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Toward an Understanding of Inhomogeneities in Structure of Lignin in Green Solvents Biorefinery. Part 1: Fractionation and Characterization of Lignin

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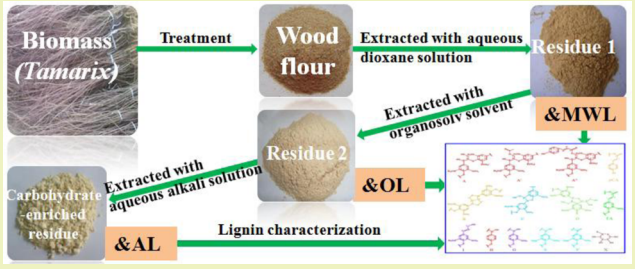
Supporting Information

ABSTRACT: To investigate the inhomogeneity of the lignin, milled wood lignin (MWL), organosolv lignin (OL), and alkaline lignin (AL) were sequentially fractionated from *Tamarix* spp. with dioxane, alkaline organosolv, and alkaline solutions, respectively. The carbohydrates associated with three lignins were comparatively analyzed, and the molecular weight and polydispersity of lignins were determined by gel permeation chromatography (GPC). Nuclear magnetic resonance (NMR) spectroscopy was used to measure the contents of the substructures of lignins. The results indicated that the alkaline organosolv extraction released a higher yield of lignin (17.7%, based on lignin in the raw material) than dioxane and alkaline solution extractions. Small amounts of carbohydrates (0.79%, w/w) were detected in the OL fraction, suggesting a significant cleavage of α-ether bonds between lignin and carbohydrates in the alkaline organosolv fractionation process. GPC results revealed that the molecular weights of lignin decreased in the order $M_{w\text{-}OL} > M_{w\text{-}MWL} > M_{w\text{-}AL}$. All lignins were characterized by predominance of β-O-4′ ether linkages (68.4−79.0%). A relatively low content of β-β′ (17.3%) resinol substructure was detected in MWL compared with that in OL (22.3%) and AL (21.0%) fractions. In addition, the component units of lignin were also investigated. HSQC spectra indicated a lower **S**/**G** (2.52) ratio of AL compared with MWL (**S**/**G**, 2.80) and OL (**S**/**G**, 3.85).



KEYWORDS: *Tamarix spp., Lignin, Biorefinery, Fractionation, Structure*

INTRODUCTION

Currently, lignocellulosic biomass is a very interesting biobased feedstock, because it is highly abundant and does not compete with food and feed. In a whole-crop biorefinery concept of biomass, the process is initiated by mechanical separation of biomass into different particle sizes, and then the raw materials are treated successively. After that, high-value chemicals and main components present in the biomass could be extracted by different solvents, such as high-value nutraceuticals, food-related products, and fragrances that could provide health and medical benefits.[1] Once the valuable chemicals are extracted, the biorefinery will focus on processing plant polysaccharides (such as cellulose and hemicelluloses) and lignin for bioderived materials and fuels.[2] Therefore, innovative separation methods are required to obtain the main components from the biomass.

Tamarix spp. (Tamaricaceae) is one of the most promising renewable plants with high yield in the world, especially in the northwest of China and Inner Mongolia.[3] It is a perennial shrub species with high medicinal value and wide applications such as materials, fuels, and chemicals.[3,4] Furthermore, it has exhibited a huge potential in papermaking and chemical production, and a relatively high content of lignin as well.[5−7]

To use the biomass, lignin appears to limit the hydrolysis of cellulose and hemicelluloses, the remove of lignin to a large extent from the feedstocks is a key step in biorefinery processing.[8] Understanding the isolation process and structural features of lignin from lignocellulosic biomass is essential. Lignin is derived from dehydrogenative polymerization of cinnamyl type lignin precursors.[8−10] Then, different C−C and C−O−C ether linkages, such as β-O-4′ and β-β′ etc. are formed by different lignin precursors. In addition, a relatively large number of other phenolic compounds have been reported to act as lignin precursors.[8,11] However, in transgenic plants, it is considered that these phenolic compounds provide a significant contribution to lignin formation with modified monolignol biosynthesis.[10−13] From the potential utilization point of view, the acquired knowledge of the lignin structure is one of the primary factors to understand the basic reaction mechanisms. However, the challenge remains in the isolation methods of lignin, because it is difficult to obtain highly representative and totally unaltered native lignin.[14] Milled

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wood lignin (MWL) represents a source of native lignin, but the yield of MWL varies by different pretreatment techniques.[15] Organosolv lignins were evaluated as antioxidants by different content of functional groups. It was found that low ethanol concentration led to more phenolic hydroxyl groups (ArOH), and new ArOH was formed during the organosolv delignification process.[16] Organosolv lignin was found to have a higher homogeneity than alkaline lignin.[17] The structure of lignin differ markedly owing to different extraction ability. There are several methods to recognize the interunit linkages of the complex lignin polymer for accurate structural analysis. Among them, advanced $^{13}C-^{1}H$ correlation two-dimensional NMR spectroscopic technique is a useful tool in the structural characterization of the whole lignin macromolecule, providing resolution of overlapping signals with higher probability in correct assignment of the signals. It can also be used for semiquantitative analysis,[18] and has enabled the discovery of dibenzodioxocins and spirodienone as two new lignin substructures.[19,20] Moreover, a new method of derivatization followed by reductive cleavage (DFRC) has been developed in elucidating the structure of lignin.[21] This method could selectively degrade β-aryl ether structures in milder depolymerization conditions with a more simplified procedure, resulting in a simple mixture of primary monomers.[21] Although the monomer yields of lignin is low than that produced by thioacidolysis, it is a flexible method owing to its simple steps, and can be used on whole wood sample.[22]

In this study, lignins in different form were successively extracted by different mild solvents from *Tamarix* spp. At each stage, lignins were separated, purified, and potentially recovered as high-value-added chemicals.[23] The resulted porous and amorphous lignocellulosic residue in the last step can be further processed, as a feedstock with enhanced absorption of enzyme for produce fuel and other useful chemicals. Therefore, it was believed that the concept of this successive extraction method in whole crop utilization makes sense in biorefinery. The structural features and physical–chemical properties of the three lignin fractions were thoroughly investigated by carbohydrate analysis, molecular weights, and the basic substructures, aiming to understand the structural features of lignin and assist in the development of lignin and biomass utilization.

