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Effect of Dilute Acid Pretreatment and Lignin Extraction Conditions on Lignin Properties and Suitability as a Phenol Replacement in Phenol-Formaldehyde Wood Adhesives

Brian K. Saulnier,¹ Mohsen Siahkamari,² Sandip K. Singh,^{1,#} Mojgan Nejad,² David B. Hodge^{1,3*}

¹ Department of Chemical & Biological Engineering, Montana State University, Bozeman, Montana 59717, United States

² Department of Forestry, Michigan State University, East Lansing, Michigan 48824, United States

³ Division of Sustainable Process Engineering, Luleå University of Technology, Luleå, Sweden 97187

Current Affiliation: Center for Environmentally Beneficial Catalysis, University of Kansas, Lawrence, Kansas 66045, United States

* To whom correspondence should be addressed. Email: david.hodge3@montana.edu

ABSTRACT

Corn stover was subjected dilute sulfuric acid pretreatment to assess the impact of pretreatment conditions on lignin extractability, properties, and utility as a phenol replacement in wood phenol-formaldehyde (PF) adhesives. It was identified that both formic acid and NaOH could extract and recover 60% to 70% of the lignin remaining after pretreatment and enzymatic hydrolysis under the mildest pretreatment conditions while simultaneously achieving reasonable enzymatic hydrolysis yields (>60%). The availability of reaction sites for incorporation of lignins into the PF polymer matrix (*i.e.*, unsubstituted phenolic hydroxyl groups) was shown to be strongly impacted by the pretreatment time and the recovery. Finally, a lignin-based wood adhesive was formulated by replacing 100% of the phenol with formic acid-extracted lignin, which exhibited a dry shear strength exceeding a conventional PF adhesive. These findings suggest that both pretreatment and lignin extraction conditions can be tailored to yield lignins with properties targeted for this co-product application.

Keywords: Lignin, wood adhesive, *p*-coumarate, biorefinery, cellulosic biofuels

INTRODUCTION

Lignocellulosic biomass represents a significant renewable resource that can serve as a potential feedstock for the production of fuels, chemicals, and materials. One approach to renewable fuels is through a chemical pretreatment of biomass followed by enzymatic hydrolysis of the polysaccharides to yield fermentable sugars that can be biologically or chemically upgraded to fuels and chemicals.¹ While the structural polysaccharide content of lignocellulose can be targeted for depolymerization and conversion at high yields, the utilization of the lignin fraction is both necessary to improve overall process economics² and challenging for applications other than direct combustion due to lignin heterogeneity and its modification during processing. Three general approaches can be applied for integrating lignin recovery and utilization into lignocellulose biorefining processes. These include utilizing a pretreatment that solubilizes a fraction of the lignin (*e.g.*, alkali or organosolv) that can be recovered and utilized,² utilizing a “lignin first” pretreatment that integrates pretreatment with lignin solubilization and depolymerization,³ and the approach that will be applied in the present work: extracting and recovering lignin from the water-insoluble “lignin cake” that remains after pretreatment and enzymatic hydrolysis using a lignin-solubilizing solvent.⁴

Phenol formaldehyde (PF) resins are commonly used as adhesives in engineered wood products, including plywood, laminated veneer lumber, and oriented strand board. These resins represent a market of more than \$3.5 billion and 34% of the total adhesive market.⁵ Lignins derived from biorefining or chemical pulping processes can serve as a potential bio-based alternative to the petroleum-derived phenol used in the synthesis of these resins. However, common challenges to the utilization of lignins in this application include low reactivity towards formaldehyde, high molecular weight, and high polydispersity.⁶ Formaldehyde can react with phenol at sites *para* and *ortho* to the hydroxyl group.⁷ Low reactivity of lignin with formaldehyde relative to phenol is mainly due to the lack of reactive sites resulting from the *para* position of aromatic rings being bound in intramolecular linkages, while the *ortho* positions are often occupied by either methoxyl groups or β -5 linkages.⁸ Due to the low reactivity of lignin with formaldehyde, resins made with non-modified lignin were only able to produce resins with acceptable mechanical properties using partial phenol replacement with <60% of the phenol replaced by lignin.⁹⁻¹⁰ To address these challenges, many studies have functionalized lignin using approaches that include methylation,¹¹ phenolation,¹² and demethylation¹³ to increase lignin

reactivity and to yield mechanical properties comparable to conventional PF resin adhesives. However, these lignin functionalization steps add additional cost to the lignin-formaldehyde resin production process.

Formulation and production of PF resins with phenol fully replaced by plant-derived lignin without the need for further functionalization reactions can result in a product more attractive for industrial use. Recent work demonstrated that a lignin-formaldehyde wood adhesive with mechanical properties similar to commercial PF resins could be formulated with 100% replacement of phenol using a NaOH extracted lignin from dilute acid pretreated corn stover.¹⁴⁻¹⁵ Grass lignins are known to exhibit high phenolic hydroxyl contents namely in the form of *p*-coumarate (*p*CA) esters.¹⁶ We suggest that the success of this study in fully replacing phenol with lignin in PF resins is due to the use of a grass lignin (corn stover) as opposed to hardwood-derived lignins used in the previously mentioned studies.⁹⁻¹⁰ It has been hypothesized that high *p*CA content is especially important for the successful replacement of phenol with lignin in phenolic resin, and we have shown that dilute acid pretreatment of corn stover can result in high levels of *p*CA being retained by the biomass while also allowing for high glucose hydrolysis yields.⁴ This previous work focused on understanding the relationship between *p*CA, lignin content, and dilute acid pretreatment conditions specifically understanding the susceptibility of the *p*CA ester bond in lignin to hydrolysis during dilute acid pretreatment. We found that *p*CA solubilization and xylan solubilization during pretreatment are directly correlated with each other and with glucose hydrolysis yields following pretreatment. We also demonstrated using a *p*CA-ester model compound that acid-catalyzed ester hydrolysis proceeds according to kinetic parameters of the same order of magnitude as xylan hydrolysis.⁴ In this study, we build on these results by using a single corn stover biomass source to (1) understand the relationship between pretreatment severity and lignin extractability, (2) understand the susceptibility of the *p*CA bond to hydrolysis during extraction using two approaches to extract lignin from the lignin cake, (3) determine if *p*CA content is the primary contributor to the “H” lignin signal in ³¹P NMR, (4) scale-up pretreatment and lignin extraction and demonstrate successful 100% replacement of phenol with corn stover lignin, and (5) characterize resin and adhesive properties and optimize resin formulation for corn stover lignin. Together, these five points represent novel contributions to the literature.

The reactivity of lignin towards formaldehyde and the resulting resin properties are dependent on the feedstock used,¹⁷ the method of biomass pretreatment,¹⁸ and the conditions used for lignin extraction/purification.¹⁹ Relevant to the present study is how *p*CA content varies between different feedstock sources, genotypes, plant tissue, and plant development stage. Prior work in our laboratory has focused on understanding both the distribution and fate of *p*CA during the biorefining of graminaceous feedstocks.²⁰⁻²² Importantly, we've identified up to 5-fold differences in the abundance of *p*CA in lignin across diverse bioenergy-relevant grasses and even nearly 3-fold differences within a single plant.²³ If the hypothesis that *p*CA content is the most important contributor to lignin suitability in PF resins is correct, then the implications are that the feedstock genotype, together with growth conditions, harvest time, and lignin processing history are all of importance for lignin suitability as a raw material for these applications. Critically, this understanding presents the opportunity to optimize feedstock selection based on lignin suitability for co-products in addition to properties such as cell wall recalcitrance or agronomic suitability.

