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Research Article

Fractionation of Lignin with Decreased Heterogeneity: Based on a Detailed Characteristics Study of Sequentially Extracted Softwood Kraft Lignin

Rui Liu, Annika Smeds, Luyao Wang, Andrey Pranovich, Jarl Hemming, Stefan Willför, Hongbo Zhang,* and Chunlin Xu*

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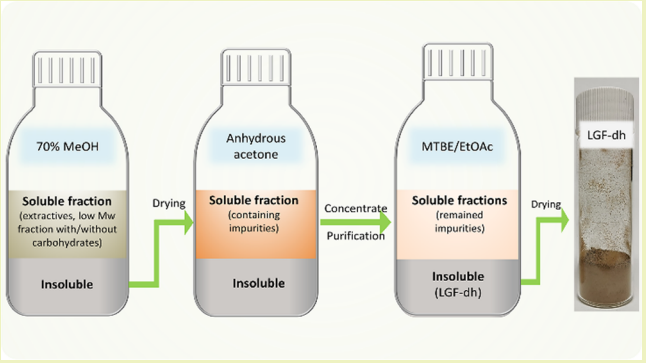
ABSTRACT: Industrial lignin fractionation is attracting increasing interest due to its enormous potential in the development of high value-added materials. However, the widely reported fractionation approaches are primarily focused on the separation of fractions with a low polydispersity index (PDI). In this study, based on the detailed characteristic examination of carefully sequential-extracted softwood Kraft lignin fractions, a novel method to isolate lignin fraction with decreased heterogeneity (LGF-dh), was established in consideration of impurities, elemental composition, molar mass distribution, carbohydrate content, functional hydroxyl content, and the content of lignin-relevant aromatic units. To characterize the mentioned properties, an elemental analyzer, SEC-MALS, GC−MS, GC-FID, Py/GC−MS, ^{31}P-NMR, and HSQC-NMR were used to compare the differences of the sequential lignin fractions that were obtained by methyl *tert*-butyl ether (MTBE), ethyl acetate (EtOAc), ethanol (EtOH), methanol (MeOH), acetone, and dioxane. Moreover, a practical and feasible three-step extraction process was proposed to separate the low heterogeneity lignin fraction from industrial lignin according to the different solubilities of each fraction in the green cosolvent system of EtOH/water, MeOH/water, and acetone/water. Overall, this work presented a comprehensive study on the properties of softwood lignin as well as proposed a feasible and convenient method to reduce the heterogeneity of lignin, which would promote its valorization.



KEYWORDS: *lignin fractionation, compositions, structural characteristics, solvent fractionation, softwood Kraft lignin*

INTRODUCTION

Plant biomass is a widely accessible and renewable resource for the development of eco-friendly products, which contribute to minimizing the negative environmental effects caused by waste products from nonrenewable petroleum-based polymers. Lignin is the most abundant aromatic material predominantly presenting in woody biomass, which devotes billion of tons of lignin annually.[1] The excellent properties of lignin have always attracted considerable research interests for the development of high-value products in different areas.[2] For example, the high energy density (9105 Btu/Ib) and numerous phenolic phenylpropane units of lignin have attracted research into the development of renewable fuels and chemicals out of lignin.[1,3,4] Moreover, the various reactive functional groups of lignin provide rich active sites ready for further modifications to satisfy the needs of the design of functional materials.[5−8] In addition, its favorable biocompatibility and amphiphilicity make lignin a promising biodegradable natural resource to be applied in the area of biomaterials and nanomedicine.[9−11] More notably, lignin and lignin-based products have generated substantial economic benefits, which has increased with an average annual growth of 2.28% from $599 million to $704 million in the last few years.[1] However, the large-scale commercial utilization of lignin is still severely limited due to the lack of well-refined industrial lignin with a narrow molecular weight distribution and a relatively uniform chemical structure.[12]

The pulping and papermaking industries are mainstays of technical/industrial lignin production. To date, more than 50% of the annual global production of lignin are originated from the softwood Kraft pulping process.[13] Unfortunately, the industrial Kraft lignin (KL) is an extremely complicated compound that could include various lignin-relevant and non-lignin-relevant matters.[14] Moreover, this kind of lignin has heterogeneous

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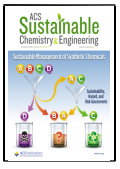

properties, especially, wide molecular weight distribution, diverse functional groups, as well as variable solubility behavior, depending on different sources and processing methods.[13] It is no doubt that these problems severely hamper the extensive application of lignin-based products with high performance. Therefore, the fundamental research on isolation of softwood KL with low heterogeneity should be studied constantly for a further development of lignin-derived high value-added material. So far, numerous fractionation methods, in short, solvent- and membrane-mediated approaches, have been developed and summarized in previous reviews.[12,15,16] Among them, solvent fractionation by single/mixture solvent and a sequential cascade of multiple solvents are the most predominant strategies to lignin fractionation to decrease the polydispersity index (PDI) of lignin fractions.[12] However, even though a great deal of effort has been devoted to lignin fractionation and purification, an effective method for obtaining low heterogeneity lignin fractions considering the impurities, elemental composition, molar mass distribution, carbohydrate content, functional hydroxyl content, and the content of lignin-relevant aromatic units is rarely reported.

