	242.9	53.5	100.0	162.6
Sn	6.3	8.3	7.4	6.5	4.7
Sr	1.2	1.0	0.1	0.1	7.4
Ti	0.8	1.4	0.5	0.1	13.2
V	<0.2	<0.2	<0.2	<0.2	1.6
Zn	62.5	59.7	30.1	16.5	99.1
C	ND[a]	ND	67.0%	ND	ND
H	ND	ND	8.2%	ND	ND
N	3.8%	2.7%	2.3%	13.2%	3.7%
O	ND	ND	22.5%	ND	ND

[a] ND – not determined.

Elemental analysis

Elemental analysis of F1 –F5 was performed using Inductively Coupled Plasma Emission Spectroscopy-Mass Spectrometry (ICP-MS) (Table 4). These ICP-MS results suggest that the most abundant minerals extracted from corn stover as a result of the EA pretreatment include Ca, Cu, Fe, K, Mg, P, S, and Si. However, in F5, relatively high levels of Al, Ca, Fe, K, Mg, Na, P, S, Si, and Zn remained insoluble after both ammonia pretreatment and enzymatic hydrolysis.

Most of the ammonia-extracted minerals were precipitated by adding ethanol and are present in F1 and F2. As a result, these fractions contain more minerals than the ethanol-soluble fractions (F3 and F4), most notably the content of K, where the difference exceeds two orders of magnitude. Adding water to the ethanol-insoluble extractives primarily enriched K, P, and S in F2.

Of the ethanol-soluble minerals present in F3 and F4, P and S are the most abundant. Ethanol extraction will therefore remove most of the inorganics from these two fractions that might otherwise interfere with catalytic processes aimed at valorizing lignin to fuels and chemicals. In this respect it is fortunate that, except for K, P and S, F3 has all mineral levels below 0.01%.

As lignin-enriched F3 offers good potential for upgrading to fuels and chemicals, it was further analyzed by CHNO analysis. The results show that this sample contains approximately 67% carbon (C), 8.2% hydrogen (H), 2.3% nitrogen (N), and 22.5% oxygen (O). Apart from a higher N content, this elemental composition is similar to those typically observed for commercial lignins.[29] Nitrogen values are typically below 1% for most commercial lignins; the hydroxycinnamoyl amides detected by NMR (Fig. 1) likely explain most of the F3 N content. Also, lipids may be part of the remaining 7.7% non-lignin portion of F3. The esters in triacylglycerides can undergo ammonolysis reactions, potentially forming amides that may then be present in F3.[30] Indeed, the multitude of correlation peaks around those labeled as fatty acids in the NMR spectrum (Fig. 1C) attest to the presence of such components in this fraction. [It should be noted that such peaks are absent from the control corn stover samples (Fig. 1A) because this lignin is derived from a material that had first been exhaustively solvent-extracted, i.e., from a pre-isolated cell wall material]. It remains premature to define the impact of nitrogen incorporation on the potential to upgrade F3 lignin. However, there

are some concerns about the formation of NO_x during the combustion of nitrogen-containing fuels derived from ammonia-extracted lignin. A possible method for removing nitrogen from lignin-derived products could be along the lines of hydro-de-nitrogenation (HDN) that is currently used to avoid catalyst poisoning during the hydro-processing of petroleum.[31] Typical HDN processing is performed in the gas-phase at temperatures around 300 –400 °C.[32] As lignin decomposition occurs within a wide range of temperatures, which could reach as high as 600 °C, gas-phase HDN cannot be applied directly to lignin as a standalone process. Instead, gas-phase HDN could be applied as a secondary treatment to remove nitrogen from volatile products generated during lignin de-polymerization and upgrade. Alternatively, hydrogenolysis of nitrogen-containing functionalities may also occur in solution-phase hydrogenolysis or hydrogenation, which typically operates under milder conditions.[33] To our knowledge, solution-phase de-nitrogenation oflignin products is still an unexplored area of research and therefore, more work is required to determine the feasibility of such processes.

The relatively minor F4 fraction, which is water and ethanol soluble, contains the highest percentage of nitrogen (13.2%) compared to the remaining fractions. Ammonolysis reactions occurring in the plant cell wall form acetamide, p-coumaroyl amide, and feruloyl amide that may contribute significantly to the nitrogen abundance in F4.[11] Sample F5 contains 3.7% nitrogen, which may be due to the proteins from corn stover, bound cellulolytic enzymes, and nitrogenous compounds produced during pretreatment.