MATERIALS AND METHODS

Raw Material. *Tamarix* spp. was collected from Inner Mongolia, China. After drying, the chipped stems (1–3 cm) were ground and the particles between 40 and 60 mesh were collected and stored. To prepare the ball-milled powder, the wood powder was dewaxed and the wax-free sample was produced according to the route in Figure 1. The final obtained wood powder was milled for 9 h in a planetary ball mill (FritschGMBH, Idar-Oberstein, Germany) in 10 min-on 10 min-off cycle.

Isolation of MWL, OL, and AL. Successive extraction of biomass and lignin precipitation is shown in Figure 1. Ball-milled *Tamarix* powder was put in a dioxane–water (96:4, v/v) solution with a solid/liquor ratio of 1:20 (g/mL), protected from light with aluminum film to avoid the lignin degradation, and stirred at room temperature for 48 h. The supernatants obtained were filtered through a glass filter crucibles (porosity 3, pore size = 16–40 μm) and evaporated to about 10 mL under reduced pressure at 40 °C. After that, the resulting solution was precipitated in deionized water (about 30 mL). The pellet produced was washed with acidified deionized water (adjusted by acetic acid to pH 2.0) for 3 times (3 × 10 mL). Then, the pellet produced was freeze-dried and named as milled wood lignin (MWL). The dried residue in the last step was successively isolated with 60%

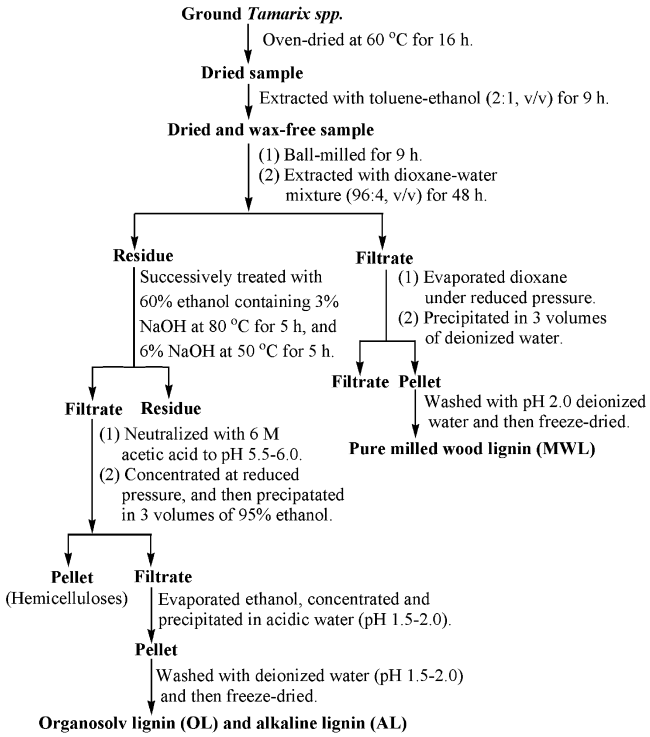

Figure 1. Scheme and conditions for fractionation of lignin from *Tamarix* spp.

EtOH containing 3% NaOH at 80 °C for 5 h at liquor to solid ratio of 1:20 (g/mL) with the heating rate of 10 °C/min. After removal of hemicelluloses, the resulting filtrates were concentrated to about 10 mL and precipitated in 30 mL acidic water. Finally, the lignin pellets produced was washed with acidified deionized water and then freeze-dried, named as organosolv lignin (OL). Alkaline lignin (AL) was successively isolated from the residue left in the OL stage by 6% sodium hydroxide (5 h, 50 °C), and the lignin fraction (AL) was obtained by the same method as described above. The experiment was done in duplicate, and values are means ± standard deviation.

Analytical Methods. The carbohydrates connected with the three lignin fractions were detected by hydrolysis with dilute H_2SO_4.[7] Lignin samples were hydrolyzed with 10% H_2SO_4 (105 °C, 2.5 h). After the mixed solution was filtered, the obtained filtrate was diluted (50-fold) and analyzed by high-performance anion-exchange chromatography (HPEAC). All the analysis was carried out at least in duplicate. Fourier transform infrared (FT-IR) spectra of the three lignins were performed on a Tensor 27 spectrometer in the range of 800–4000 cm^{-1} with 32 scans.[7] The molecular weights of the MWL, OL, and AL fractions were determined by gel permeation chromatography (GPC, Agilent 1200, USA) with a refraction index detector (RID).[24] Lignin sample was dissolved in tetrahydrofuran with a solid/liquid ratio of 2:1 mg/mL. After that, 10 μL of the filtered solution was analyzed by the HPLC system. Tetrahydrofuran (THF) was used as the eluent for GPC analysis. Elemental analysis was performed on a vario MACRO cube (Elementar Analysensysteme, Germany), and the oxygen content of the lignins was determined by subtracting a sum of these elements from 100%. The NMR experiment was carried out on a Bruker Avance 400 MHz instrument.[25] For ^{1}H NMR analysis, lignin was acetylated and 20 mg was dissolved in 0.5 mL of $CDCl_3$. HSQC cross-signals were analyzed and assigned by comparing with published literature.[25−27] The DFRC protocol was carried out by Lu and Ralph.[21] The degraded lignin fragments were simultaneously detected through GC-FID and GC–MS (Agilent 7890A/5975C) using the same 30 m × 0.25 mm (0.25 μm film thickness) HP-5MS column with He as carrier gas. The amounts of lignin monomers were determined according to the individual response factors (**H**, **G**, and **S** for 1.3, 1.2, and 1.6, respectively), which were from the pure compounds.