Besides the impacts of pretreatment on lignin, any additional purification, extraction, or recovery steps are important parts of a lignin's processing history which can affect the subsequent lignin properties. While not studied as extensively as pretreatment, some studies have been conducted investigating lignin properties after additional purification processes. Previous work demonstrated that when compared to kraft lignin, lignin extracted from sugarcane bagasse using an acetosolv process displayed lower molecular mass, higher thermal stability, higher phenolic and total hydroxyl contents, and more *p*-hydroxyphenyl units.²⁴ Other work compared lignin extraction by organic acids to extraction via organic alcohols and showed higher lignin recovery yields using mixtures of formic and acetic acid versus acetic acid alone or ethanol and methanol. They also identified minor differences in the molecular weights of lignins extracted using the different solvent systems and found higher contents of *p*-hydroxyphenyl units in lignin extracted with organic acid versus organic alcohol.²⁵ These results illustrate the importance of feedstock, pretreatment method, and lignin extraction conditions on the suitability of certain lignins for incorporation into PF resins based on the lignin's properties. Higher *p*-hydroxyphenyl content within lignin allows for more open reactive sites in the *ortho* position of the aromatic ring and thus increased reactivity with formaldehyde allowing for complete phenol replacement in PF resins.²⁶⁻²⁷ The molecular weight distribution of a specific lignin has also been shown to have an impact on lignin reactivity towards formaldehyde, with lower molecular weight lignins

demonstrated to be more reactive towards formaldehyde.²⁸⁻²⁹ One study suggests that this effect of increasing lignin reactivity as molecular weight decreases is due to higher phenolic hydroxyl content resulting from the cleavage of β -O-4 bonds in low molecular weight lignin fractions.³⁰ Prior work linked lignin thermal stability to degree of incorporation into PF resins and hypothesized that a high molecular weight lignin contains fewer end chains which will result in lower volume mobility, causing more energy for chain rotation, resulting in higher T_g values.³¹ It was suggested that lower T_g values for a certain lignin indicate more free volume, opening a higher possibility for cross-linking formation in PF resins.³¹ Other work suggested that lignins with a higher thermal decomposition temperature can be incorporated into PF resins to heighten their thermal stability allowing for their use in high-temperature applications.³²

For the present study, we investigate how dilute acid pretreatment conditions for corn stover impact lignin properties, lignin solubility/extractability, *p*CA content of the lignins through each processing step, and the resulting incorporation of these lignins into the polymer matrix of phenol-formaldehyde resins. Specifically, corn stover is subjected to a range of dilute acid pretreatment conditions (*i.e.*, varying acid loading, temperature, and time) followed by enzymatic hydrolysis and lignin extraction/recovery utilizing either formic acid or NaOH. Based on the results of this screening select conditions are identified to scale up the lignin generation to yield sufficient material for testing in materials applications. These lignins are next used to synthesize PF resins and corresponding wood adhesives, with 100% of the phenol replaced with lignin. Finally, the physical and mechanical properties of these resins and adhesives are characterized and linked to differences in the chemical properties of the source lignins.

MATERIALS AND METHODS

Biomass Source and Small-Scale Dilute Acid Pretreatment and Enzymatic Hydrolysis

A commercial hybrid corn stover was obtained from Idaho National Laboratory after harvest in 2014 in Story County Iowa. The corn stover was first milled to pass a 2-inch screen using a Vermeer bale grinder BG-480 followed by milling with a Bliss Hammermill to pass a 1-inch screen, and finally milled to pass a 20-mesh using a Wiley mini-mill (Thomas Scientific). Aqueous, dilute sulfuric acid pretreatments were conducted in 300-mL stainless steel sample cylinders (Swagelok, 316L-HDF4-300) submerged in fluidized aluminum oxide heated to the desired temperatures (Techne SBS-4, Cole-Parmer). Pretreatments were performed at solid

loadings of 20% (mass dry corn stover/liquid volume) with initial values for pH ranging from pH 1-3 adjusted using 6 M H₂SO₄, times ranging from 1 to 6 hours, and temperatures ranging from 150 to 200 °C corresponding to combined severity factors (CSF) of 1.0 to 4.0 calculated using the equation developed by Chum et al.³³ Heat-up was considered in the pretreatment time, while samples were air-cooled to room temperature following the targeted pretreatment time. The conditions used for pretreatment are presented in **Table 1**. Following pretreatment, corn stover was subjected to enzymatic hydrolysis using CTec2 (Novozymes A/S, Bagsvaerd, Denmark). Protein content of the commercial enzyme cocktail was determined using the Bradford assay (Thermo Fisher Scientific, Waltham, MA) with BSA as the protein standard. Protein loadings were determined based on quantified protein content and biomass glucan content. Hydrolysis was conducted at 10% biomass loading (wt/vol) with two different enzyme loadings (15 and 30 mg protein/ g glucan). Samples were hydrolyzed at a constant pH of 5.0 using a 1 M citrate buffer within 15-mL centrifuge tubes for 72 hours at 50 °C. A tetracycline solution was added to impede bacterial growth (0.045 mg/mL). Hydrolysis liquor samples were taken at 24, 48, and 72 hours. Glucose concentrations in the hydrolysate were quantified using HPLC (Agilent Technologies, 1260 Infinity II) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) using detection by RID and used to calculate glucose hydrolysis yields.

Small-Scale Lignin Extraction and Recovery

Lignin was extracted from hydrolysis residues using both NaOH and formic acid extraction methods. NaOH extraction was conducted in 40-mL stainless steel synthesis reactors (Shenzen Lange Ltd., Huizhou, China) using solid loadings of 20% (wt/vol) hydrolysis residue to 1 M NaOH corresponding to a biomass to NaOH loading of 200 mg NaOH/g biomass. Reactors were submersed in a silicon oil bath and heated on a hot plate to 85 °C for 1 hour. Lignin was precipitated from the resulting alkaline liquor by adding concentrated H₂SO₄ dropwise until a pH <2 was achieved. Lignin was separated from the solution via vacuum filtration (Whatman Grade 42 Ashless Filter Paper), washed with DI water until neutral pH was reached, and dried at room temperature. Formic acid extraction was conducted using the same reactor system as the NaOH extraction method. Solid loadings of 10% (wt/vol) hydrolysis residue to 88% (w/w) formic acid was used. Reactors were refluxed at 101 °C for 1 hour. Formic acid lignin solution was collected, and solid residue was washed with an additional 50 mL of formic acid. This formic acid was

added to the previously separated formic acid solution, and lignin was precipitated by the addition of 300 mL DI water. Lignin was removed from solution via centrifugation and washed with additional DI water.

Scale-Up of Pretreatment, Hydrolysis, and Lignin Extraction

Pretreatment scale-up was performed at the pretreatment conditions corresponding to a CSF of 2.0 from **Table 1**. Corn stover was hammer-milled to pass a 3-mm screen (Model 158, Pellet Masters, Chippewa Falls, WI). Milled corn stover and dilute sulfuric acid solution were mixed in 16-inch 3 NPT 316 threaded stainless-steel pipes, sealed with pipe fittings and pipe dope, and allowed to equilibrate overnight. Sealed pipes were submerged in a water bath inside a 20 L stainless steel digester (RegMed model AU/E-20, São Paulo, Brazil) and heated to 150 °C and held for 1 hour. After pretreatment, the biomass was washed with water in a home brewing mesh filter bag and dried at 50 °C.