Thermogravimetric analysis (TGA)

The thermal stabilities of the various fractions obtained in this study were measured by TGA as shown in Fig. 2. This figure shows very distinct profiles associated with the thermal decomposition of the di fferent fractions. A substantial mass loss was recorded for all samples at a temperature range between 100 and 450 °C, after which no major decomposition was observed. The water-soluble fractions (F2 and F4) showed significant thermal decomposition at temperatures ranging from 100 to 200 °C, a pattern that was not observed in the remaining water-insoluble fractions (F1, F3, and F5). These water-soluble fractions are rich in carbohydrates, probably monomeric and small oligomeric sugars, that can be thermally decomposed over these temperature ranges.[34–36]

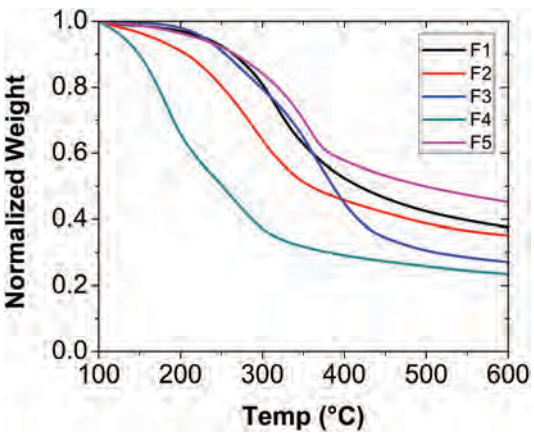


Fig. 2 Thermogravimetric curves for the various biomass fractions (F1 – F5) derived from the EA pretreatment of corn stover. The heating ramps for all the samples were set at 10 °C min^{-1}.

The water-insoluble fractions, F1 and F3, are significantly more thermostable than the water-soluble samples. Fraction F1 experiences 5% weight loss at temperatures below 250 °C followed by an abrupt decrease in weight (40%) before reaching temperatures around 375 °C. F1 lost about 60% ofits total weight due to thermal decomposition. On the other hand, F3 showed an unusual decomposition pattern for a sample that contains approximately 92% lignin. The initial thermal stability in relation to the other samples is explained by the low carbohydrate content as well as the low amounts of volatile organic compounds. The onset temperature of 220 °C was followed by a rapid decrease in mass up to about 420 °C, resulting in a mass loss of approximately 70% in F3. Organosolv lignin, for example, at 10 °C min^{-1}, behaves quite di fferently, as it is possible to observe an onset temperature around 350 °C (130 °C higher than in F3) followed by a very slow decrease in mass.[37] At temperatures around 420 °C, only about 30% of the organosolv lignin was lost in TGA (as opposed to 70% for F3).[37] Sample F3 leaves behind a much lesser amount of solid char products following pyrolysis, as only 26% of the sample remained in the TGA tray at 600 °C. At these temperatures, about 50% of the organosolv lignin was pyrolyzed to gases or volatile liquids whereas the remaining 50% required higher temperatures to continue thermal decomposition.[37]

In contrast to F3, F5 contains high levels of unhydrolyzed ("recalcitrant ") carbohydrates. Cellulose and hemicellulose decomposition occurs at 315 –400 °C and 220 –315 °C, respectively, whereas lignin decomposes at a temperature range from 350–600 °C, depending on the origin of the lignin. Therefore the thermal decomposition occurring between 220 °C and 375 °C should be mostly associated with cellulose and hemicelluloses present in F5. Lignin decomposition should occur after 375 °C, where it is possible to see a very small weight loss with increasing temperature. A similar TGA profile is observed from Maplewood.[37]

In summary, the results from this study show that the low molecular weight F3 product stream pyrolyzes at lower temperatures, leaving behind relatively low amounts of biochar. In contrast, higher molecular weight lignins with residual recalcitrant carbohydrates require higher temperatures to decompose to volatile products while also producing a considerable fraction of biochar.

These results suggest that the F3 product stream may be the most interesting candidate for thermochemical conversion to liquid fuels via pyrolysis compared to F5. Because the F3 product stream is ethanol soluble, it could potentially be used in solution for homogeneous and heterogeneous catalytic reactions to further upgrade it to fuels and chemicals. As the native β-aryl ether units remain intact in the F3 product

stream, conversion processes that target these units can be applied. On the other hand, apart from fuel and chemical production, the F5 product stream may be more attractive for polymer additives (e.g., as polyols) or as precursors for carbon fibers due to its high molecular weight. However, it may be important to develop inexpensive lignin separation techniques to further improve the quality offraction F5 for some specific applications. Ultimately, more work is required to fully evaluate the potential of these lignin streams for various applications. Technologies for lignin conversion to value-added products are still in the developmental stage, and the use of lignin streams with distinct properties may play a crucial role in developing diverse lignin applications.