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Table 1. Neutral Sugar and Uronic Acid Content (%) (± 0.02%)[a] of the Lignin Fractions

	sugars							
lignin fraction[b]	rhamnose	arabinose	galactose	glucose	xylose	glc acid[c]	gal acid[d]	total
MWL	0.01	0.10	0.52	2.02	0.49	0.04	0.06	3.24
OL	0.03	0.34	0.06	0.22	0.07	0.06	0.01	0.79
AL	0.01	0.38	0.07	0.13	1.12	0.14	0.01	1.86

[a]Pooled standard error. [b]MWL, milled wood lignin; OL, organosolv lignin; AL, alkaline lignin. [c]Glucuronic acid. [d]Galacturonic acid.

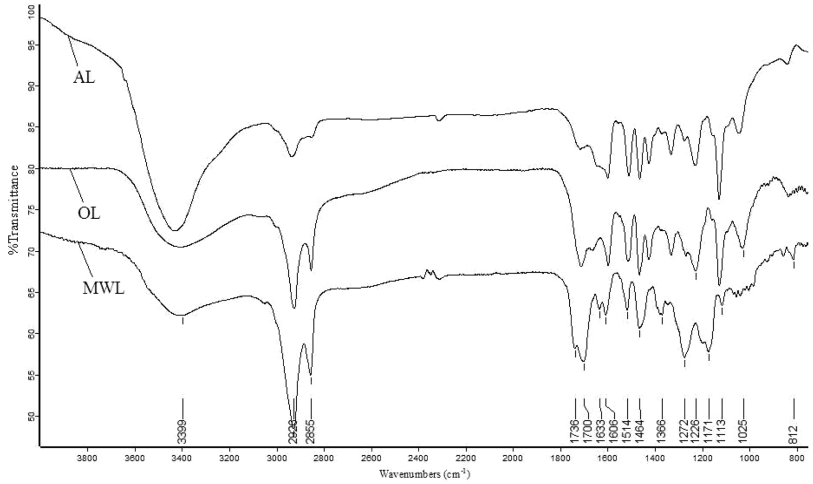

Figure 2. FT-IR spectra of the three isolated lignin fractions.

RESULTS AND DISCUSSION

Yield and Purity. In this work, the dioxane−water solution extraction yielded 6.7% (% original lignin, w/w) of MWL after purification. The relatively lower yield was due to the mild extraction and milling conditions. However, the structure of MWL is more close to the native form of lignin of the plant.[28] In general, a high yield of MWL could be obtained when increasing the milling time; however, the strength of mechanical milling resulted in damage of the lignin structure.[29] Therefore, for a more detailed structural characterization, the residue was first fractionated with 60% EtOH containing 3% NaOH and then extracted with 6% NaOH solution, respectively. It was found that high yields of lignins were obtained by alkaline ethanol and aqueous alkaline solvents extractions, amounting to 17.7% and 15.3% (based on the original lignin in the raw material), respectively. The low content of the carbohydrates associated with OL and a relatively high yield of OL have indicated that alkaline ethanol treatment selectively removes lignin from the middle lamella (ML). This finding was in accordance with the report by Hallac et al.,[30] who declared that ethanol organosolv pretreatment selectively removes lignin from ML. Results also revealed that the successive alkali extraction could further destroy the rigid network of the residue and increased the AL yield. Moreover, the relatively high yields of OL and AL preparations indicated that alkaline solvents extraction was effective in cleavage of linkages such as benzyl ethers and benzyl esters between lignin and carbohydrates, and make the cellulose fiber enzyme accessible as well.[31] These results are proved by HSQC analysis and FT-IR spectra. Very small signals corresponding to carbohydrates were observed in OL and AL HSQC NMR spectra, suggesting the cleavage of linkages between lignin and carbohydrates.

The carbohydrates content of the three lignin fractions was analyzed by sugar analysis (Table 1). The contents of glucose and xylose were found to be 0.22 and 0.07% of the dry OL lignin fractions, which was lower than that in the MWL fraction. This feature was found to be a consequence of cleavage of ether bond between lignin and carbohydrates in alkaline organosolv extraction. The OL contained a lower content of carbohydrates (0.79%) than the AL (1.86%) and MWL (3.24%) fractions. These findings suggested that the dioxane extraction under the conditions given favored the release of lignin−carbohydrate complex (LCC), whereas more ether bonds between lignin and carbohydrates were broken in the alkaline organosolv and aqueous alkaline solution extractions. Evidently, glucose (2.02%) and galactose (0.52%) were detected and found to be the major carbohydrates that were connected with the MWL fraction. Results showed that the OL fraction was characterized by a high content of arabinose (0.34%) and glucose (0.22%), and the xylose and arabinose contents of AL fraction were 1.12% of and 0.38%, respectively, suggesting that high amount of xylose was associated with the AL fraction. In addition, mannose and galacturonic acid have been detected at a trace-level of concentrations in the three lignin fractions.

FT-IR Spectroscopy. The spectra of these samples showed typical syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) lignin absorption bands (Figure 2): (i) the wide absorption peaks at 3399 and 2926 and 2855 cm^{-1} are arising from the O—H stretching vibrations in aromatic and aliphatic OH groups and C—H stretch in $-CH_3$ and $-CH_2$ groups, respectively, (ii) bands observed at 1606, 1514, and 1464 cm^{-1} are assigned to an aromatic ring stretching mode of phenyl-propane skeleton are present in all spectra, (iii) bands at 1330−1326 cm^{-1} and at 1272−1263 cm^{-1} represent the aromatic ring breathing of S and G lignin units, respectively.