Hydrolysis was performed to yield “lignin cake” or the lignin-rich hydrolysis residue following enzymatic hydrolysis and was conducted in a five-gallon polyethylene bucket using 10% (wt/vol) solid loadings and a 25 mg protein/g glucan enzyme loading comparable to our previous work.³⁶ The bucket was heated to 50 °C in an incubator for 72 hours, stirring periodically for the first 24 hours, with periodic pH adjustment to pH 5.0 using the dropwise addition of 6 M H₂SO₄. Following hydrolysis, lignin cake was washed 4 times using 1 L DI water in a Beckman Coulter Avanti JXN-26 centrifuge and dried at 50 °C for 48 hours.

Formic acid lignin extraction was conducted using 10% (wt/vol) solids, 260 g of lignin cake was added to 2.6 L of 88% (w/w) formic acid in a 5-L glass round bottom flask (USALab, RBF5L1N) submerged in an oil bath heated by a hot plate (**Supplemental Fig. S1**). The round bottom flask was equipped with a condenser and the formic acid solution was allowed to boil at reflux for 1 hour. Remaining solids were washed twice with ~40% w/w formic acid, and lignin was precipitated by the addition of 13 L of DI water. Lignin was separated from the solution and washed with additional water via centrifugation (Avanti JXN-26, Beckman Coulter).

NaOH lignin extraction was conducted in a stainless-steel digester (RegMed model AU/E-20, São Paulo, Brazil) equipped with 4 intermediate 1.5 L reactor vessels (Lid T4T-20). Lignin cake was submerged in 1 M NaOH and heated to 85 °C for 1 hour. Black liquor was separated from remaining solids using a home brewing mesh filter bag and a cider press. Liquor was adjusted to

pH <2 using H₂SO₄ to precipitate lignin. Lignin was recovered via centrifugation (Avanti JXN-26, Beckman Coulter), and washed with DI water.

Biomass and Lignin Characterization

Untreated corn stover, pretreated corn stover, hydrolysis residues, and extracted lignins were analyzed via NREL LAP TP-510-42618 to determine structural polysaccharide and Klason lignin contents.³⁴ Sugars were quantified via HPLC with an RID detector using a Bio-Rad Aminex HPX-87H column, a mobile phase of 5 mM H₂SO₄, a flow rate of 0.6 mL/min, and a column temperature of 65 °C. These samples were further analyzed for moisture and ash content by drying at 105 °C for 4 hours and measuring mass loss due to moisture, and subsequently measuring remaining ash content gravimetrically after combusting the biomass at 620 °C for 4 hours according to a method modified from the NREL laboratory analytical procedure LAP TP-510-42622. Alkaline hydrolysis was used to determine the amount of *p*-coumarate contained within pretreated biomass and extracted lignin using the same approach as in our prior work.⁴ Gel permeation chromatography (GPC) was used to determine the weight and number average molar masses of extracted lignins according to previously published methods³⁵ using a calibration curve constructed from Readycal polystyrene standards (Fluka Analytical) in the 250-70,000 Da range. Elemental analysis of select recovered lignins was performed by ICP-OES with samples prepared according to AOAC 985.01. Recovered lignins were characterized by ³¹P NMR to quantify the abundance of lignin functional groups (phenolic hydroxyl, aliphatic hydroxyl, and carboxylate groups) as in our prior work.¹⁵

Preparation of Resins and Adhesives

Phenol-free lignin-based resins were prepared according to our previous work.¹⁴ Briefly, 25 g of recovered lignin was first dissolved in 85 mL of 1M NaOH and then transferred to a 250 mL three-necked round bottom flask equipped with a condenser. Formaldehyde was gradually added to the flask until a 1:2 molar ratio of formaldehyde to lignin phenolic hydroxyl was achieved. Using a digital hot plate with constant stirring, the flask was heated from room temperature to 65 °C over 30 minutes followed by incubation at 65 °C for 30 minutes. Next, 40 mL of 1M aqueous NaOH was added. The resulting mixture was gradually increased to 90 °C and the temperature was held constant for 2 more hours. After cooling the resin using an ice bath, some of the resin

was frozen to prevent additional polymerization, while the rest was utilized to characterize chemical and physical properties. The developed resins were used to prepare lignin-based adhesives utilizing the same process used for commercial phenol-formaldehyde glue mix formulation as described in our prior work.¹⁵ Adhesives are composed on a weight basis of 66% thawed resin combined with 8% wheat flour, 16% distilled water, 8% plywood extender (Modal), and 2% NaOH pellets. The lignin-based adhesives were stirred for 5 minutes using a high-speed mixer at 800 rpm achieve a homogeneous mixture.

Resin and Adhesive Property Characterization

Properties of developed resins and adhesives were evaluated according to ASTM-recommended test methods. All properties were measured in triplicate except for solid content which was tested in five replicates. For each sample solid content was measured according to ASTM D4426-01. In labeled pans 1 g of each resin was heated to 125 °C for 105 minutes. After heating, samples were cooled in a desiccator, weighed, and percent solid content was calculated. The pH of resins and adhesives was measured using a Mettler Toledo S220 digital pH meter. Rheology of formulated resins was analyzed using a Discovery HR-1 hybrid rheometer (TA Instrument) equipped with a 40-mm parallel plate geometry and a 1000 μm gap at 23 °C. Rheometer tests were performed at constant shear rates of 1000 (s^{-1}) to determine the viscosity of resins and adhesives. Gelation time was determined following the method reported by Pizzi and Mittal.³⁷ A glass test tube containing 1 g of each resin sample was submerged in a beaker of boiling water. The resin was then stirred with a glass rod until a string formed around the rod. The gelation time was recorded from the time the test tube was immersed in boiling water to the time that the string was formed. The free formaldehyde level of resins was determined using the hydroxylamine hydrochloride method according to European Standard DIN EN ISO 9397. The alkalinity of resin and adhesive samples was measured according to Lorenz and Christiansen's recommended procedure.³⁸ Using an estimated amount of NaOH in each sample, different amounts of resin or adhesive (2 to 10 g) were added to 100 mL distilled water. This solution was titrated to pH 3.5 using 0.1 N hydrochloric acid solution. The alkalinity is calculated based on the amount of resin used and the hydrochloric acid volume.

The adhesives were tested for strength of adhesion using a lap shear strength test according to ASTM D5868-01. For each measurement 0.12 g of adhesive was applied to one-fourth (25.4 x

25.4 mm²) of the hardwood veneer samples (25.4 mm x 102 mm x 5.6 mm). Single-lap joint samples were prepared using a digital hot press (Carver, Inc., Model 3895.4PL1000). The automatic press instrument enables adhesives to cure between veneer samples by adjusting the temperature of the press plates and the required pressure. Utilizing the pressing conditions recommended by industry for commercial adhesives, the veneer samples were pressed for 4 min at 175 °C under 1400 kPa. The adhesion strength (tensile strength at maximum load) of produced adhesives was measured using an Instron 5565 Universal testing equipment with a loading rate of 12.7 mm/min.

RESULTS AND DISCUSSION

Biomass Pretreatment and Enzymatic Hydrolysis

For the first part of this study, corn stover was subjected to dilute sulfuric acid pretreatments performed over a range of times, temperatures, and initial pH values with the goal of generating lignins with substantial differences in properties. This set of materials was utilized to achieve two goals. First, the diverse lignins were used to develop a better understanding of how these differences in lignin properties impact the extractability/solubility during a lignin recovery process from a hydrolysis residue (“lignin cake”). Second, through the characterization of both lignin recovery yields and lignin properties combinations of conditions for pretreatment and lignin extraction can be identified that may yield lignins that are optimal for application as a phenol replacement in phenol-formaldehyde wood adhesives.

