Electronic Supplementary Information

Improved understanding of technical lignin functionalization through comprehensive structural characterization of fractionated pine kraft lignins modified by the Mannich reaction

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Dialysis as a method for purifying lignin amines

Dialysis was initially explored as a method for removing impurities that could be contributing to elemental analysis overestimation. The goal was to see if nitrogen contents of dialysed aminated lignins would better agree with NMR data. When using dialysis to purify whole aminated pine kraft lignins in preliminary experiments, product recovery yields averaged 52% by mass after accounting for addition of amine groups. The presence of amine groups directly contributed to the hydrophilic nature of lignin amines, further improving their diffusion across the regenerated cellulose membrane (**Figure S1)**.

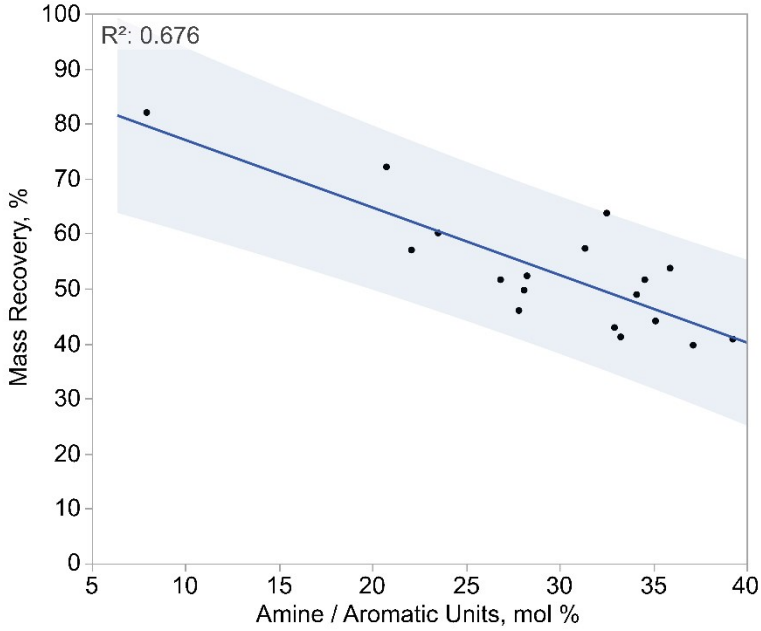

Figure S1. Percent of starting lignin recovered after dialysis as a function of extent of amination per elemental analysis. Recovery is inversely related to diffusion through dialysis membrane. The shaded area represents the 95% confidence interval.

It was thought that dialysis losses would be mitigated by starting with a lignin sample of sufficiently high average molecular weight. A non-modified HMW lignin sample was dialyzed for three days until no lignin was observed in the dialysate by UV-Vis. Four percent by weight of HMW lignin was removed during this pretreatment. After Mannich reaction of four samples with DMA at a pH of 5, two were immediately freeze-dried and two first underwent dialysis until neutral pH was achieved. Under both work-up methods, quantification by NMR gave similar results, with 26.3 mol% amine/aromatic unit using freeze-drying and 21.3 mol% using both dialysis and freeze-drying. One explanation for the apparent reduction in amine substitution following dialysis is that 39% of the product mass was removed through the process even having pretreated the HMW lignin to prevent this. The loss of product was not as severe as when starting with whole lignin, but the increased polarity of lignin molecules after modification was apparently effective at increasing diffusion across the hydrophilic dialysis membrane. Even with the loss in

mass, this test helps to confirm that the NMR analysis only quantifies lignin-amine bonds given the agreement between the two samples. Furthermore, elemental analysis overestimated amine substitution by four times, even after purifying with dialysis. So, the use of elemental analysis and dialysis once again skewed the data through loss of product when assuming all nitrogen was contained in attached amine groups.