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These results confirm that the lignin aromatic structure unchanged remarkable by the isolation procedures. Figure 2 revealed an increase in the hydroxyl groups (3399 cm^{-1}) of AL fraction than that of MWL and OL fractions. This was due to the effective cleavage of ester bond between lignin and carbohydrate. In addition, it can be deduced that less condensation reaction between lignin monomer occurred in the alkaline extraction step. In general, the band intensity at 2926 and 2855 cm^{-1} decreased from MWL to OL to AL, indicating a decrease amount of carbohydrate associated with lignin fractions. The results were in accordance with data supplied by purity analysis. In particular, a shoulder absorption band in the MWL spectra at 1736 cm^{-1} is assigned to the acetyl, *p*-coumaric, or uronic ester groups, which is unconjugated carbonyl (C═O) with the aromatic ring. The decrease of this carbonyl signal in the OL and AL spectra implied that both alkaline ethanol and aqueous NaOH extraction significantly cleaved these ester bonds. In addition, in the wavenumber ranging from 1700 to 800 cm^{-1}, the spectra showed similar peak forms but different absorption intensities. The absorption band at 1366 cm^{-1} is assigned to the characteristic stretching of aromatic ring in lignin. The band at 1328 cm^{-1} is an indicative of S type lignin absorption. The high intensity peaks at 1272 cm^{-1} in MWL was due to G type lignin ring breathing with C—O stretching, indicating more G unit was presented in MWL than OL and AL. Furthermore, the small sharp band at 1171 cm^{-1} is attributed to the CH_2 and C═O bonds of the carbohydrates,[32] whereas this peak was rather weak in OL and AL, indicating that less carbohydrates were associated with lignin. This observation was confirmed by the purity analysis. From the spectra of MWL and OL, a higher intensity of bands assigned to **S** than **G** units can be observed. The calculating of the corresponding IR peaks indicated a high value of **S**/**G** ratio for OL. Moreover, the absorption peak observed at 1113 cm^{-1} is attributed to the aromatic ring bending mode of **S** unit. Besides, bands around 920 (**G** unit) and 832 cm^{-1} (**S** unit) are assigned to aromatic out-of-plane C—H bending.[27]

Molecular Weight. The molecular weights decreased in order of $M_{w\text{-OL}} > M_{w\text{-MWL}} > M_{w\text{-AL}}$ (Table 2). The lowest molecular weight of AL (M_w = 1870 g/mol) indicated that the lignin macromolecule was substantially degraded under the alkaline conditions. As anticipated, the ball milling resulted in a relatively low molecular weight MWL (M_w = 2395 g/mol). The data showed that a short time of planetary ball milling was an ideal time to receive information on the native lignin. It can be deduced that high M_w of lignin fraction is easy to accessible when increasing the milling time. This phenomenon will lead to lignin association effects, resulting to the formation of high M_w fraction.[33] Moreover, the ball milling may affect the β-aryl ether and other lignin substructures, resulting in extensive cleavage of the lignin moieties.[34] Clearly, the OL fraction showed a high M_w (3415 g/mol), which was in accordance with a previous finding.[35] In addition, it was observed that the three lignins exhibited relatively narrow molecular weight distributions (MWD, Figure 3), which ranged between 1.90 and 2.88. The OL lignin fraction obtained by alkaline organosolv solution extraction showed a higher proportion of high M_w component compared with the AL and MWL fractions. However, AL fraction displayed a wide MWD curve with the low M_w value, suggesting a homogeneous distribution of the lignin molecular weights.

Table 2. Weight-Average (M_w), Number-Average (M_n) Molecular Weights and Polydispersity (M_w/M_n) of the Lignin Fractions

	lignin fraction		
	MWL	OL	AL
M_w	2395	3415	1870
M_n	1020	1185	980
M_w/M_n	2.34	2.88	1.90

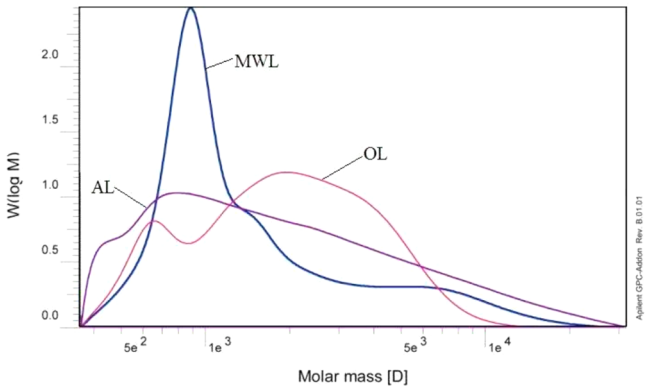

Figure 3. Molecular weight distributions of MWL, OL, and AL.

Elemental Analysis. It can be seen from Table 3 that the carbon content decreased from MWL (70.6%) to OL (63.2%) and to AL (60.1%), and the OL fraction has a relatively high content of hydrogen (9.3%). A possible reason for the high carbon content in MWL is that the condensation reaction may occur at some point in the fractionation procedure. However, N and S content were ranged from 0.2 to 1.4% and 0.3 to 0.7%, respectively. Nitrogen content of lignins reflects contamination by the protein residues, implying the strong chemical bond between proteins and precipitated lignin, and the protein residues are difficult to remove by the extraction or washing processes.[36] As shown in Table 3, the MWL exhibited a low content of associated nitrogen (0.2%), indicating that MWL fraction was less contaminated with protein. The relatively high contents of nitrogen in OL and AL fractions may be explained by the fact that proteins linked to lignin in the starting ball-milled *Tamarix* powder material. The methoxyl content determined from the three lignin fractions is presented in Table 3 along with the calculated C_9 formulas. The methoxyl contents were calculated from the ^{1}H NMR spectra of acylated lignin according to the published literatures.[37,38] Results showed that the amounts of methoxyl for MWL (14.7%) and AL (15.1%) fractions were comparable, but OL showed a high content of methoxyl (21.4%), implying that the alkaline ethanol extraction may preserve more methoxyl group in the lignin. In addition, more S unit in OL fraction indicated high amount of methoxyl group. It was also found that OL exhibited a high value of M_w (191.51 g/mol) of C_9 unit compared with MWL and AL, indicating a relatively low M_w value for MWL and AL lignin units.