The four pretreatment conditions are outlined in **Table 1**. The three moderate conditions at 150 °C chosen in this study span a range of CSFs expected to result in high glucose hydrolysis yields^{4, 39} as well as moderate modification of lignin properties and solvent extractability.⁴⁰ A fourth significantly more severe condition at higher temperature (200 °C) and the lowest initial pH (1.0) corresponding to a CSF of 4.0 was chosen to assess the effects of high-severity pretreatments on the subsequent lignin properties as well as enzymatic hydrolysis yields. The pretreated biomass was tested for its response to enzymatic hydrolysis and xylan solubilization. These results are presented in **Figure 1**, while complete composition profiles are found in **Supplemental Figure S2**. One key observation is that increasing values of CSF are not necessarily correlated to increasing hydrolysis yields. One reason for this is that the mildest pretreatment in terms of CSF (*i.e.*, CSF = 1.0) was also the longest pretreatment (6 hr) and gave

the strongest response of the three pretreatments performed at 150 °C. Xylan is distributed on the surface of the cellulose fibers as well as being present in the inter-fibrillar space⁴¹ and has been shown to act as a barrier inhibiting the access of enzymes to cellulose.⁴² The presence of lignin has also been shown to inhibit glucose hydrolysis by hindering enzymes from penetrating the cell wall to gain access to cellulose⁴³ and through the non-specific binding of cellulase to lignin.⁴⁴ Dilute acid pretreatment improves cellulose accessibility by melting and redistributing lignin rather than solubilization and removal.¹ Xylan removal is one of the key outcomes of dilute acid pretreatment and the correlation between xylan removal and enzymatic hydrolysis yields in pretreated corn stover is well-established.^{4, 39} This study reinforces this finding showing a strong correlation between xylan removal and enzymatic hydrolysis yields following dilute acid pretreatment (**Fig. 1B**).

Lignin Extraction, Recovery, and Characterization

Challenges to utilizing the water-insoluble lignin within enzymatic hydrolysis residue (*i.e.*, “lignin cake”) for biobased materials and chemicals include (1) the high level of contamination with other “non-lignin” components (unhydrolyzed polysaccharides, ash, cellulase protein) that necessitates purification, (2) the relatively poor solubility of the lignins in many solvents that would be necessary for both purification/separation of the lignins from the lignin cake, and for processing in many of the target applications, and (3) the substantial modification of the lignins during pretreatment that may limit its utility in many applications. To address these challenges, two approaches (NaOH and formic acid treatment) were next applied to extract, solubilize, and recover the lignin from the hydrolysis residue. The lignin recovery yields are assessed and select properties of the recovered lignins are characterized (molar mass, abundance of *p*CA and other functional groups, and contamination of the recovered lignins with polysaccharides and ash).

Lignin solubility is key for its utilization in many chemical and materials applications that involve chemical modifications such as cross-linking, functionalization, or depolymerization. Pretreatments of lignocellulose using NaOH are well-established technologies⁴⁵ and target lignin (as well as hemicellulose) removal primarily through two mechanisms. First, alkali contributes to the chemical cleavage of β -aryl ethers in lignin at elevated temperature as well as hydrolysis of ester bonds (*e.g.*, ferulate and *p*-coumarate) within and between lignin and xylan in grasses such as corn stover.⁴⁶ Second, these lignin and xylan fragments are soluble in aqueous alkali, which

facilitates their removal from the plant cell wall. Consequently, NaOH treatments may be employed after enzymatic hydrolysis for lignin extraction and recovery for lignin utilization in co-products applications.⁴⁶⁻⁴⁷ One advantage of utilizing NaOH extraction and solubilization of lignin is that lignin-formaldehyde resins are ultimately formulated under alkaline conditions and potentially this approach could remove a lignin precipitation/recovery step. As a potential disadvantage of NaOH lignin extraction, previous studies have shown that NaOH treatment of lignin results in reduction of phenolic hydroxyl content especially the reduction of *p*-hydroxyphenyl subunits,⁴⁸ presumably due to the alkali-catalyzed hydrolysis esters of *p*CA pendant groups on lignin.⁴ Notably, our prior work utilized NaOH extraction to isolate lignin from polysaccharide-rich residues such as lignin cake.¹⁴ A formic acid lignin extraction is also employed as it is hypothesized that this may result in a higher retention of *p*-hydroxyphenyl and *p*CA for incorporation into PF resins. Formic acid lignin purification and fractionation has become the subject of increasing research in recent years.⁴⁹ Formic acid extraction processes have been shown to be successful in separating lignin from carbohydrates and ash components in multiple types of biomass while retaining high levels of phenolic hydroxyls and *p*CA within the lignin structure.⁵⁰⁻⁵¹ These high-purity lignins with an absence of polysaccharides and inorganics while retaining reactive phenolics such as *p*-hydroxyphenyl and *p*CA exhibit promise for lignins with ideal properties for reaction with formaldehyde and creation of PF resins.

The formic acid and NaOH lignin extractions used in this study were both conducted under relatively low temperature (101 °C and 85 °C, respectively) and atmospheric pressure. Extraction and recovery under these conditions resulted in high lignin recovery yields from the lignin cake in the 60-70% range for both formic acid and NaOH extractions under all pretreatment conditions except the most severe condition at CSF 4 (**Fig. 2A**). Importantly, these yields of 70% under relatively mild extraction conditions are significant relative to prior work. As one example, increasing CSF from 0.5 to 1.5 increased lignin recoveries from ~45% to 57% in dilute acid-pretreated and enzymatically hydrolyzed corn cob using ball-milling, additional acid hydrolysis, and extraction with acidic dioxane-water mixtures.⁴⁰ As another example, prior work demonstrated that despite significant lignin solubilization, direct alkaline pretreatment of corn stover under relatively comparable conditions to those used in the present work resulted in approximately 35% recovery of the lignin.⁵² One possible implication of this is that prior dilute acid pretreatment act synergistically with subsequent alkaline solubilization to improve the

recoverability of the lignin. One notable exception to the high lignin recovery yields is the for the most severe pretreatment (CSF = 4.0) of corn stover, with yields of less than 20% (**Fig. 2A**). It can be hypothesized that under these severe conditions, the lignin becomes more highly condensed and “char-like”, both reducing its solubility in aqueous alkali and formic acid, and reducing its chemical utility. Acid hydrolysis residue following furfural production from corn cob is comparable to these high-severity pretreated materials, and it’s notable that low lignin yields (less than 30%) using alkali extraction have been identified from these materials as well.⁵³ Overall, these results suggest that some prior acid pretreatment may improve lignin recoverability relative to only alkaline pretreatment, and harsh acid pretreatments render the lignin less extractable. The results for recovered lignin molar mass (**Fig. 2B**) show that both number average ($\bar{M}_N$) and weight average ($\bar{M}_W$) molar masses increase as CSF increases from 1.0 to 2.0. At the most severe CSF (4.0) the values for recovered lignin molar mass drop by approximately 50% relative to those recovered from other pretreatment conditions. Coupled with the low lignin recovery yields from most severe CSF, it can be hypothesized that the bulk of the lignin becomes highly cross-linked and extraction solvent-insoluble such that only a small low-MW subset of the lignin is recoverable from this sample.