Experimental

Untreated corn stover

Corn stover (Pioneer 36H56) was harvested in September 2009 in Wisconsin (USA) and oven dried at 60 °C for approximately 2 weeks. The biomass was further passed through a 5 mm screen installed in a Christy hammer mill (Christison Scientific LTD, England) and stored at 4 °C in heat-sealed bags prior to utilization. The moisture content of the dried and milled corn stover was approximately 6% on a wet weight basis. The biomass composition analysis was performed using NREL protocols NREL/TP-510-42618 and NREL/TP-510-42620. On a dry weight basis, the untreated corn stover contained approximately 38% glucan, 23% xylan, 1% galactan, 3% arabinan, 14% Klason lignin, 2% acid-soluble lignin, 5% ash and 15% extractives (i.e., ethanol- and water-soluble compounds).

EA pretreatment of corn stover

EA pretreatment was conducted in high pressure, stainless steel tubular reactors, as previously described. [3] In each reactor, 40 g of corn stover (dry weight basis), containing 10% moisture (wet weight basis) was reacted with 240 g of ammonia for 30 min at 120 °C. The EA extractives were drained from the collector and further concentrated under vacuum using a rotary evaporator (BUCHI Labortechnik AG, Switzerland) using a water bath set at 70 °C. The EA extractives were further dried using a freeze drier (Labconco, Kansas City, MO, USA). The dry weights of the extractives and EA-pretreated biomass were recorded and the dried samples were stored at 4 °C in sealed containers to reduce exposure to moisture.

EA extractives ' fractionation and production oflignin-rich streams from the EA process

Freeze-dried EA extractives were solubilized in 100% ethanol using a 1 : 20 (w/v) extractives-to-solvent ratio for 30 min under continuous mixing conditions. The ethanol-insoluble fraction was filtered using a fiberglass filter installed in a Millipore vacuum filter holder (EMD Millipore, Billerica, MA, USA). The filtrate was further washed with fresh 100% ethanol to remove residual ethanol-soluble components adsorbed to the solid fraction. The solid fraction was air dried for 2 h in the hood to evaporate the residual ethanol from the sample. The dried ethanol-insoluble sample was weighed and placed in a beaker containing distilled water in a 1 : 30 (w/v) extractives-to-water ratio and stirred for 30 min. The resulting suspension was vacuum filtered using a fiberglass filter and the filtrate washed with water to remove the residual water-soluble components. The water-insoluble fraction resulting from this separation was transferred to a pre-weighed container and dried using a freeze dryer (Labconco, Kansas City, MO, USA). The dried sample was weighed and labeled as Fraction 1 (F1). The water soluble fraction was collected in a round-bottom flask and concentrated using a rotary evaporator (BUCHI, Labortechnik AG, Switzerland) under vacuum, while not allowing it to reach dryness. The sample was then transferred to a pre-weighed container and freeze dried. The dried sample was weighed and labeled as Fraction 2 (F2).

The ethanol-soluble fraction was transferred to a pre-weighed round-bottom flask and dried using a rotary evaporator at 60 °C under vacuum. Distilled water in a 1 : 30 (w/v) extractives-to-water ratio was added to the dried ethanol-soluble fraction and mixed for 30 min to solubilize water-soluble extractives. The suspension was vacuum-filtered and washed with distilled water to remove additional water-soluble components adsorbed to the water-insoluble fraction. The water-insoluble fraction was transferred to a pre-weighed container and freeze dried. The dry weight of the sample was recorded and labeled as Fraction 3 (F3). The filtered water-soluble fraction was further concentrated using a rotary evaporator under vacuum at 80 °C, while not allowing it to reach dryness. The concentrated fraction was transferred to a pre-weighed container and freeze dried. The dried sample was weighed and labeled as Fraction 4 (F4). All freeze dried samples were stored at 4 °C in sealed plastic containers prior to their use in subsequent measurements.