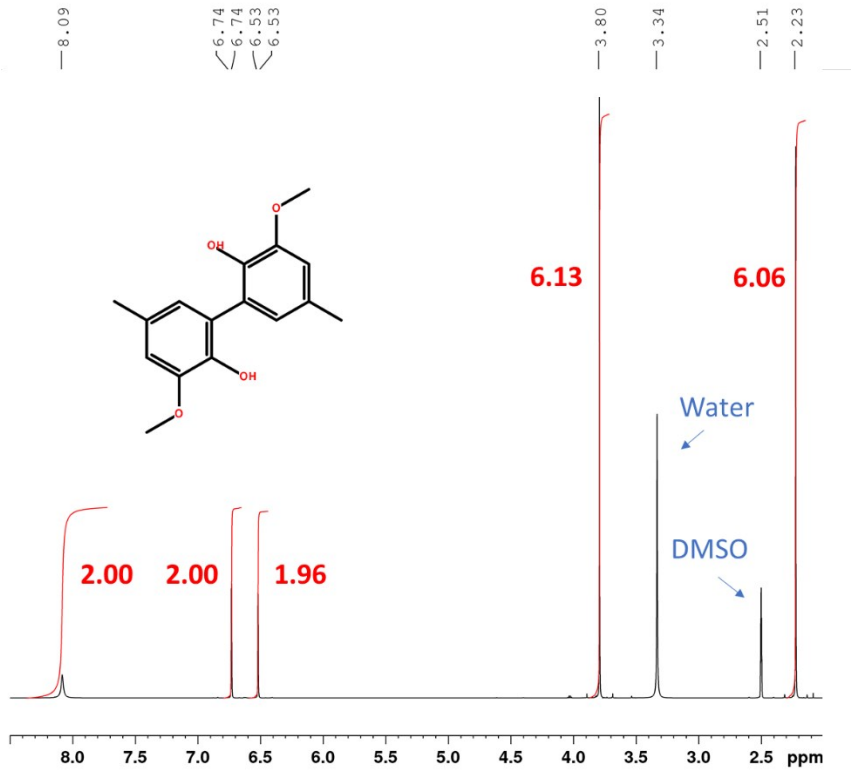

Figure S2. ^{1}H NMR spectrum for product isolated from methyl guaiacol oxidative coupling (3,3'-dimethoxy-5,5'-dimethylbiphenyl-2,2'-diol).