In our view, to be familiar with the chemical compositions of KL as well as the structural characteristics of their fractions is necessary to establish a fractionation method for low heterogeneity lignin. Considering the highly heterogeneous nature of softwood KL and the advantage of sequential solvent extraction to reduce lignin polydispersity,[17−19] the effort of the present study was focused on the understanding of the structural characteristics of sequentially extracted softwood KL and on setting up a practical fractionation process for obtaining a lignin fraction with low heterogeneity and low PDI. In brief, sequential fractionation using MTBE, EtOAc, EtOH, MeOH, acetone, and dioxane was carried out to isolate different compositions/fractions. An elemental analyzer, GC/MS, SEC-MALS, GC-FID, ^{31}P-NMR, HSQC, and Py-GC/MS were used to determine the elemental composition, extractive identification, molar mass distribution, sugar moiety residues, functional hydroxyl groups, and lignin-relevant substructures of the lignin fractions. Eventually, a simple and economically feasible method was established to obtain LGF-dh with a moderate molar mass and narrow polydispersity based on their different solubilities in serval green cosolvent systems.

MATERIALS AND EXPERIMENTS

Materials. Softwood KL powder (UPM BioPiva 350) was obtained from UPM Forest (Finland). HPLC-grade solvents of methyl *tert*-butyl ether (MTBE), ethyl acetate (EtOAc), methanol (MeOH), acetone, and AR-grade dioxane were purchased from Sigma-Aldrich. Ethanol (EtOH, 99.5 p-%) was bought form Altia Industrial. Other chemical agents used in this work were analytical and HPLC/GC grade.

Lignin Fractionation. Several steps were included for the fractionation of lignin: removal of inorganic and water-soluble substances, removal of lignin-relevant and nonrelevant extractives, and sequential organic solvent fractionation of lignin. Nylon membrane filters (pore size 0.22 μm) were used to separate the soluble and insoluble fractions throughout the process. (1) *Removal of inorganic and water-soluble substances*: KL was first repeatedly washed with an aqueous hydrochloric acid (HCl) solution at pH 2.5 and then deionized water was used to eliminate the HCl residue. The washing solution was concentrated and freeze-dried to obtain water-soluble substances for further analyses. (2) *Removal of lignin-relevant and nonrelevant extractives*: The water-washed lignin was further dispersed into a 25 times volume of MTBE to extract soluble matters for multiple times until a nearly colorless liquid was obtained. The substances soluble in MTBE were collected followed by rotary evaporation to remove the solvent, and the collection was further dried in a vacuum oven and stored in a cold room for further studies. (3) *Sequential solvent fractionation*: The solvent sequence used in our experiment was based on the solubility of fractions as obtained from our pre-experiment as well as from previous reports.[20−22] After being washed and extracted by MTBE, ethyl acetate (EtOAc), EtOH, MeOH, acetone, and dioxane were used in order. It should be emphasized that repeated extraction by every solvent should not be terminated until an almost colorless liquid is obtained. These sequentially extracted samples were marked as KL_{EtOAc}, KL_{EtOH}, KL_{MeOH}, $KL_{Acetone}$, $KL_{Dioxane}$, and $KL_{Residue}$. The yield percentage of each fraction was calculated based on the raw lignin dried in a vacuum oven for 48 h.

Elemental Analysis. An organic elemental analyzer (Thermo Scientific) was used to determine the carbon (C), hydrogen (H), and sulfur (S) percentage contents, and the percentage of oxygen (O) was calculated by subtracting C, H, and S from 100%.

Extractives Analysis. Ten milligrams of each extractive were dissolved in 1 mL of pyridine, and then, 100 μL of solution was taken out and dried before silylation. The silylation reaction was carried out by 150 μL of the mixture of pyridine, *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), and trimethylchlorosilane (TMCS) at a ratio of 1:4:1 under 70 °C for 45 min. Then, 10 μL of silylated samples were detected by a chromatography instrument with a mass selective detector (EI, 70 Ev), (HP 5973 GC/MS System, USA). An HP-1 capillary column (25 m × 0.2 mm I.d., film thickness 0.11 mm) and the carrier gas helium were used. The temperature was increased from the initial temperature of 60 °C to the final temperature of 290 °C with an increase rate of 8 °C/min. A modified version of the Agilent ChemStation software, the spectral databases Wiley 11th/NIST 2012 and the database created by the Laboratory of Wood and Paper Chemistry (Åbo Akademi University) were applied to recognize the recorded substances.