Enzymatic hydrolysis (EH) of EA-pretreated corn stover

Enzymatic hydrolysis (EH) was performed at 6% glucan loading, using 15 mg of enzyme per gram of glucan in a 5 L bioreactor set to control the mixing speed at 120 RPM, at a temperature of 50 °C and pH 4.8 for 72 h. The enzymes utilized in this work were Cellic® CTec2 (138 mg protein per ml, batch no. VCNI0001) and Cellic® HTec2 (157 mg protein per ml, batch no. VHN00001), generously provided by Novozymes (Franklinton, NC, USA). The enzymatic cocktail was also supplemented with Multifect Pectinase (MP) (72 mg protein per mL, batch no. 4861295753), a gift from Genencor (Pala Alto, CA, USA). The protein concentration for the enzymes was determined using the Kjeldahl nitrogen analysis method (AOAC Method 2001.11, Dairy One Cooperative Inc., Ithaca, NY, USA). The enzyme ratios utilized in this work was 50% Cellic® CTec2, 25% Cellic® HTec2 and 25% MP in a dry protein weight basis.

After EH, the resulting suspension was centrifuged at 8000 RPM for 30 min in a Beckman Coulter Avanti J-26XP centrifuge, equipped with a rotor model JLA 8.1000 (Beckman Coulter, Inc., Brea, CA, USA), to separate the residual solids

from the liquid hydrolysate. The liquid hydrolysate was decanted to a volumetric cylinder and the volume was recorded. The unhydrolyzed solids were washed twice with distilled water. In each washing step, the volume of distilled water used was equal to the volume of hydrolysate generated during EH. This washing step was performed by the sequential re-suspension of the solids, centrifugation and decantation. The solution resulting from the washing steps was transferred to a volumetric cylinder and the volumes were recorded for mass balance calculations. Samples of the hydrolysate and water washing solutions were prepared for glucose and xylose analysis using an HPLC system equipped with a Bio-Rad Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) as previously described.[38]

Lignin mass balance

A mass balance study on lignin was performed around the EA pretreatment, EA extractive fractionation, and enzymatic hydrolysis. The dry weight loss observed during pretreatment of corn stover was calculated by measuring the weight of the biomass before and after pretreatment, together with the moisture content using a moisture analyzer A&D MX-50 (A&D Engineering, Inc., San Jose, CA, USA). Compositional analysis was performed on corn stover before and after EA pretreatment using the standard NREL protocols, NREL/TP-510-42618 and NREL/TP-510-42620. Nitrogen analysis was performed using a nitrogen analyzer (Skalar PrimacsSNC, Breda, The Netherlands).

Lignin extraction yield was calculated by the difference in the total lignin weight before and after pretreatment, divided by the total lignin weight of the untreated sample. The percent recovery of each fraction was normalized with respect to the total lignin present in the corn stover and total extracted lignin during EA pretreatment.

2D-HSQC NMR analysis

Crude extracts generated from EA pretreatment of corn stover (30 mg) were dissolved in DMSO-d_6/pyridine-d_5 (4 : 1, v/v, 600 μL) and transferred into 5 mm NMR sample tubes. Enzymatic lignin (EL) from corn stover, described previously,[39] was similarly prepared, and the whole corn stover material, after fine milling, was subjected to gel-NMR as previously described.[39–41] NMR spectra were acquired on a Bruker Biospin AVANCE 700 MHz spectrometer fitted with a cryogenically-cooled 5 mm TXI gradient probe with inverse geometry (proton coils closest to the sample). The central DMSO solvent peak was used as internal reference (δ_C, 49.5; δ_H, 3.49 ppm). Adiabatic HSQC experiments (hsqcetgpsisp2.2) were carried out using the parameters described previously.[40,41] Typical matched Gaussian apodization in F2 (LB = −0.5, GB = 0.001) and squared cosine-bell apodization and one level of linear prediction (32 coefficients) in F1 were used for processing. Volume integration of contours in HSQC spectra (processed without using linear prediction) was carried out using Bruker's TopSpin 3.1 (Mac) software with no correction factors; i.e., the data represent volume integrals only; end groups (such as p-coumarate and tricin) are severely over-estimated by these methods due to their relaxation rate properties compared to the internal units of a chain.[40]

For quantitation of lignin aromatic distributions, only the carbon/proton-2 correlations from G and G′ units and the carbon/proton-2/6 correlations from S and S′ units were used, and the G and G′ integrals were doubled; other aromatic integrals are reported relative to the total lignin aromatics (G + G′ + S + S′ = 100%).