Lignins generated using both extraction methods over the entire pretreatment range were next subjected to characterization with respect to lignin purity/composition, *pCA* content, and phenolic hydroxyl content. This was performed with the goal of identifying coupled pretreatment and lignin recovery conditions that can (1) maximize lignin recovery yields and (2) generate lignins with properties suitable for subsequent use in PF resins. Specifically, the identified conditions will be used for subsequent scale-up for producing lignins of sufficient quantity for production and property testing of PF resins and the resulting adhesives. The “ideal” lignin properties for this application include high phenolic hydroxyl content and/or *pCA* content, and low content of ash and polysaccharides. Results show that the recovered lignins all exhibited less than 2% by weight ash content with formic acid extracted lignin exhibiting ~10 to 20-fold higher ash than the NaOH lignin (**Fig. 3A**). This discrepancy disagrees with previous literature,⁵⁰ and may be due to different washing methods employed after the two lignin extractions. All lignins contained less than 5% polysaccharide content by weight except for the NaOH lignin from the CSF 1.5 condition (**Fig. 3B**). It is unknown why this lignin showed such high sugar content after lignin extraction.

The NaOH and formic acid-extracted and recovered lignins were next subjected to ^{31}P NMR characterization to determine the relative abundance of functional groups in the lignin (**Fig. 3B**) and, importantly, phenolic hydroxyl content, which is critical for lignin incorporation into PF resins. It should be noted that not all lignins generated in this study were analyzed due to poor solubility in the solvents used during derivatization and NMR characterization. In agreement with previous literature lignins extracted with formic acid showed reduced aliphatic hydroxyl content and increased phenolic hydroxyl content when compared to NaOH extracted lignins (**Fig. 3B**).⁵⁰⁻⁵¹ This supports our previous hypothesis that formic acid lignin will exhibit more favorable properties compared to NaOH lignin for implementation in PF resins. Prior literature on solvent extraction of dilute acid-pretreated corn cob found that increasing CSF from 0.5 to 1.5 increased the total phenolic hydroxyl content from 2.6 to 3.1 mmol/g,⁴⁰ which is the same magnitude as our results.

The presence of *p*CA ester groups on lignins derived from graminaceous feedstocks have been hypothesized to be a key feature of their improved incorporation into PF resins relative to wood lignins derived from other processes (**Fig. 4A**).^{4, 15} However, these labile ester linkages are highly susceptible to alkaline- and acid-catalyzed hydrolysis during both pretreatment and lignin recovery stages. In the present work, *p*CA content in the extracted lignins was quantified using alkaline saponification (**Fig. 4B**). The results show several key trends. First, the dilute acid pretreatment can hydrolyze a significant amount of the *p*CA from the lignin (16%-96% removal, **Fig. 4B**). A second key trend is that the formic acid-extracted lignins have a significantly higher *p*CA content than the NaOH-extracted lignin. This indicates that the combination of temperature, time, and NaOH concentration required to solubilize the lignin is also capable of hydrolyzing the majority of the *p*CA esters remaining in the lignin. However, the formic acid extraction can solubilize a significant fraction of the lignin (greater than 60%, **Fig. 2A**) while preserving approximately 50% of the *p*CA esters remaining on the lignins (**Fig. 4B**). A third and final trend is that, as identified in our prior work,⁴ *p*CA hydrolysis during dilute acid pretreatment approximates first-order reaction kinetics with respect to *p*CA content and temperature and time rather than pH (or CSF) are the strongest predictors of *p*CA hydrolysis (**Fig. 4C**). Fitting trendlines to the 150 °C and the 200 °C data it is possible to estimate rate constants as 0.148 hr⁻¹ and 1.632 hr⁻¹, respectively. However, developing correlations using only two data points for the 200 °C condition is not expected to provide an accurate estimate, but is useful for providing an

order of magnitude assessment. Combining these results with prior work on corn stover *p*CA hydrolysis at 180 °C is able to yield order of magnitude estimates for activation energy (E_a) and pre-exponential constant (A) for this reaction system (**Fig. 4C**).

The *p*-hydroxyphenyl aromatic hydroxyl content of a lignin is a key hypothesized component of the utility of these lignins as a phenol replacement in PF resins. However, from the literature it's not clear how much of this can be attributable to *p*CA esters within the lignin. For the current work, this relationship was explored for the samples for which data is available using *p*CA determined by base-catalyzed hydrolysis (saponification) followed by HPLC quantification and the *p*-hydroxyphenyl contents determined by ^{31}P NMR. A comparison of these results shows a strong correlation between these two measured quantities (**Fig. 5**). Specifically, this shows that the *p*CA may contribute to more than 40% of the *p*-hydroxyphenyl content of this lignin for the highest *p*CA-containing samples, while the *p*CA-poor samples still have a significant level (~55 mmol/g lignin) of *p*-hydroxyphenyl functional groups.

Scale Up of Pretreatment, Hydrolysis, and Lignin Extraction and Recovery

Scale up of lignin production was next performed in order to generate sufficient quantities of lignin for subsequent synthesis of lignin-based resins and testing of lignin-based adhesives. Based on the conditions previously screened for biomass pretreatment and lignin extraction/recovery, an “ideal” set of conditions can be identified to meet economic objectives including high yields for both lignocellulosic sugar-derived biofuels and lignin-based co-products as well as lignin properties suitable for these co-products. Specifically, these criteria could be envisioned as comprising (1) high sugar hydrolysis yields (**Fig. 1**), (2) high lignin recovery yields (**Fig. 2**), and (3) high *p*-hydroxyphenyl or *p*CA contents in the recovered lignins (**Figs. 3B and 4A**). For the first criterion (hydrolysis), pretreatments at CSF 1.0, 2.0, and 4.0 were capable of glucose hydrolysis yields greater than 60% (**Fig. 1**). For the second selection criterion (lignin recovery yields), both NaOH and formic acid extraction could give recovery yields greater than 60% at CSF 1.0, 1.5, and 2.0 (**Fig. 2**). Finally, for the third selection criteria (*p*-hydroxyphenyl or *p*CA content), it is clear (**Fig. 4B**) that only the formic acid-extracted lignin from CSF 1.0, 1.5, and 2.0 retain much of the original *p*CA (20-40% of the original) compared to the NaOH-extracted lignin (less than 8%). However, both NaOH- and formic acid-extracted lignins retain a large portion of the phenolic *p*-hydroxyphenyl content (**Fig. 5**) that may be

hypothesized to be present in lignin structures other than *p*CA esters. The formic acid-extracted lignins from CSF 1.0, 1.5, and 2.0 all had comparable levels of *p*-hydroxyphenyl contents (0.86-0.88 mmol/g lignin, **Figs. 3B and 5**). Based on these selection criteria, the CSF 2.0 pretreatment was chosen for scale-up of lignin generation for subsequent PF resin and adhesive synthesis, characterization, and testing. Both formic acid and NaOH extraction methods were used after pretreatment for comparative results. While the formic acid-extracted lignin is expected to represent the “ideal” lignin, the NaOH-extracted lignin was chosen to test the hypothesis that the adhesive mechanical performance will be compromised due to the lower *p*-hydroxyphenyl and *p*CA contents in this lignin relative to the formic acid-extracted lignin.