Molar Mass Detection. The weight-average molar mass (M_w), number-average molar mass (M_n), and PDI were determined by size exclusion chromatography equipped with a multiangle light scattering detector (SEC-MALS, Wyatt Technology) was employed to record the molecular weight distribution as described in previous report.[23] The lignin stock solution was 10 mg/mL in DMSO/0.05 M LiBr solution. The same solution was also used as the eluent at a flow speed of 0.5 mL/min. Data were processed with the ASTRA software, version 7.3.2.

Carbohydrate Content Detection. Ten milligrams of each lignin fraction were placed into a pear-shaped bottle, suspended in 2 mL of a 2 M methanolysis reagent (2 M HCl in methanol). After being vortexed to form a uniform suspension, a methanolysis reaction was performed at 105 °C for 3 h. Then, the sample was taken out and cooled down to room temperature before adding 100 μL of pyridine to neutralize excess HCl. Next, 1 mL of an internal standard solution containing 0.1 mg/mL resorcinol, pentaerythritol, and sorbitol in methanol was added. Then, the sample was dried under a nitrogen flow and in a vacuum oven. After this, silylation was carried out using 150 μL of pyridine as the solvent and 150 μL of hexamethyldisilazane (HMDS) and 75 μL of TMCS as the silylation agents. The supernatant was analyzed by a gas chromatography instrument with flame ionization detection (GC-FID). The calibration solution was treated using the same process and each sample was repeated with three replicates. Moreover, chromatography−mass spectrometry (GC−MS) was also used to identify monomeric sugars, avoiding a possible interference from the nonsugar substance for accurate calculation.

NMR Analysis. The structural features of lignin were determined using NMR analysis. ^{31}P- and HSQC-NMR were carried out to study the functional hydroxyl groups, carbohydrate linkages, and lignin structures of sequential fractions according to a previous report.[24] (1) *^{31}P-NMR analysis*: Briefly, 20 mg of lignin powder was first dissolved in 400 μL of a mixture of anhydrous pyridine/deuterated chloroform ($CDCl_3$) (1.6:1, v:v). Then, an internal standard of 100 μL of e-HNDI (0.12 M in pyridine/$CDCl_3$) and 50 μL of the relaxation reagent of $Cr(acac)_3$ (11.4 mg/mL in pyridine/$CDCl_3$) was added into the lignin solution. After intensive mixing, 100 μL of the phosphorylating reagent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) was added and the solution was mixed thoroughly before analysis. (2)

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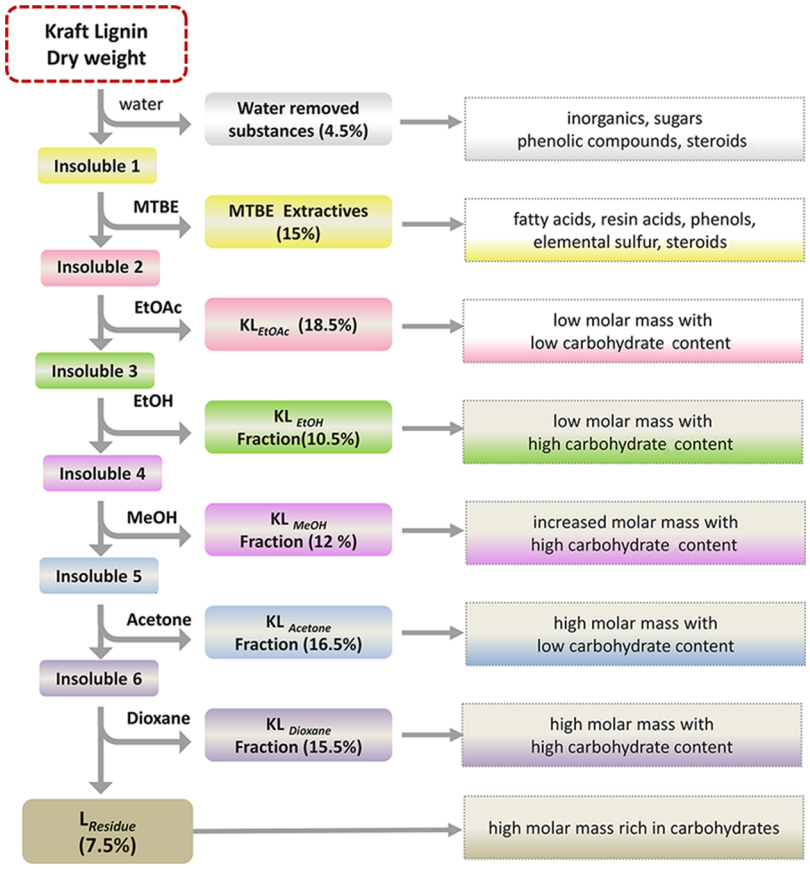

Figure 1. Illustration of the sequential fractionation approach. The yield and the main properties of each fraction are also described.