Quantitative ^{13}C NMR Spectra. The structural features of lignins were comparatively analyzed (Figure S1, Supporting Information), and the integral range from 162.0 to 102.0 ppm was set as a reference value that equal to 6.12.[34,39] According to Holtman et al.,[40] the degree of condensation was calculated by three minus the value of integration for the protonated

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Table 3. Elemental Analysis and Methoxyl Content of the Lignin Fractions

	elemental analysis (±0.3%)[a]						
lignin fraction	C%	H%	N%	S%	OCH_3 (%) (±0.2%)[a]	C_9 formula	C_9 unit (g/mol)
MWL	70.6	6.8	0.2	0.3	14.7	$C_9H_{8.97}O_{1.55}(OCH_3)_{0.79}$	166.26
OL	63.2	9.3	1.4	0.7	21.4	$C_9H_{9.83}O_{1.97}(OCH_3)_{1.36}$	191.51
AL	60.1	7.3	1.3	0.6	15.1	$C_9H_{10.9}O_{2.86}(OCH_3)_{0.97}$	167.83

[a]Pooled standard error.

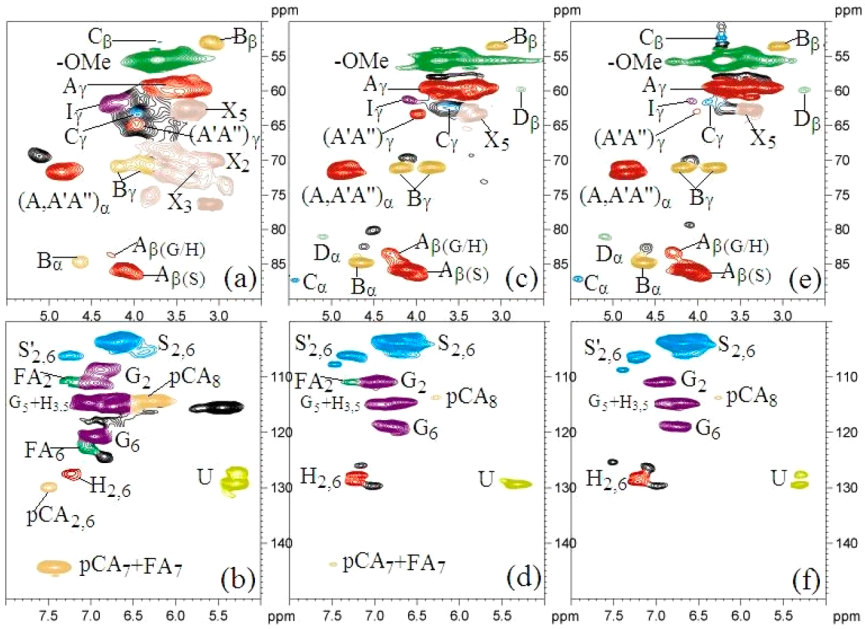

Figure 4. Expanded HSQC spectra (in DMSO-d_6, 25 °C) of the MWL, OL, and AL fractions. (a, c, and e): the side-chain regions (δ_C/δ_H 50−90/2.5−5.5) of the MWL, OL, and AL fractions, respectively. (b, d, and f): the aromatic regions (δ_C/δ_H 100−150/5.0−8.0) of MWL, OL, and AL fractions, respectively.

aromatic region (125−103 ppm). The MWL and AL fractions showed integration for the protonated region with the same value of 2.38/Ar, yielding degree of condensation of 0.62 (3.00−2.38). However, the value of OL of this region was 1.08, indicating a relatively high degree of condensation (1.92). The amounts of aromatic carbon−carbon per aryl in MWL, OL, and AL were 1.36, 1.97, and 1.43, respectively. Results indicated that MWL has a low degree of condensation, suggesting that the condensation reaction occurred during the ball milling process. It was revealed that a relatively high amount of condensed lignin was extracted in the alkaline ethanol extraction process, leading to form large fragment of lignin, which was in accordance with the results of M_w analysis. In addition, the amounts of oxygenated carbons per aryl in MWL, OL, and AL were 2.32, 2.19, and 2.29, respectively. The sharp signal at 178.6 ppm in spectra of MWL was attributed to the carbonyl (C═O) groups. The regions of 176.0−171.0 and 168.0−165.0 ppm were due to aliphatic COOR and conjugated COOR groups, which yielded values of 0.07/Ar and 0.18/Ar, respectively. The presence of **S** units was observed at 152.2 (C-3/C-5, etherified), 148.3 (C-3/C-5, nonetherified), 139.4 (C-4, etherified), and 104.2 ppm (C-2/C-6). The **G** units were detected by the signals at 149.4 (C-3, etherified), 148.3 (C-4, etherified, in erythro β-O-4′), 145.6 (C-4, nonetherified), 134.5 (C-1, etherified), 121.1 (C-6), 115.9 (C-3/C-5, etherified), and 111.2 ppm (C-2). Moreover, a signal of *p*-hydroxyphenyl (**H**) units at 128.0 ppm (C-2/C-6) was also present in the spectrum. In addition, **H** units were also detected in the three lignin fractions. These signals observed from the ^{13}C NMR spectra indicated that *Tamarix* spp. were **GSH** monomers type lignins. This result was in agreement with the observed characteristics of lignins found in the FT-IR analysis.