The scale-up of pretreatment, enzymatic hydrolysis, and extraction was completed and additional characterization was performed to validate that processes performance at larger scale was comparable to smaller lab scale, and that lignin properties were comparable. The glucose hydrolysis yields for the scaled-up experiments ($55.4\% \pm 1.5\%$) were within the expected range of the small-scale results (**Fig. 1A**). Additionally, analysis of recovered lignins for functional groups by ^{31}P NMR, molar mass by GPC, xylan content, and alkaline saponification for *p*CA contents all showed close correspondence (generally within 5%) between the large-scale and small-scale lignins (data not shown). One exception was for the ash content (**Supplemental Table S1**), where the formic acid-extracted lignin had a lower ash content at the large scale (0.46% vs. 1.61%) and the NaOH-extracted lignin had a higher ash content at the large scale (0.97% vs. 0.26%). Potential reasons for these discrepancies may be due to slight differences in the lignin washing procedure between scales. One final interesting finding from the lignin characterization results was relatively high N content of the recovered lignins, which were 1.13% for formic acid-extracted lignin and 1.53% for NaOH-extracted lignin (**Supplemental Table S2**). Assuming that the majority of this N is structural protein in the corn stover, this yields a significant amount of protein in each of these lignins: 5.6% protein for formic acid-extracted lignin and 7.6% protein for NaOH-extracted lignin assuming a nitrogen-to-protein conversion factor of 4.95 for corn stover.⁵⁴ Many non-lignin compounds (*e.g.*, cutin/suberin, fat/waxes, and protein) can contribute to the Klason lignin measurement⁵⁵ and it is likely that a significant fraction of these may propagate through the pretreatment, extraction, and recovery steps to end up enriched in the final recovered “lignin”.

PF Resin and Adhesive Synthesis, Characterization, and Testing

Lignin-formaldehyde resins were next synthesized using the formic acid-extracted lignin and the NaOH-extracted lignin followed by characterization. These resins exhibited significant differences in many of the characterized properties (**Table 2**) due to differences in lignin chemical composition (including impurities such as ash, xylan, and protein), and structure (molar mass and functional groups). Due to the nature of the lignin extraction process, the resin formulated with the NaOH-extracted lignin had a higher pH and alkalinity than the resin formulated with formic acid-extracted lignin. Because of its higher phenolic hydroxyl content (**Fig. 3A**) more formaldehyde was used for the formulation of resin made with the formic acid-extracted lignin, which resulted in this resin exhibiting a lower solid content than the resin utilizing the NaOH-extracted lignin (**Table 2**). Resin solid content and viscosity are important properties for the subsequent resin adhesive performance. If adhesives exhibit low viscosity and solid content during the curing stage most of the adhesive will penetrate between layers in the wood during pressing leading to severely impaired adhesive performance.⁵⁶ The formic acid-extracted lignin contained more reactive sites for reaction with formaldehyde than NaOH-extracted lignin primarily due to higher *pCA* content (**Fig. 4**). These additional reactive sites made the formic acid lignin more reactive with formaldehyde leading to a lower free formaldehyde content in the resulting resin (**Table 2**). High free formaldehyde content in PF resin has been shown to be correlated to increased formaldehyde emissions during the manufacture of engineered wood products.⁵⁷ This release of formaldehyde both during manufacture and from the finished product can lead to adverse health effects in both manufacturing workers and end product users.⁵⁸ These results show that feedstock selection and lignin processing history can be used to tailor the reactivity of lignin with formaldehyde in order to create resins with low levels of free formaldehyde. The formic acid-extracted lignin also exhibited a faster gelation time than NaOH-extracted lignin (**Table 2**). This is likely also due to the increased number of available reactive sites in the formic acid-extracted lignin leading to increased crosslinking in the lignin-formaldehyde matrix causing accelerated polymer gelation.⁵⁹ Gelation time is an important metric that can influence resin curing parameters such as press time.⁶⁰ This relationship between lignin reactivity towards formaldehyde and resin gelation time shows how lignin processing conditions can be used to create lignin-formaldehyde resins with optimized curing parameters.

After characterizing these original resins, adhesives were made according to both the formulation applied in our previous work,¹⁴⁻¹⁵ and using a modified formulation (**Supplemental Table S2**) in an attempt to improve the physical properties of the lignin-formaldehyde resins for improved adhesive performance. This modified formulation resulted in improved viscosity and solid content when compared to the original resins (**Table 3**). Finally, the performance of these adhesives on wood was quantified using a lap shear strength test and was compared with results from previously published lignin-formaldehyde (or a PF control) adhesives from our group (**Fig. 6**). These results include the adhesive performance using the “original formulation” for the resin for an industrial lignin cake derived from dilute acid pretreatment of corn stover using NaOH extraction as well as the conventional phenol-formaldehyde control.¹⁵ Several key results can be observed. Namely, the wood adhesive shear strength from formic acid-extracted lignin made with the conventional adhesive formulation from the present work was statistically indistinguishable from prior work as well as the PF control. Notably, many other lignin sources were unable to achieve comparable performance to the control adhesive.¹⁵ Another key finding is that this performance was obtained with a relatively high-ash lignin (0.46-0.96% ash), indicating that these levels of impurities (*e.g.*, ash, protein, xylan) can be tolerated. A final observation is that the lignin-formaldehyde resin formulated using the formic acid-extracted lignin and the modified adhesive preparation exhibited a lap shear strength significantly higher (based on a paired t-test at a 95% confidence interval) than the conventional PF resin as well as the NaOH-extracted lignin using the same optimized adhesive formulation (**Fig. 6**). Overall, these are significant and validates the strategy for optimizing lignin properties for this target application while simultaneously achieving high sugar yields and high lignin recovery yields.

CONCLUSIONS

The feedstock, pretreatment, and method of lignin extraction/purification all influence the properties of lignins extracted from a biorefinery process. Structural and chemical properties of a specific lignin, such as phenolic hydroxyl content and molecular weight effect the ability of a lignin to be incorporated into PF resins for wood adhesives and the subsequent material properties of the formulated adhesives. Our previous studies have shown that lignin generated from NaOH extraction of dilute acid pretreated corn stover can be used to formulate PF resins with 100% of phenol replaced, which exhibit material properties that are comparable to a

conventional PF adhesive.¹⁵ We have previously hypothesized that the improved lignin incorporation in this study is largely due to high amounts of *p*CA present within grass lignins, presenting additional phenolic hydroxyls for reaction with formaldehyde. In a previous study, we identified dilute acid pretreatment conditions that result in high hydrolysis yields while retaining high *p*CA levels within the biomass.⁴ In this study, we expand on those findings by using corn stover to investigate a wide range of dilute acid pretreatment conditions and lignin extraction using NaOH and formic acid. A number of key findings can be highlighted. First, it was shown that relatively mild pretreatment conditions could result in lignin extraction yields of 60-70% using both lignin extraction approaches while simultaneously achieving glucose hydrolysis yields >60%. These yields are encouraging relative to prior literature. The harshest pretreatment condition resulted in low lignin extraction and recovery yields (<20%) as a likely consequence of lignin cross-linking during pretreatment. The main impurities in the recovered lignins were xylan (1.5-13%), ash (<2%), and potentially protein (up to 7.6% based on N content). The *p*CA content following pretreatment was demonstrated to be a strong function of pretreatment time and kinetic parameters were estimated for the acid-catalyzed hydrolysis of *p*CA esters during pretreatment. Formic acid lignin extraction was shown to preserve significantly more *p*CA than NaOH extraction. The “*p*-hydroxyphenyl” or “H” lignin content was shown to be positively correlated to *p*CA content, demonstrating that *p*CA can comprise a significant fraction of the “H” lignin in biomass. Finally, a combination of pretreatment and lignin extraction were scaled up to generate lignin-formaldehyde resins and wood adhesives and to test physical and material properties. It was shown that both formic acid- and NaOH-extracted lignins could yield wood adhesives with dry shear-strength properties comparable to a conventional industrial phenol-formaldehyde (PF) adhesive formulation and that an optimized adhesive formulation could yield an adhesive with statistically higher shear strength than the control. Overall, this work demonstrates that for a target application such as lignin-based wood adhesives, both pretreatment and lignin extraction conditions can be selected to yield lignins with optimized properties.