HSQC-NMR measurements: For HSQC, 80 mg of lignin in 0.5 mL of deuterated DMSO-d_6 was prepared. The experiments were carried out with the Bruker Pulse Program "hsqcetgpsi" with a relaxation delay of 2.0 s, acquisition time 0.13 s, 256 increments, and 80 scans per increment. The spectral width was 8012 Hz (from −3.3 to16 ppm) in the F2 direction and 20,750 Hz (from −7.5 to 157.5 ppm) in the F1 direction. HSQC spectra were recorded at 298 K on Bruker 500 MHz instruments with a smartprobe. All data were processed with the Topspin software (version 4.0.6, Bruker).

Py-GC/MS Detection. The pyrolysis/gas chromatography−mass spectrometry (Py/GC−MS) analysis was performed using a Pyrola 2000 pyrolyzer connected to a GC system (Agilent 7890B) and a mass selective detector (Agilent 5977B). The GC system was equipped with a 30 m × 0.25 mm (L × I.D.), 0.25 μm film thickness ZB-35 capillary column. About 100 μg of each sample was added onto the platinum filament and pyrolyzed at 650 °C with a residence time for 2 s. The injector temperature of the GC system was kept at 280 °C, and the oven temperature was programmed from 50 °C (0.5 min) to 320 °C (4 min) with a heating rate of 8 °C/min. The mass spectra were operated in electron ionization mode at 70 Ev to obtain a signal with m/z from 35 to 700.

Method to LGF-dh Lignin Fractionation. The raw KL was first extracted with 20 times its volume (w:v) of 70% MeOH/water overnight. The soluble substance in the cosolvent system was separated by centrifugation (15,000 rcf, 5 min, 20 °C). Then, the insoluble part was collected and dried to a constant weight by a vacuum oven at 45 °C before the fractionation with 20 time its volume (w:v) of acetone (HPLC-grade) for two times and vigorously stirred for 2 h at each time. Next, the acetone soluble fraction was collected by centrifugation and then concentrated to a sticky liquid by rotary evaporation at 50 °C. After this the collected acetone fraction was purified with 20 times its volume (three times in our experiment) of EtOAc/MTBE mixture (v:v = 1:5) until the liquid became colorless. The target fraction that was insoluble in the EtOAc/MTBE mixture was collected by suction filtration and dried in a vacuum oven at 45 °C. All the extraction work in this study was conducted at room temperature.

RESULTS AND DISCUSSION

This section consists of two parts. First, in order to have a general understanding of the composition of the KL, the fundamental properties of the sequentially extracted lignin were discussed in terms of their extractives, elemental content, molar mass distribution, carbohydrate content, functional hydroxyl groups content, and their pyrolysis products. In the second part, based on a further study of these sequentially extracted lignin in the cosolvent system of ethanol/water, methanol/water, and acetone/water, a feasible approach was proposed for LGF-dh fractionation.

Characteristic of Sequentially Extracted Lignin. In order to have a general understanding about the components of initial KL, a sequential solvent extraction order was used as show in Figure 1. The solvent sequence was established according to their increasing ability to dissolve raw lignin in our preliminary experiment.

Extractives Analyses. Technical lignin from the Kraft chemical pulping process may contain a variety of nonlignin substances that originate from the wood itself, alien reaction products, and residual inorganic salts. Due to the absence of quality standards and strict quality reports, it is of utmost importance to know the components of the commercial technical lignin generally to use it in further applications. The conductivity and pH value of a 1% (w:v) raw lignin (KL_{Raw}) suspension in distilled water was 123 ± 1.56 μS/cm and 4.08 ± 0.02, respectively, which gave an obvious indication that inorganic/organic ionic compounds may have been remained

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in the raw lignin. After being repeatedly washed with water, the conductivity decreased to 8.96 ± 0.6 μS/cm, and the pH value slightly increased to 4.56 ± 0.06. In total, 4.5% of the materials, including inorganic substances and low molecular weight organic matters, were eliminated from the raw lignin by washing with water (Figure S1).