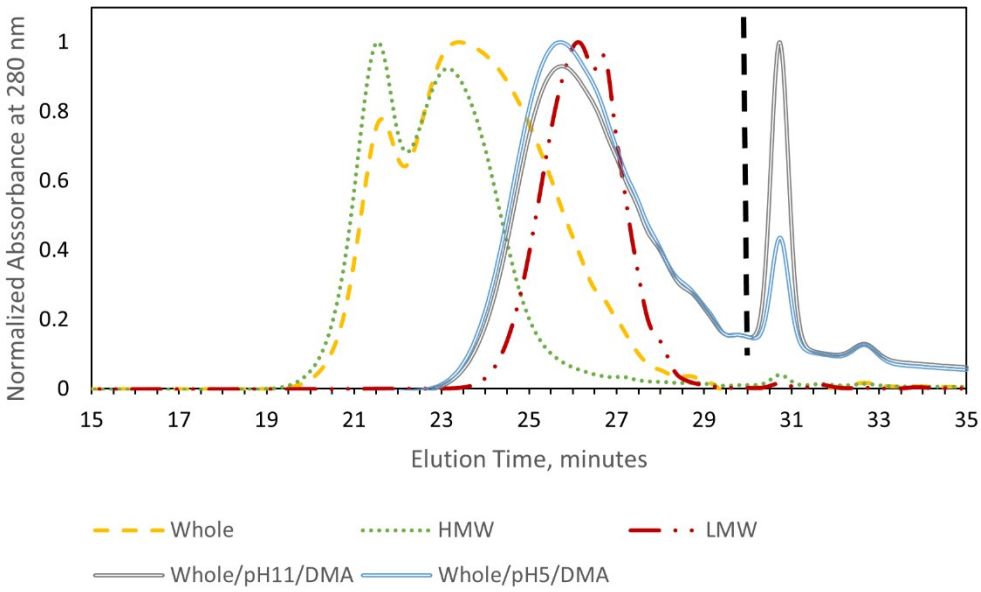

Figure S3. Gel permeation chromatogram of whole and fractionated BCL as well as two samples of aminated BCL. The aminated BCL was not entirely soluble in THF, hence the shifted molecular weight distribution and raising baseline, so it was not reported. Amination was expected to slightly increase average molecular weight from the introduction of amine groups, but it would be useful to check for inter-unit condensation as well.

Further discussion on the NMR spectroscopy of lignins

Quantification of ^{13}C NMR spectra requires that peak area be proportional to the number of carbon nuclei. This is not usually the case for broadband-decoupled spectra because the Nuclear Overhauser Effect (NOE) and T1 relaxation times are different for each carbon nucleus.[1] To overcome these, an inverse-gated decoupled pulse sequence can be applied that minimizes or eliminates NOE and a pulse delay greater than five times the T1 relaxation time can be used to ensure all nuclei return to equilibrium.[1–3] If nuclei do not return to equilibrium (meaning the Boltzmann distribution at equilibrium) prior to the next pulse, then the corresponding spectral peak height will be too low. In other words, if the delay between pulses is not long enough, then the quantification of nuclei with long T1 relaxation times will be underestimated. To account for this, T1 relaxation times were measured (Figure S4). Chromium(III) acetylacetonate was used as a common NMR relaxation agent to shorten T1 relaxation and times. The highest T1 relaxation times were for aromatic and methoxyl carbon nuclei of HMW lignins at 0.4 s (Figure S4). The pulse delay for all ^{13}C 1D experiments was set to 0.4 s * 5 = 2 s.

Sufficient signal-to-noise ratio (SNR) is needed to ensure an acceptable accuracy is achieved. Probe type, spectrometer field strength, sample concentration, number of scans, and processing of collected FIDs all contribute to the SNR. Quantitative ^{13}C analysis of softwood kraft lignin has been demonstrated using a concentration of 12% to 16% (plus 0.01 M chromium(III) acetylacetonate) with a 5 mm broadband inverse (BBI) probe, 1.2 s acquisition time, 1.7s delay, and 20k to 25k scans.[4] Other ^{13}C NMR analyses of various lignin types have used similar parameters with a slightly higher concentration of 20%.[2,5,6] In this study, 20% concentration was used with a 5 mm BBO probe (more sensitive to ^{13}C), 1.5 s acquisition time, 2 s delay, and 19k scans. ^{13}C spectra were thought to be of sufficient quality given that similar equipment, sample prep, and acquisition and processing parameters detailed in prior protocols were used. However, it has recently been suggested that an aromatic signal (163 ppm to 98 ppm) to noise (10 ppm to 0 ppm) ratio of 200 is ideal and is only achievable by doubling the sample concentration to 40%.[3] In the current study, aromatic region SNR averaged 25, much lower than 200. The authors have not seen SNRs reported before this, but it is assumed that they were close to 25 given the similarities in instrumentation and methodology. At least two recent lignin analytical studies have adopted a higher concentration (36% to 44%) for collecting quantitative ^{13}C NMR spectra (Balakshin 2016 Holz and Lancefield 2018 Chem Sci).[7,8] It is recommended that higher sample concentrations (200 mg per 500 μL) be used in the future. Derivatization and/or different solvents may need to be evaluated to dissolve aminated lignins at this higher concentration.