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SUPPORTING INFORMATION

Supporting information is available for this work. This includes the apparatus for solvent extraction of lignin (**Fig. S1**), the composition of the raw and pretreated corn stover (**Fig. S2**), the elemental composition of the lignins generated during scale-up (**Table S1**), and the composition of the adhesive components used in this work (**Table S2**).

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TABLES

Time	Initial pH	Temperature	CSF
6.0 h	3.0	150 °C	1.0
1.8 h	2.0	150 °C	1.5
1.0 h	1.25	150 °C	2.0
2.0 h	1.0	200 °C	4.0

Table 1: Conditions used for aqueous dilute sulfuric acid pretreatment screening at the 100 mL scale.

Lignin Source		pH	Solid Content (%)	Free Formaldehyde Content (%)	Viscosity (mPa·s)	Alkalinity (%)	Gelation Time (min)
Pretreatment	Extraction						
CSF 2.0	Formic Acid	10.7 (0.1)	19.3 (0.1)	0.37 (0.02)	8.3	2.4 (0.1)	18.3 (0.2)
CSF 2.0	NaOH	11.3 (0.1)	21.7 (0.1)	0.56 (0.04)	8.4	2.9 (0.1)	20.4 (0.3)

Table 2: Properties of lignin-formaldehyde resins.

Adhesive Formulation	Lignin Source	pH	Solid Content%	Viscosity (mPa·s)
Original Formulation	Formic Acid-Extracted	13.3 (0.1)	27.5 (0.1)	210
		12.5 (0.1)	29.5 (0.3)	420
Modified Formulation	NaOH-Extracted	13.12 (0.2)	30.3 (0.1)	626

Table 3: Properties of original and modified lignin-formaldehyde adhesives

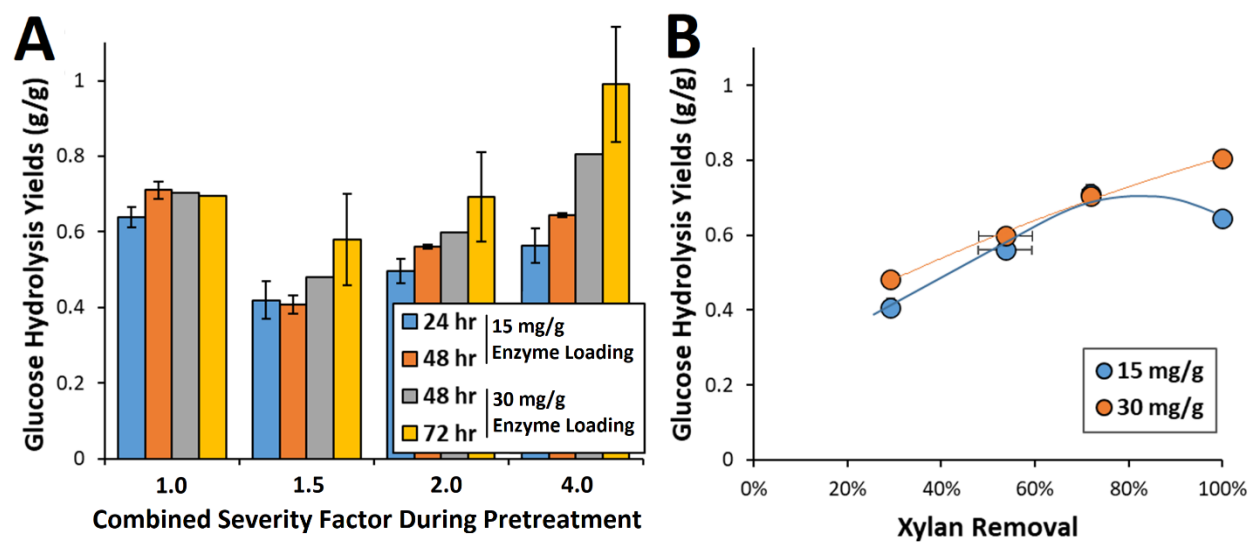


Figure 1: Impact of pretreatment conditions on (A) subsequent enzymatic hydrolysis yields and (B) relationship between hydrolysis yields and xylan removal for 48-hr hydrolysis times at two enzyme loadings.

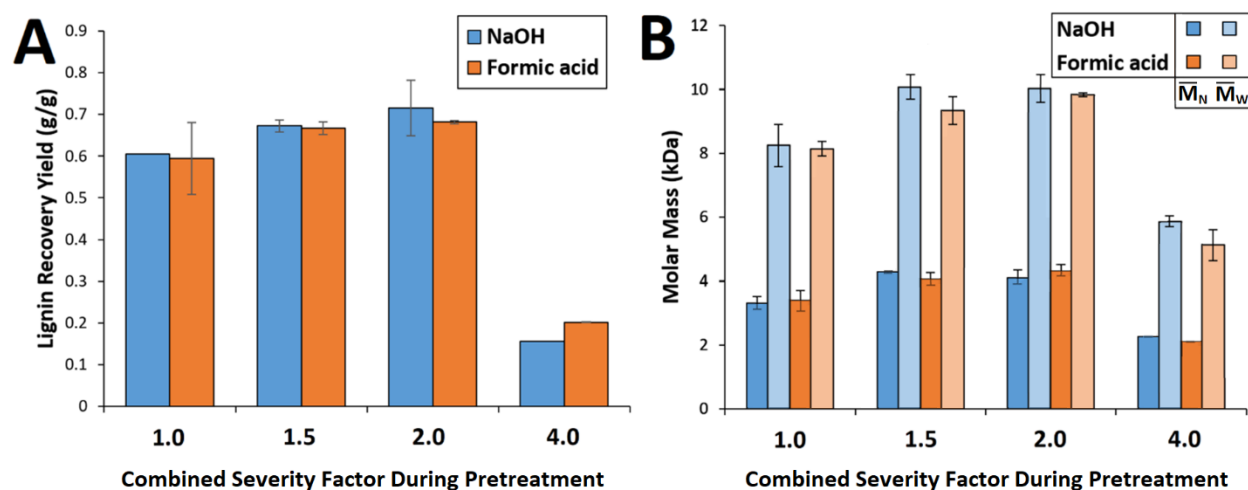


Figure 2: Impact of pretreatment conditions on (A) lignin extraction and recovery yields from the resulting corn stover lignin cake and (B) recovered lignin number average molar mass using either NaOH or formic acid lignin extraction.