MTBE has been reported to be an excellent solvent to remove extractives from lignin.[25] As shown in Figure 1, 15% of all substances was extracted by MTBE from insoluble 1. In order to identify the MTBE extractives, GC/MS was employed to recognize its compositions. As expected, a large variety of representative extractives in softwood were detected (Figure S2, Table S1), mainly involving vanillin, guaiacols, coniferyl alcohols, long-chain fatty acids, alkanes, resin acids including abietic-type acids, dehydroabietic acid, neoabietic acid, and pimaric-type acids, isopimaric acid, which are representative products in softwood.[26] The rich lipophilic extractives extracted by MTBE reasonably explain the higher carbon and hydrogen content but lower oxygen content of MTBE extractives when compared with other fractions (Table 1). These phenols related

Table 1. Elemental Content of C, H, S, and O in Different Lignin Fractions in Weight Percentages (%)

sample	C (%)	H (%)	S (%)	O (%)
KL_{Raw}	61.75	5.87	1.86	30.42
KL_{MTBE}	67.46	6.65	3.82	22.07
$KL_{MTBE\ purified}$	64.42	6.14	1.22	28.12
KL_{EtOAc}	66.38	5.85	1.46	26.21
KL_{EtOH}	64.38	5.96	1.42	28.14
KL_{MeOH}	65.06	5.82	1.21	27.81
$KL_{Acetone}$	66.26	5.90	1.23	26.51
$KL_{Dioxane}$	64.41	6.12	1.15	28.22
$KL_{Residue}$	58.15	5.75	0.91	35.09

to lignin could have been released from the wood itself, as well as could have been degraded from lignin polymers during Kraft delignification and then coprecipitated with lignin during the acidification of black liquor. In terms of substances containing carboxyl groups, for example, fatty acids and resin acids, they could be deprotonated during the delignification process resulting in a high solubility in black liquor under the strong alkaline environment. After being protonated during the acid precipitation procedure, these substances would be coprecipitated abundantly. Moreover, similar extractives were obtained by MTBE from other commercial softwood lignins (Figure S3 and Table S2, Figure S4 and Table S3), LignoBoost KL (CRUDE LIGNIN LIGNOBOOST SW) was obtained from MetGen, Finland, and lignin alkali that was bought from Sigma-Aldrich (CAS:8068-05-1, a KL according to supplier's website).

Plants can produce thousands of different types of compounds, including fatty acids, resin acids, steroids, alkanes, alkaloids, polysaccharides, and numerous simple phenols and polyphenols.[27] Therefore, it is inevitable that various nonlignin and lignin-relevant substances could be extracted from technical softwood KL. Herein, removing these nonlignin substances and lignin-relevant phenols is important to understand the structural properties of KL, as well as to design an optimized approach for LGF-dh fractionation and their valorization.

Apart from extractives that originate from the wood itself, nonwood substance impurities in KL should be of concern. The presence of sulfhydryl groups in KL is widely acknowledged. While there is no exact sulfur content related to sulfhydryl groups in KL, but usually it could be 1.5−3% depending on the conditions of delignificaiton.[28,29] The sulfur content of different fractions quantified by an organic elemental analyzer is listed in Table 1. After water washing and MTBE extraction, the sulfur content decreased from 1.86% in raw KL to 1.22% in MTBE-purified lignin. A reasonable explanation is that sulfur related inorganic/organic substances have been removed by water washing and MTBE extraction. Moreover, the lignin fractions extracted by sequential solvents had different sulfur contents, 1.46%, 1.42%, 1.21%, 1.15%, and 0.91% in KL_{EtOAc}, KL_{EtOH}, KL_{MeOH}, $KL_{Acetone}$, $KL_{Dioxane}$, and $KL_{Residue}$, respectively (Table 1), which was inversely proportional to their molar mass (discussed later). The results suggested that the sulfur content may vary remarkably in industrial KL with different molecular weight distributions.

Interestingly, the sulfur content in MTBE extractives increased observably, up to 3.82%. The detection of cyclo-octasulfur (S_8), and hexasulfur (S_6) from MTBE extractives of KL and LignoBoost lignin by GC/MS, as well as the successfully separation of elemental sulfur from MTBE extractives (Figure S5), strongly indicated that the insidious elemental sulfur contamination could result in an overestimated sulfur content of KL. Elemental sulfur is one of the sulfur contaminants in KL that has been reported in previous studies.[29,30] The negative influence of elemental sulfur on the application of KL has been discussed in previous reports.[29] For example, a report showed that S_8 extracted from pulp and paper mills was acutely toxic to luminescent bacteria in the Microtox test, as well as to zebrafish and perch larvae.[31] Daniel et al. also revealed that a sulfur contaminant would decrease the bio-oil yield, the production distribution, and increase the char content during the pyrolysis process of KL.[32] The highly hydrophobic S_8 could dissolve reasonably well in nonpolar solvents like aliphatic ethers due to its zero hydrogen bond acidity and zero hydrogen bond basicity.[33,34] MTBE used in this study was an effective aliphatic ether being capable of removing nonlignin substances as well as elemental sulfur.