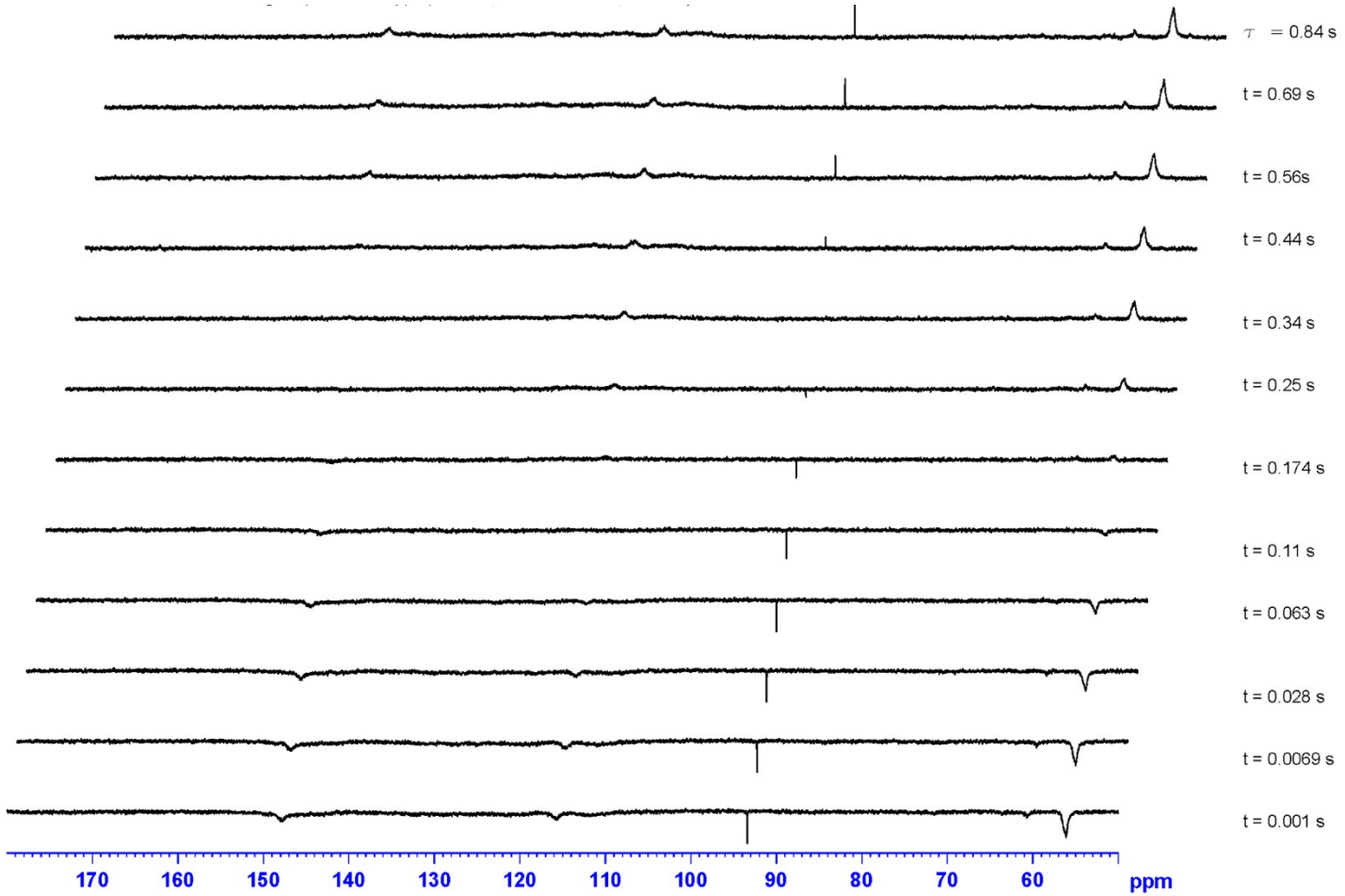

Figure S4. ^{13}C NMR spectra from an inversion-recovery experiment on HMW lignin in DMSO-d6 to determine T1 relaxation times of the aromatic and methoxy regions. 0.016M chromium(III) acetylacetonate was added to the sample to generate the shown spectra. Calculated T1 relaxation times were 0.4 s for both the aromatic (160 ppm to 100 ppm) and methoxy (55 ppm) regions and 0.5 s for trioxane. Without adding relaxation agent, T1 relaxation times were calculated to be 0.9 s for aromatic carbons and 2.96 s for the internal standard, trioxane.