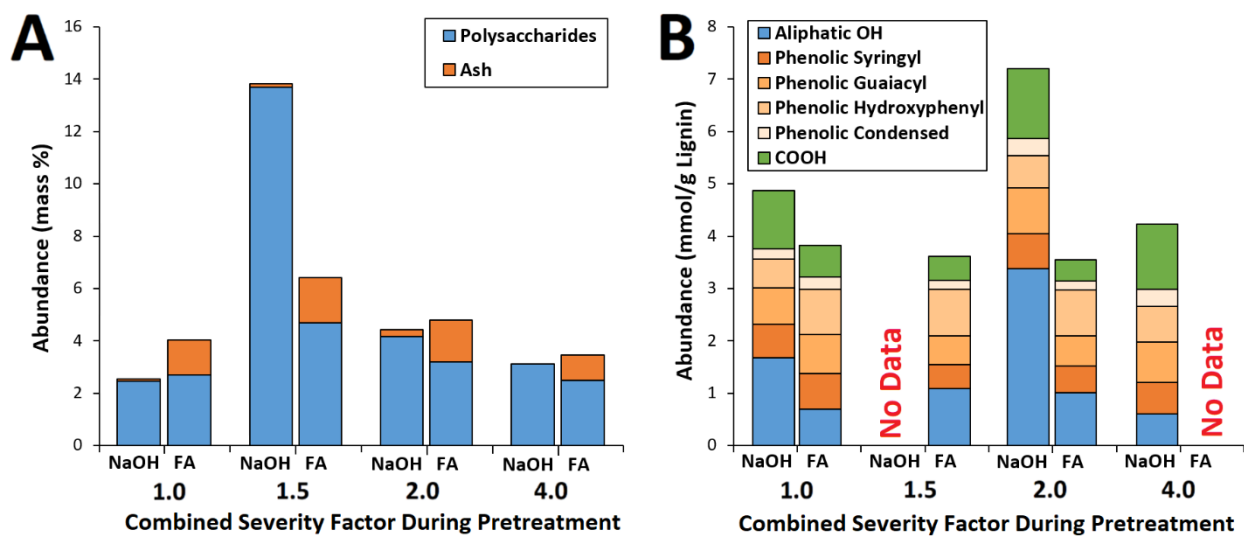


Figure 3: Chemical properties of diverse lignins recovered from corn stover lignin cakes using NaOH or formic acid (FA) extraction including (A) composition of non-Klason lignin components and (B) functional groups as determined by ^{31}P NMR.

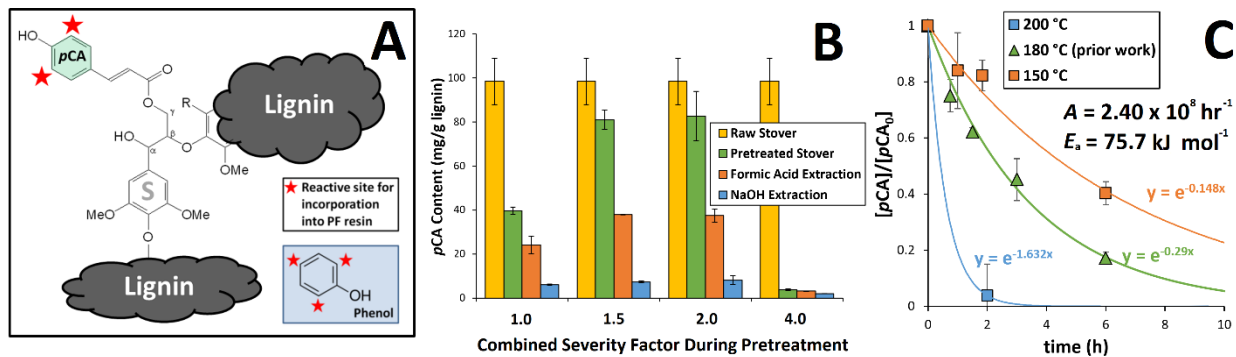


Figure 4: Esterified *p*CA in grass lignins (A) and the relationship between (B) pretreatment and extraction conditions on *p*CA solubilization and (C) pretreatment temperature on *p*CA hydrolysis kinetics.

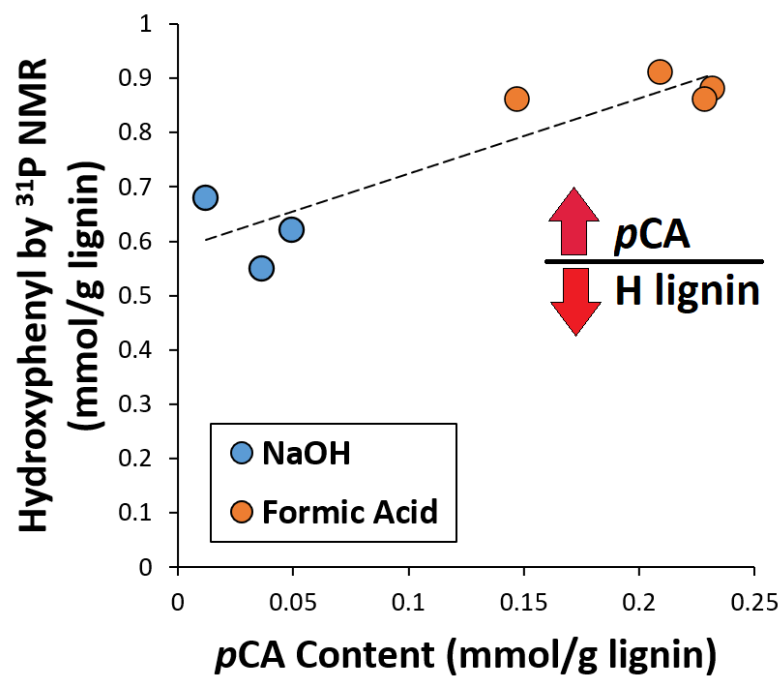


Figure 5: Relationship between $p\text{CA}$ content and ^{31}P NMR-determined p -hydroxyphenyl monomer.

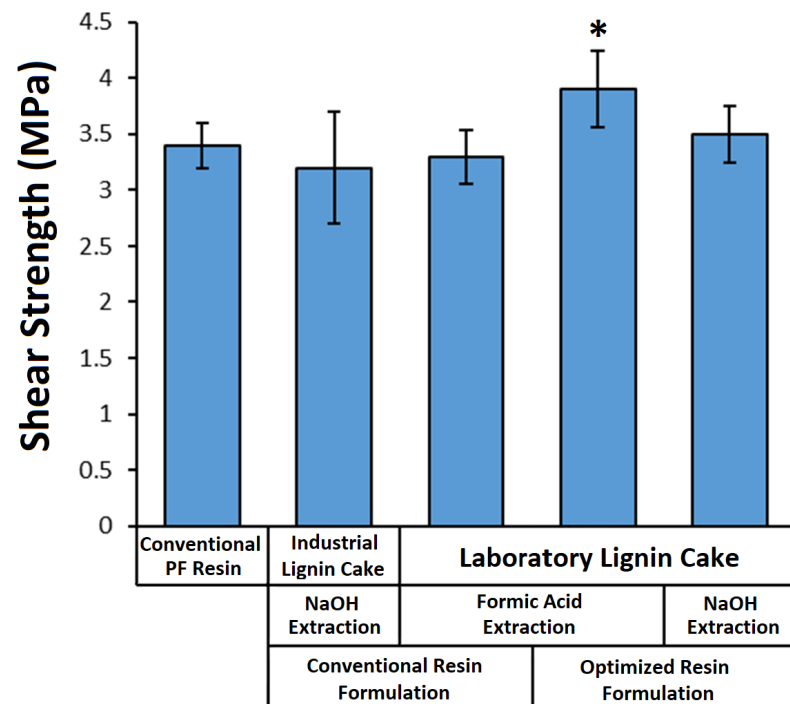


Figure 6: Lap shear strength of adhesives isolated from dilute acid-pretreated corn stover lignin cake with results from our prior work.¹⁵