Molar Mass Analyses. Due to the absence of reliable calibration standards, the detection of lignin molecular weight is still a great challenge.[23] Fortunately, SEC-MALS equipped with fluorescent filters has been proven to be a useful tool to monitor the absolute molar masses of lignin.[23,35] In this study, the molar mass of lignin fractions detected by SEC-MALS are presented in Figure 2 and in Table 2. The raw lignin sample had a M_w of 14,170 g/mol, a M_n of 1659 g/mol, and a high PDI of 8.54. Moreover, one could clearly conclude a tendency that the fractions with increased molar mass were obtained using the sequential organic solvent extraction. With regard to the MTBE extractions, due to the abundance of existence of lipophilic extractives, the small molar mass distribution is reasonable. Because the $KL_{Residue}$ could not be totally dissolved in DMSO/0.05 M LiBr, the Mw and Mn values only represent the soluble portion of $KL_{Residue}$ by SEC-MALS.

Carbohydrate Content. A previous study has shown that the total carbohydrate content of softwood KL fractions would change when extracted using sequential organic solvents.[17] To determine the amount of remaining sugar moieties, we further determined the carbohydrate content by GC-FID analysis of the lignin fractions after acid methanolysis (Figure S6). Moreover, to avoid the interference of nonsugar signals that may overlap with the sugar signals, GC/MS was also used to identify the silyl derivatives (Figure S7). The content of different sugar moieties is summarized in Table 3. In general, KL_{EtOAc} and $KL_{Acetone}$

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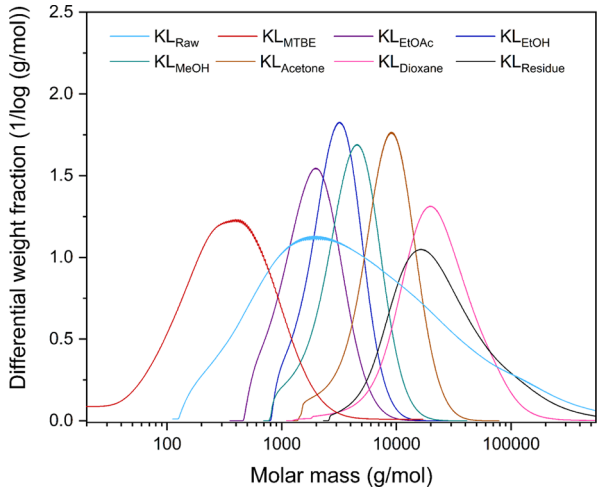

Figure 2. Molar mass distribution of lignin fractions, detected by SEC-MALS.

Table 2. Calculated Molar Mass Distribution of Lignin Fractions Detected by SEC-MALS, M_w, M_n, and PDI

sample	M_w (g/mol)	M_n (g/mol)	PDI (M_w/M_n)
KL_{Raw}	14,170	1659	8.54
KL_{MTBE}	934	532	1.75
KL_{EtOAc}	2870	2476	1.16
KL_{EtOH}	4185	3306	1.27
KL_{MeOH}	6132	4641	1.32
$KL_{Acetone}$	12,490	9547	1.31
$KL_{Dioxane}$	27,590	16,500	1.67
$KL_{Residue}$	49,640	17,000	2.92

presented much lower carbohydrate content compared to other fractions. However, the results indicated that arabinose (Ara) and galactose (Gal) were the main sugar moieties among the KL_{EtOH}, KL_{MeOH}, and $KL_{Dioxane}$, except the $KL_{Residue}$, which has a higher content of xylose than arabinose. These two remaining sugar moieties are probably derived from galactoglucomannan and arabinoxylan that is chemically bonded between the fiber wall lignin and the hemicelluloses in softwood species.[36] In terms of uronic acids, the intensity of the GlcA peaks was higher than those of GalA, implying that GlcA was the dominant uronic acid moiety in lignin fractions (Figure S6). The total carbohydrate content increased in the following order, KL_{EtOAc}, $KL_{Acetone}$, KL_{EtOH}, KL_{MeOH}, $KL_{Dioxane}$, and $KL_{Residue}$. This result was perfectly in accordance with the previous report stating that with EtOAc, the obtained lignin fraction with a lower molecular weight and a small amount of carbohydrate content could be extracted.[17,20] Moreover, a similar trend that acetone was a good solvent for extracting higher molar mass lignin with lower carbohydrate content and the fraction after an acetone treatment that was rich in carbohydrates was also observed.[17,20] Notably, the highest carbohydrate content, which was measured in $KL_{Residue}$ would be an unfriendly factor for its solubility in organic solvent, even in 0.05 M LiBr/DMSO.[37] More interestingly, carbohydrate cross-peaks in HSQC spectra showed a significant difference between fractions with lower/higher content of neutral sugar moieties (discussed later). As previously described above, the extractions obtained by MTBE contained abundant lipophilic extractives, it was not characterized any longer.