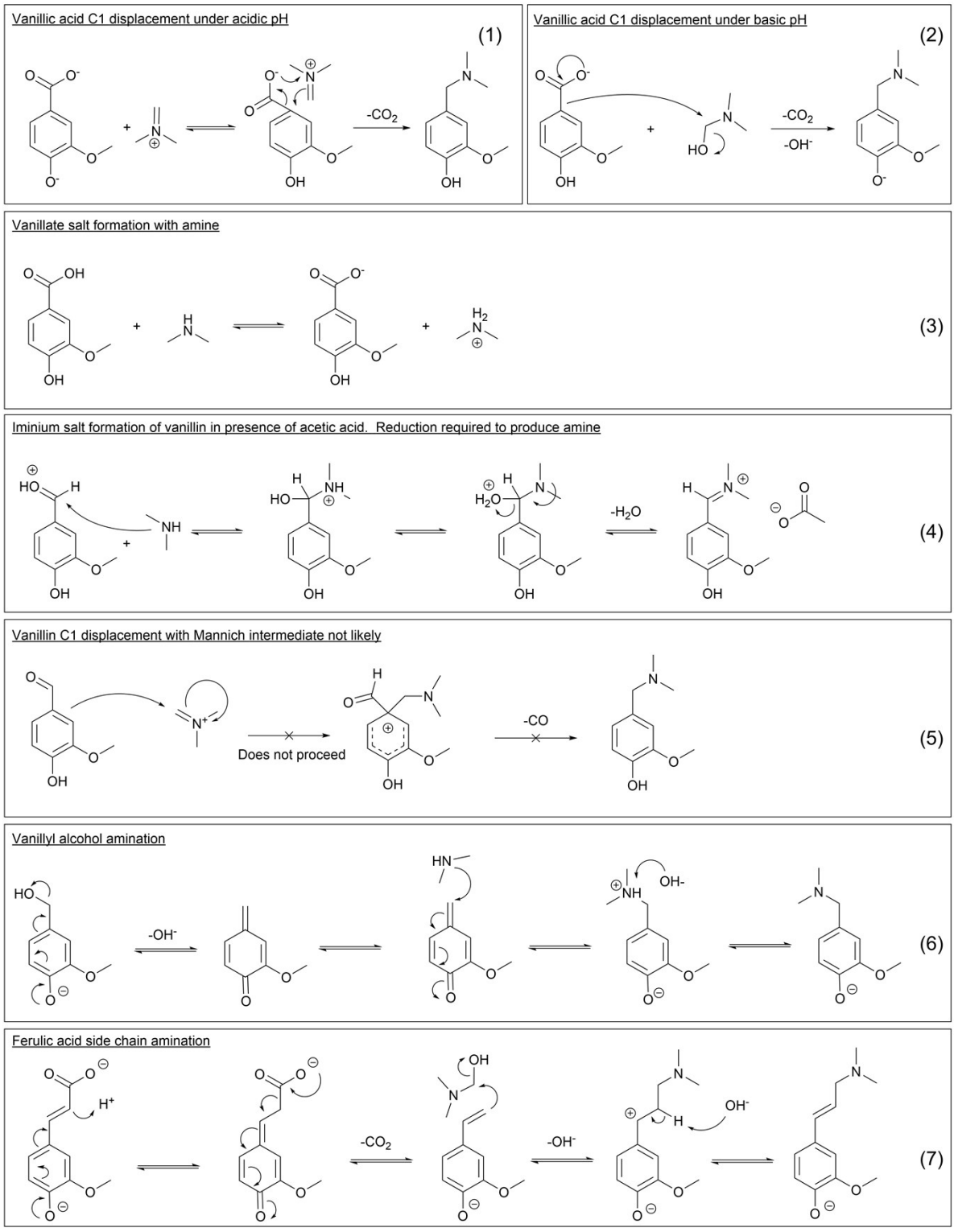

Figure S5. Proposed mechanisms for amination of lignin side chain. All products have been experimentally verified.[9–11]