The **S**/**G** ratio is an important feature in lignin characterization. Generally, most hardwood plant lignin is composed of **S** and **G** type units, and containing less amount of of *p*-hydroxyphenyl unit than softwood plant.[41] The methods for estimate the amounts of **S** and **G** units of plant have been given by Landucci[42] (1985) and Lapierre et al.[43] However, the method to calculate **S** and **G** units by Chen[39] (1998) has been considered to be more precise. Meanwhile, in order to more accurately predict the values, the content of condensed **G** unit should be taken into account, and the signal of second carbon atom of the guaiacyl aromatic ring (**G**-2) should be integral at 113.0−110.0 ppm. In this region, the **G**-2 showed small value for the condensed part and can be neglected. The $S_{2,6}$ unit can be calculated from the integral at 101.0−108.9 ppm. Therefore, the values of the **S**/**G** ratios of MWL, OL, and AL were 1.25, 2.01, and 1.28, respectively. It is well-known that lignin in middle lamella typically has a lower S/G ratio than lignin from the secondary wall. This indicated that in extraction process, the MWL fraction was mainly derived from middle lamella of the ball-milled sample, and the residual lignin with abundant syringyl unit was subsequently extracted by the alkaline organosolv process.[44] Moreover, it was found that almost the

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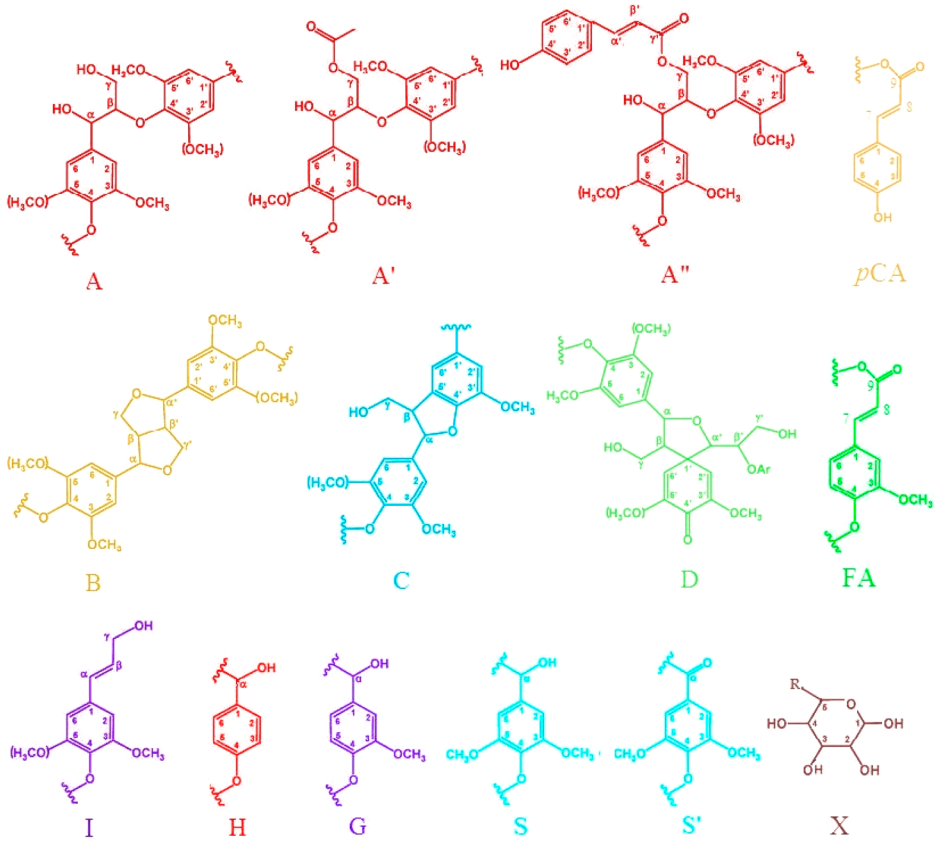

Figure 5. Main classical and acylated substructures, involving different side-chain linkages, and aromatic units identified by 2D NMR of the MWL, OL, and AL fractions of the *Tamarix* spp. lignin: (**A**) β-O-4′ linkages; (**A**′) β-O-4′ linkages with acylated γ-carbon; (**A**″) β-O-4′ linkages with *p*-coumaroylated at γ-carbon; (**B**) resinol structures formed by β-β′, α-O-γ′, and γ-O-α′ linkages; (**C**) phenylcoumarance structures formed by β-5′ and α-O-4′ linkages; (**D**) spirodienone structures formed by β-1′ and α-O-α′ linkages; (***p*CA**) p-coumaric acid; (**FA**) ferulic acid; (**I**) *p*-hydroxycinnamyl alcohol end groups; (**H**) *p*-hydroxyphenyl unit; (**G**) guaiacyl unit; (**S**) syringyl unit; (**S**′) oxidized syringyl unit bearing a carbonyl group at C_α (phenolic); (**X**) xylopyranose (R, H). See Table S1 for signal assignment.

same amount of G and S units lignin were extracted in the AL fraction. The contents of methoxyl group were estimated from the integral at 57.3−54.4 ppm with the result of 1.3/Ar, 2.2/Ar, and 1.4/Ar, respectively. The result was proved by a published report by Whiting et al.,[45] who found that lignin from the secondary wall contains higher methoxyl group than lignin from middle lamella.[45] In addition, the β-O-4′ substructure can also be observed in the spectra of the three lignin fractions, corresponding to the signals at 86.5, 72.1, and 59.5 ppm. Moreover, the cluster at 51.4−47.8 ppm belongs to the signals of the substructure of resinol and phenylcoumaran.