^{31}P-NMR Results. ^{31}P-NMR provides a convenient method for quantifying the functional hydroxyl groups of lignin.[38] In this study, the content of aliphatic groups, phenolic groups, 5-substituted and carboxyl groups is presented in Table 4. As discussed in the previous section, $KL_{Residue}$ had the highest content of hemicelluloses, resulting in its poor solubility in pyridine and DMSO; thus, it was not analyzed further. Generally, the phenolic hydroxyl group content of each fraction increased with the decrease in molecular weight. However, the phenolic −OH amount of $KL_{Acetone}$ (3.61 mg/g) was slightly higher than KL_{MeOH} (3.32 mg/g), although the latter has a lower molecular weight distribution. Another clear result was that the aliphatic hydroxyl group content of each fraction showed a similar tendency of the carbohydrate content in each lignin fraction, implying that sugar hydroxyls could be an important source of aliphatic −OH groups in our softwood KL sample. It is noteworthy that because a large number of compounds with hydroxyl groups had been removed by MTBE, the ^{31}P-NMR spectra shapes of the lignin fractions highly resembled each other (Figure S8), which was different from a previous report, where softwood KL directly extracted by EtOAc presented a ^{31}P-NMR spectrum with different peak shapes when compared to other fractions.[22]

HSQC-NMR Analyses. HSQC spectra of KL_{EtOAc}, KL_{EtOH}, KL_{MeOH}, $KL_{Acetone}$, $KL_{Dioxane}$, and raw lignin (KL_{Raw}) are presented in this study. Moreover, the HSQC spectra in the form of an oblique mode, which displays more recorded signals are shown in Figure S10. The lignin-relevant signal assignments and the counterpart chemical structures are presented in Figure S11 and Table S4 according to previous reports.[39,40] In Figure 3 of the HSQC spectra, several differences could be observed. First, the cross-peaks in the alkyl region ($\delta C/\delta H$: 10∼40/0∼2.5 ppm) declined in numbers and intensity as the sequential extraction proceeded. Initial lignin without any purification gave numerous mixed signals (Figure S9) due to the interference of various extractives, such as fatty acids, resin acids, and alkanes,

Table 3. Sugar Moiety Content in Each Lignin Fraction Determined by GC-FID[a]

fractions	neutral sugar (mg/g)							uronic acid (mg/g)		
	Ara	Xyl	Rha	Glc	Gal	Man	total neutral sugars	GlcA	GalA	total carbohydrates (mg/g)
KL_{EtOAc}	NC	0.16	NC	0.33	0.51	NC	1.00	1.16	NC	3.16
KL_{EtOH}	5.51	1.23	NC	0.51	2.34	0.25	9.84	1.35	0.72	11.91
KL_{MeOH}	5.62	2.27	NC	0.45	2.02	0.39	10.75	1.07	0.17	11.99
$KL_{Acetone}$	1.34	0.32	NC	NC	0.42	NC	2.08	0.78	NC	2.86
$KL_{Dioxane}$	2.34	1.89	NC	0.56	4.71	0.38	9.89	11.00	0.39	21.28
$KL_{Residue}$	30.89	67.43	0.47	20.64	137.06	7.57	264.06	12.86	NC	276.92

[a]Ara, arabinose; Xyl, xylose; Rha, rhamnose; Glc, glucose; Gal, galactose; Man, Mannose; GalA, galacturonic acid; GlcA, glucuronic acid; NC, noncalculated for its low content.

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Table 4. Phenolic, Aliphatic, and Carboxylic Hydroxyl Contents of Each Lignin Fraction Determined by ^{31}P-NMR

fractions	phenolic −OH (mmol/g)				aliphatic −OH (mmol/g)	carboxylic −OH (mmol/g)	total −OH (mmol/g)
	C_5-substituted −OH	guaiacyl −OH	*p*-hydroxylphenyl −OH	total phenolic −OH			
KL_{EtOAc}	1.99	2.37	0.16	4.52	1.61	0.40	6.53
KL_{EtOH}	1.76	1.84	0.16	3.77	3.03	0.33	7.13
KL_{MeOH}	1.50	1.64	0.18	3.32	2.97	0.06	6.35
$KL_{Acetone}$	1.95	1.52	0.14	3.61	1.96	0.17	5.74
$KL_{Dioxane}$	1.53	1.39	0.02	2.94	2.92	0.17	6.04
$KL_{Residue}$	not analyzed because of its poor solubility						

that have been detected by GC/MS. After having been purified by MTBE repeatedly, such signals still appeared, especially in KL_{EtOAc}, but their intensities were decreased gradually through the sequential extraction. In addition, additional signals at the aromatic region assigned to extractives or quinone at $\delta C/\delta H$ of 122.01/5.69, 120.02/5.32, 127.53/5.3, 127.57/5.11, and 129.26/5.32 ppm were captured only from KL_{EtOAc} and KL_{EtOH}.[39,40]