Hydroxyl Content Analysis using ^{31}P NMR Spectroscopy

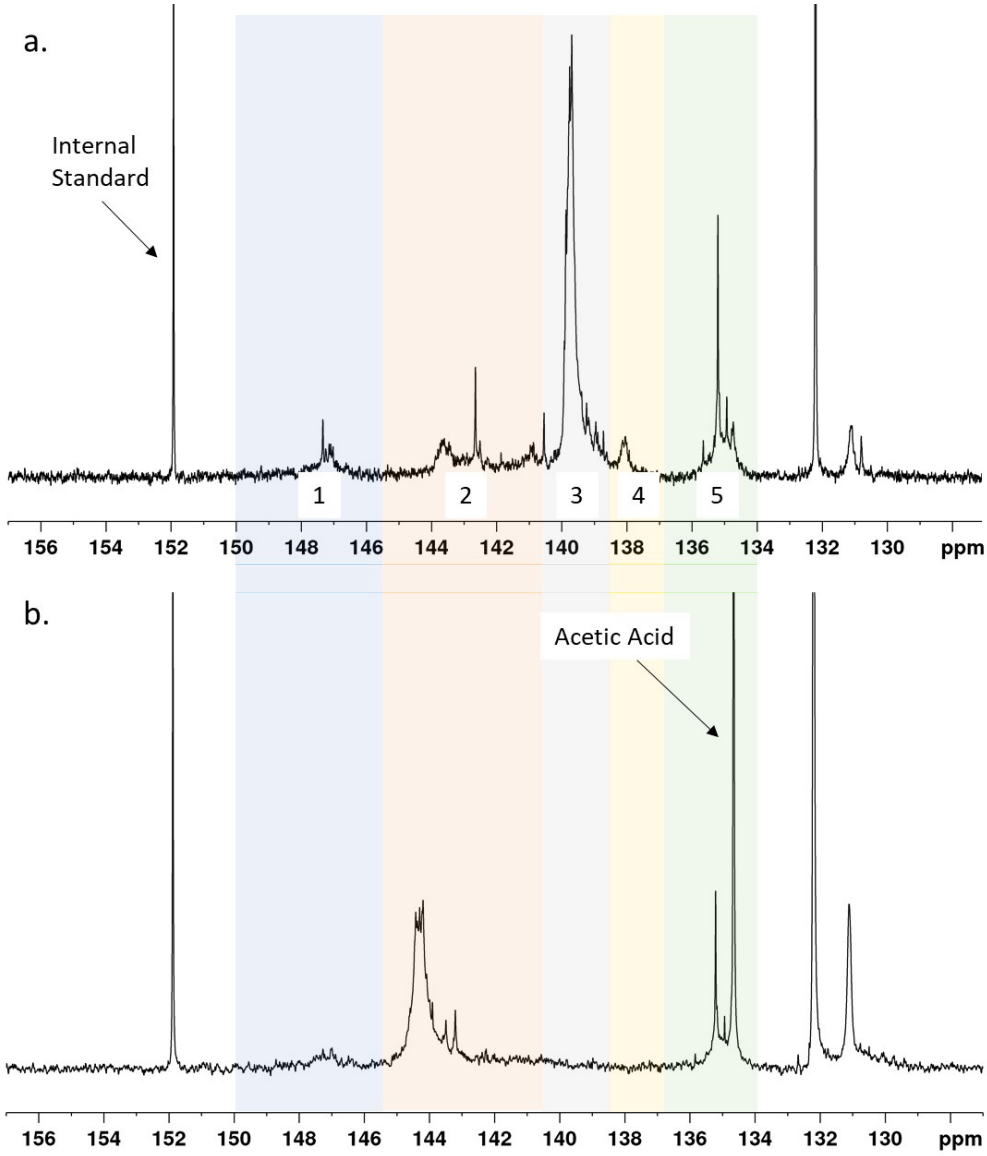

Figure S6. ^{31}P NMR spectra for a.) LMW lignin fraction and b.) LMW lignin fraction after Mannich reaction at pH 11 using dimethylamine. Regions are numbered as follows and assigned based on a previous report: 1) aliphatic OH, 2) *ortho* di-substituted phenolic OH, 3) *ortho* mono-substituted phenolic OH, 4) *ortho* non-substituted phenolic OH, and 5) carboxylic acid OH.[12]