^{31}P-NMR analysis

Quantitative ^{31}P NMR analysis of phosphitylated ball milled lignin (BML) (25 mg) was accomplished by using a pyridine/$CDCl_3$ (1.6 : 1, v/v) solvent, cyclohexanol as an internal standard and 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) as the derivatization agent following literature methods.[42] The ^{31}P NMR spectra were acquired using an inverse-gated decoupling pulse sequence with a 25 s pulse delay and 128 scans. NMR spectra were acquired on a Bruker Biospin AVANCE 400 MHz spectrometer fitted with a 5 mm broadband probe operating at 161.951 MHz.

Gel-permeation chromatography (GPC)

The isolated lignin samples (100 mg) were treated with a mixture of pyridine and acetic anhydride (1 : 1, v/v, 4.00 mL) with stirring at room temperature for 24–36 h. The reaction mixture was diluted with ethanol (30 mL) and stirred for 30 min and then concentrated under vacuum. The acetylated lignin samples were dissolved in chloroform (2 mL) and added dropwise into diethyl ether to precipitate the sample and then centrifuged. The precipitate was washed with diethyl ether and centrifuged three times. After air drying, the acetylated samples were dried for 24 h in a vacuum oven at 40 °C prior to GPC analysis.

Molecular weight determination was conducted using a Polymer Standards Service (PSS) GPC Security 1200 system equipped with four Waters Styragel columns (HR0.5, HR2, HR4, HR6) at 30 °C, an Agilent isocratic pump, an Agilent auto-sampler, an Agilent degasser, an Agilent refractive index (RI) detector and an Agilent UV detector (270 nm) using THF as the mobile phase (1.0 mL min^{-1}) with injection volumes of 20 μL.

The weight-average molecular weight (M_w) values of the derivatized lignin samples were acquired using a relative calibration curve and this relative calibration curve was created by fitting a third order polynomial equation to the retention volumes obtained from a series of narrow molecular weight distribution polystyrene standards (1.36×10^6, 5.38×10^5, 3.14×10^4, 7.21×10^3, 4.43×10^3, 5.80×10^2 g mol^{-1}). The curve fit had an R^2 value of 0.9984.

Elemental analysis

The inorganic elements in the samples were determined by Inductively Coupled Plasma Emission Spectroscopy (ICP) according to the methodology previously employed by Allison et al. (2000).[43] Nitrogen, carbon, hydrogen and oxygen analysis

for fraction F3 was performed by Galbraith Laboratories, Inc., Knoxville, TN, USA.

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) analysis

Thermal gravimetric analysis (TGA) data from fractions F1 to F5 were obtained using a PerkinElmer Simultaneous Thermal Analyzer (STA) 6000. Samples were put into a ceramic crucible with a lid. The ramp rate was 10 °C min^{-1} from 25 to 600 °C. When the temperature reached 600 °C, it was held under isothermal conditions for 2 min. Nitrogen was used as the flushing gas set at a flow rate of 50 mL min^{-1} throughout the test.

Conclusions

Characterization of various lignin-rich streams generated during EA processing of corn stover was reported herein. EA pretreatment performed on corn stover with 10% (w/w) moisture at 6 : 1 ammonia-to-biomass ratio and 120 °C for 30 min, extracted approximately 44% (w/w) ofits lignin, which was further fractionated using inexpensive solvents, water and ethanol, to generate a lignin-rich product stream (F3) that was practically free of carbohydrates. This lignin-rich product stream (F3) was characterized by its good yield, low molecular weight, ethanol solubility, high (92%) lignin content, and high proportion ofintact, native lignin functionality (e.g., β-O-4 linkages). Also, F3 exhibits a relatively low decomposition temperature with products that may prove interesting on further study. Fraction F3 also contained low levels ofinorganics, potentially benefitting catalytic processes by reducing catalyst poisoning.

The ammonia-insoluble lignin-rich product stream (F5) also shows good chemical properties such as high molecular weight and preserved functionalities such as β-aryl ether units. However this stream may require further processing to reduce its carbohydrate content and thereby improve its quality as a lignin product/feedstock for additional uses.

An important goal of this study was to provide good quality lignin, useful as a starting material for valorization to improve biorefinery economics. The F3 stream, as well as F5 stream, can provide such feedstocks for lignin-based conversion to fuels and chemicals. These streams contain 2 and 4 wt% of nitrogen, respectively, which could lead to NO_x formation during combustion of compounds derived from those lignin fractions. However, methods such as HDN can be integrated into the processing of F3 and F5 lignins to reduce the levels of nitrogen in the products derived from these fractions.

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