2D HSQC NMR Spectra. 2D-HSQC spectra provide structural information on the whole lignin macromolecule,[46−48] and was collected as shown in Figure 4. It can be observed that the corresponding intensities of MWL spectra were much higher than that of OL and AL spectra. This phenomenon was probably owing to the different molecular weights and associated sugar contents of different lignin fractions. The three spectra of MWL, OL, and AL fractions were comparatively studied, and the C_γ−H_γ in γ-acetylated β-O-4′ substructure $(A'/A'')_\gamma$ were observed at signal δ_C/δ_H 63.3/3.98 (A′ and A″). It was found that the intensity of this signal for OL and AL were much weaker than that of MWL, indicating that the naturally γ-acylated β-O-4′ linkages in the organosolv and alkaline lignin were less condensed. However, as can be seen from the spectra, more resinol structures (B) were appeared in the OL and AL spectra, as well as the $C_{2,6}$−$H_{2,6}$ correlations of *p*-hydroxyphenyl unit at δ_C/δ_H 127.5/7.23. The AL fraction displayed a very neat spectrum as compared to MWL and OL spectra, probably owing to a small molecular weight and trace amounts of associated sugar content. The spectra of the aliphatic (nonoxygenated) region (δ_C/δ_H 0−50.0/0−4.0) were not discussed in detail because of the irrelevant signals. The side-chain regions (δ_C/δ_H 50.0−90.0/2.5−5.5) and the aromatic regions (δ_C/δ_H 100.0−150.0/5.0−8.0) were depicted, and the main cross-signals (Table S1, Supporting Information) were assigned by published literature.[25,47,49,50] The structural characteristics observed in the lignin fraction are depicted in Figure 5.

Side-Chain Region. In the side-chain region, methoxyl and side chains in β-O-4′ substructure (**A**) were the most prominent signals. The C_γ-H_γ correlation in structure **A** was observed at δ_C/δ_H 60.7/3.48. The C_γ−H_γ correlations were also appeared this region, indicating that small amounts of β-O-4′ substructure is acylated at γ-carbon in aryl ether linkages. It worth noting that γ-acylated **G** and **S** type lignin units have been found in many kinds of biomass species.[51] In addition, other substructures such as resinol (**B**) C_α−H_α (δ_C/δ_H 84.9/4.64), C_β−H_β (δ_C/δ_H 53.4/3.13), and double C_γ−H_γ (δ_C/δ_H 71.4/3.82 and 4.18) correlations were observed. Lower amounts of phenylcoumaran (β-5′/α-O-4′) substructure (**C**) were also found in the lignin spectra, and with the signals being

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observed at δ_C/δ_H 86.6/5.49 (C_α–H_α) and 51.8/3.76 (C_β–H_β). Small signals at δ_C/δ_H 81.1/5.09 and 60.1/3.25 for C_α–H_α and C_β–H_β correlations respectively corresponding to the spirodienone (β-1′/α-O-α′) substructure (**D**) were also observed. As proved by purity analysis, lignin was contaminated with carbohydrate. The signals of carbohydrate ($\mathbf{X}_2$, $\mathbf{X}_3$, and $\mathbf{X}_5$ of β-D-xylopyranoside unit) can also be detected in the lignin fractions by HSQC spectra. However, as can be observed in Figure 4, the signal intensities of $\mathbf{X}_2$, $\mathbf{X}_3$, and $\mathbf{X}_5$ in OL and AL were much lower than that in the MWL, indicating a low content of carbohydrate associated with lignin fraction and the cleavage of ether bond between lignin and carbohydrate. In the spectra, it was also proved by the fact that the signals of the benzyl ether structure were missing.

The relative abundances of the main interunit linkages present in the MWL, OL, and AL fractions obtained in this work were calculated as shown in Table 4. A characteristic of the MWL preparation was the high amounts of β-O-4′ interunit substructure, and was estimated more than 79.0% of all lignin substructures, following by resinol (17.3%) and small percentages of phenylcoumaran (2.2%) and spirondienones (1.5%) substructures. Although the alkaline organosolv and alkali treatment destroyed a small number of the β-O-4′ substructures, leading to relatively low amounts of β-O-4′ substructure of OL (69.7%) and AL (68.4%). However, high amounts of β-β′ substructure of OL (22.3%) and AL (21.0%) was observed. It was revealed that a relatively high amount of the β-O-4′ type lignin substructure in the MWL fraction was acylated at the γ-position (**A**′/**A**″). The extent of acylation was calculated using the $\mathbf{A}'_\gamma/\mathbf{A}''_\gamma$ integral, and it was estimated to be 7.9% in the MWL. This value seems reasonably correct, and could represent all the side-chain linkages, because the resinol substructure (**B**) is only formed via nonacylated monolignols, and phenylcoumaran and spirondienone substructures were relatively minor of the side chains.[52] On the contrary, the AL fraction has a trace amount of acylated β-O-4′ lignin substructure ($\mathbf{A}'_\gamma/\mathbf{A}''_\gamma$), probably due to the cleavage of ester groups by aqueous NaOH. Martínez et al.[48] also reported that acylated lignin units were found in all the woody angiosperms, attaining up to 45% acylation of **S** unit, and it was also found to be absent from gymnosperm lignin. In addition, the phenylcoumaran (**C**) and spirodienone (**D**) substructures were calculated to range from 2.2 to 5.6% and 1.5 to 5.0%, respectively.

Table 4. Lignin Structural Characteristics from Integration of ^{13}C–^{1}H Correlation Signals in the HSQC Spectra of the MWL, OL, and AL Fractions

characteristics	lignin fraction		
	MWL	OL	AL
linkages (% side chains involved)			
β-O-4′ aryl ether (**A**, **A**′, **A**″)	79.0	69.7	68.4
resinol (**B**)	17.3	22.3	21.0
phenylcoumaran (**C**)	2.2	3.5	5.6
spirodienone (**D**)	1.5	4.5	5.0
acetylation degree (major γ-acetylation)	7.9	1.5	trace
syringyl to guaiacyl ratio			
S/**G** (HSQC)[a]	2.80	3.85	2.52
S/**G** (DFRC)[b]	2.08	1.37	1.43

[a]**S**/**G** ratio is obtained by the following equation: $\mathbf{S}/\mathbf{G} = 0.5IS_{2,6}/IG_2$. [b]**S**/**G** ratio is obtained according to the corresponding area of the peaks.