Table S1. Original Data for Hydroxyl Group Characterization by ^{31}P NMR Spectroscopy for Lignin Fractions before and after Mannich Reaction.

Lignin sample[b]	mol / 100 mol aromatic units ± 95% CL[a]					
	Aliphatic OH	Phenolic OH				COOH[c]
		Di-substituted	Mono-substituted	Non-substituted	Total	
HMW	32	28	24	6.1	58	7.6
HMW /DMA/5	34 ± 1	48.8 ± 0.9	7.6 ± 0.4	2.5 ± 0.7	59 ± 1	ND
HMW /DEA/5	41 ± 2	49 ± 1	8.1 ± 0.3	2.7 ± 0.5	60 ± 2	3 ± 2
LMW	11	24	55	6.1	85	17
LMW/DMA/5	12 ± 2	57 ± 5	3.7 ± 0.7	1.5 ± 0.6	62 ± 6	11 ± 3
LMW/DMA/11	13 ± 3	54 ± 12	3.8 ± 0.5	2.0 ± 0.3	60 ± 12	9 ± 3

a. Where provided, the second value represents the 95% confidence limit calculated from an estimated standard deviation using student's t-distribution. Sample size = 3.
b. The sample designation is: MW fraction of lignin / amine species / pH of Mannich reaction.
c. Values for aminated samples calculated by subtracting the large, overlapping acetic acid peak.

For HMW samples, free phenolic OH abundance increased by up to 6% and LMW total phenolic OH content decreased by 5% to 37% according to original integrations of ^{31}P NMR spectra (**Figure S6**, **Table S1**). No reaction likely accounts for these differences, so experimental error was suspected to be the cause. Error may be introduced at several points throughout ^{31}P NMR analyses of lignins (sample prep, incomplete phosphitylation, poor spectral sensitivity or resolution, integration, or calculation errors). Troubleshooting was performed to rule out possibilities. It was determined that sample weight measurements and calculations were the most likely sources of error. Specifically, as these analyses involved lignin modifications, the exact mass gain must be known to correctly report abundances per unit original lignin. It is essential to know how much of the final product's mass is attributed to original lignin. Total free phenolic hydroxyl content changed relatively more for LMW as compared to HMW samples per ^{31}P NMR. If the actual phenolic hydroxyl content did not change, an underestimation could be caused by a lower-than-actual product weight caused by the loss of volatile lignin molecules during freeze drying. This explains why LMW samples had an apparent decrease phenolic content, but not HMW samples.

Other explanations were also considered. The lignin amine results were normalized so the total aliphatic and phenolic hydroxyl contents (per unit original lignin) matched corresponding starting materials (normalized values shown in **Table 5**). This was a valid normalization for a few reasons. Aliphatic hydroxyl group content should not change in pink kraft lignin at the Mannich reaction conditions. Only vanillyl alcohol has been shown to undergo C1 displacement and no vanillyl alcohol was present according to HSQC spectra. Aliphatic hydroxyl content increased according to the original data. This fact supports the assumption that the amination conditions are too mild

to cause elimination of γ-hydroxyl group as formaldehyde discussed in the previous section above. Loss of or condensation of phenolic hydroxyl groups is very unlikely. Carboxylic acid groups were shown to decrease by HSQC spectra and by model compound studies, so those groups were not included in the data normalization.

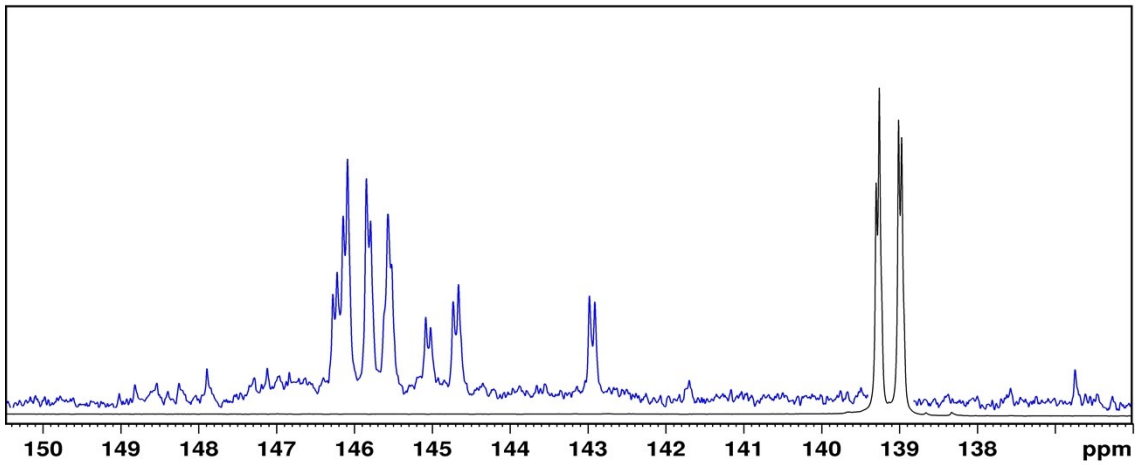

Figure S7. ^{31}P NMR spectra for 4-methyl catechol (black) and products after Mannich reaction (blue). Hydroxyl groups of the products are all shifted downfield into the di-substituted phenolic hydroxyl region (145.5 ppm to 140.3 ppm) and aliphatic hydroxyl region (150 ppm to 145.5 ppm).

Table S2. Comparison of results derived from different techniques across different studies.

Reference	Lignin Type	pH	Amine	Dialysis	Product Recovery, wt%	Analytical Method	Amine/Aromatic Units, mol%	Comments
Results from Du 2014[13]	Softwood Kraft Lignin from Lignoboost	5	DMA	Yes, 1 kDa	N/A	Total Nitrogen	36.0	Converted to common units assuming aromatic unit molar mass of 180 g/mol
					N/A	^{13}C NMR	28.0	using Ar-CH2-N structure
Results from Wang 2017[9]	Softwood Kraft Lignin from Lignoboost	5	DMA	No	N/A	Total Nitrogen + ^{31}P NMR	80.4	Converted to common units assuming aromatic unit molar mass of 180 g/mol
		7			N/A		26.2	
		9			N/A		19.5	
Results from Wang 2018[14]	Alkali Lignin, depolymerizd to LMW. Assumed to be non-woody due to H-unit content and hydroxycinnamic acids	5	DMA	Yes, 500 Da	75	CHN Analysis	65.0	10 C/mol Ar unit given 1 mol methoxyl group/mol Ar unit
						2D HSQC	62.0	using Ar-H, note that 2D HSQC is not a quantitative technique so while this value matches CHN, accuracy is not assured
						^{13}C NMR	28.7	using Ar-CH2-N structure
This study	Pine Kraft Lignin from Lignoboost, HMW fraction	5	DMA	No	61, corrected for amine addition	CHN Analysis	78.2	
						2D HSQC + 13C NMR	73.2	using Ar-H
						2D HSQC + 13C NMR	21.3	using Ar-CH2-N structure
	Pine Kraft Lignin from Lignoboost, HMW fraction	5	DMA	Yes, 1 kDa	106, corrected for amine addition	CHN Analysis	101.7	
						2D HSQC + 13C NMR	62.7	using Ar-H
						2D HSQC + 13C NMR	26.3	using Ar-CH2-N structure

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