Aromatic Region in HSQC NMR Spectra. The **S**-lignin unit exhibited a prominent signal (δ_C/δ_H 104.7/6.72) corresponding to the $C_{2,6}$–$H_{2,6}$ correlation; however, the **G**-lignin unit displayed different signals for C_2–H_2, C_5–H_5, and H_6–C_6 correlations. The double C_5–H_5 signals revealed certain degree of heterogeneity among the **G**-lignin unit especially affecting the C_5–H_5 signal, which was probably owing to the different substituents at the fourth carbon of the aromatic ring. Signal assigned to $C_{2,6}$–$H_{2,6}$ correlations in oxidized (C_α-oxidized) syringyl unit (**S**′) were found in the three lignin fractions. This fact indicated that these moieties existed in the native *Tamarix* spp., although their provenance from the milling process cannot be completely ruled out.[50] The small signal at δ_C/δ_H 114.8/7.02 is assigned to $C_{3,5}$–$H_{3,5}$ correlation in the **H** unit. The $C_{2,6}$–$H_{2,6}$ of the **H** unit were clearly distinguished at δ_C/δ_H 127.5/7.23. In the spectra of MWL, *p*-coumaric acid structures (***p*CA**) were observed by very prominent signals, which were observed at δ_C/δ_H 130.2/7.49, 118.1/6.85, 144.4/7.42, and 114.3/6.27 for $C_{2,6}$–$H_{2,6}$, $C_{3,5}$–$H_{3,5}$, C_7–H_7, and C_8–H_8 correlations, respectively. The strong signal of ***p*CA** was probably derived from lignan, which was probably extracted from the mixed bark of *Tamarix* wood powder.[53,54] However, the cross-signals for the ***p*CA** substructure were much lower in OL and AL fractions. Moreover, the structure of ferulic acid (**FA**) ester was also observed in the MWL spectra. However, the signals at δ_C/δ_H 144.4/7.42 and 114.3/6.27 are arising from C_7–H_7 and C_8–H_8 correlations of **FA**, which were overlapped with cross-signals of $\mathbf{pCA}_7$ and $\mathbf{pCA}_8$, respectively. In addition, additional signals at δ_C/δ_H 129.4 and 127.8/5.29 (**U**, Figure 4) in the three lignin fractions were probably due to signals of unsaturated fatty acid or lignin, which need further confirmation.[41]

The relative abundances of the **G** and **S** type lignin units were calculated, and the results are shown in Table 4. As expected, the **S** unit was the main lignin monomer in the three lignins, and the **S**/**G** ratios were calculated to be 2.80, 3.85, and 2.52 for MWL, OL, and AL, respectively. It was found that the aqueous alkaline solution extraction resulted in a low **S**/**G** ratio as compared to the alkaline organosolv and dioxane solution treatments. In general, the **S**-rich lignin fragment was more easily extracted than the **G**-rich lignin fragment during the three isolation processes. It was observed that the **S**/**G** results calculated by DFRC method were higher than that by ^{13}C NMR, but both of the results give the same order of **S**/**G** ratio values. However, HSQC NMR signal intensities are much clearer to observe. Therefore, the overall distribution of **S** and **G** signals were calculated by a 3-D integral image, and the results were more accurate than that by ^{13}C NMR analysis. From these results, it should be emphasized that the **S** unit type alcohol is quite prevalent in the β-ether unit, and resinol type substructure (**B**) is usually highly originated from the **S** unit in S and G unit lignin.[48]

As shown in Table 4, the **S**/**G** ratios evaluated by the DFRC method were lower than that by HSQC. However, these two methods are different because the DFRC method only detects α- and β-aryl ethers linked lignins, and the major degradation products were acylated coniferyl alcohol and sinapyl alcohol. The DFRC procedure can also provide structural information, and the primary monomers as isomeric mixtures could be produced. The isomers of **G** and **S** monomers were detected from the MWL fractions (Figure S2, Supporting Information),

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and result showed that the content of **S** unit was higher than that of **G** unit. In addition, it was found that MWL showed a relatively high value of **S**/**G** ratio (2.08) by DFRC.

CONCLUSIONS

Alkaline organosolv and aqueous alkaline solutions were effective in isolating lignins from the *Tamarix* spp. The relatively high yields of OL and AL preparations indicated that organosolv fractionation and alkaline solvents extraction were effective in cleavage of linkages such as benzyl ethers and benzyl esters between lignin and carbohydrates. GPC results indicated that a high molecular weight lignin was obtained (OL, M_w = 3415 g/mol) by the alkaline ethanol extraction; however, aqueous alkaline solution extraction released a low molecular weight of lignin with narrow polydispersity. HSQC results indicated that alkaline solvent extraction lead to a decrease of the **S**/**G** ratio as compared to the alkaline organosolv and dioxane solution treatments. In addition, the **S**-rich lignin fragment was more easily to extracted than the **G**-rich lignin fragment during the three isolation processes. This study has revealed that a high amount of lignin can be successfully isolated by the mild solvents fractionation processes, and thus provides a meaningful way for lignin production, which could also promote the research of biomass utilization.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.5b00809.

Detailed chromatography conditions for GC−MS analysis and quantitative ^{13}C NMR spectra of MWL, OL, and AL (Figure S1), GC−MS analysis of the monomeric DFRC products from MWL (Figure S2), and main lignin $^{13}C-^{1}H$ correlation signals in the HSQC spectra (Table S1) (PDF).

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Notes

The authors declare no competing financial interest.

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