In terms of aliphatic chain regions in lignin, the most conspicuous finding was that signals assigned to carbohydrates were missing in KL_{EtOAc} and $KL_{Acetone}$. The fact that carbohydrates are covalently linked to lignin to form lignin−carbohydrate complexes (LCCs) has been verified.[41,42] The main types of LCC linkages in wood are believed to be phenyl glycoside linkages (PhGly), ester linkages (LCC-γ-esters), and benzyl ether linkages (BE). The quantitative analysis of carbohydrates by GC-FID in different lignin fractions showed that the content of carbohydrates varied in different fractions in this study. According to previous reports, PhGly gave a group of signals from the carbohydrate C-1 atom at 104−99/4.8−5.2 ppm,[28] but no such obvious signal could be recognized in this region in our lignin fractions, although hexose was detected by GC-FID and GC/MS. Benzyl ester (α-ester), which would give a signal at 75/6.1 ppm was also absent in all samples.[28] Notwithstanding, even though the signal for CH_2-γ in γ-esters could be detected at 65.5−62/4.5−4.0 ppm,[28] it was unreasonable to claim the presence of benzyl α-ester because γ-acetyl lignin moieties always overlap with this signal. BE LCC structures could be detected at the region of $\delta C/\delta H$ 81.5−80/5.3−4.3. There are two types of BE linkage based on the studies of lignin−carbohydrate model compounds: (i) a cross-peak at 80−81/4.5−4.7 ppm assigned to BE1-linkages between the α-position of lignin and primary OH groups at C-6 of Glc, Gal, and Man and C-5 of Ara, and (ii) a cross-peak at $\delta C/\delta H$ 81−80/5.1−4.9 resulting from BE2 linkages of the α-position of lignin and secondary −OH groups of carbohydrates, normally of the lignin−xylan type.[28] In the spectra, the BE1-linkage ($\delta C/\delta H$ 81.19/4.58) could be well recognized by HSQC-NMR in all fractions, but this type of cross-peak could overlap with γ-acylated β-O-4′ substructures linked to a G-type unit, which also gave a signal at around $\delta C/\delta H$ 81.0/4.75 ppm. Since galactose is the richest hexose moiety in all lignin fractions that were analyzed by GC-FID, the main BE1-linkage could be an α-position of lignin and primary OH groups at the C-6 of galactose. In addition, lignin samples extracted by EtOH showed strong signals for arabinose but a very weak indication of xylose (Figure 3 and Figure S10). However, MeOH- and dioxane-extracted lignin has intense carbohydrate signals of xylosyl (Xly) and arabinosyl (Ara) units at 101.57/4.30 (Xyl_1), 72.46/3.09 (Xyl_2), 73.78/3.28 (Xyl_3), 75.21/3.53 (Xyl_4), and 63.61/3.30 & 3.89 (Xyl_5) and 107.86/4.80 (Ara_1), 81.42/3.89 (Ara_2), 77.02/3.69 (Ara_3), 86.81/4.34 (Ara_4), and 61.77/3.41 (Ara_5).[18] Therefore, we tentatively proposed that solvents with higher hydrogen bonding abilities would be better for extracting lignin fractions with high carbohydrate content, simply, LCC, and relevant research work is ongoing in our group.

Py-GC/MS Analyses. Py-GC/MS is an effective tool for studying the chemical composition of lignin by analyzing the pyrolysis fragments.[43,44] The striking differences in the HSQC spectra prompted us to perform a Py-GC/MS examination so that more structure characteristics of these fractions could be obtained. Lignin is acknowledged as an aromatic polymer; herein, the percentage of total content of aromatic units may be an important metric of the lignin purity. The peak area percentage of G-units, H-units, methylated lignin units, and other aromatic compounds was well identified by Py-GC/MS (Table 5). The top four richest fragments are G-type units, namely, 4-methylguaiacol, guaiacol, 4-vinylguaiacol, and *trans*-isoeugenol in each fraction, and more details about these formed fragments can be found in the Supporting Information. Unexpectedly, the proportion of the lignin basic units and other aromatic units in each lignin fraction varied noticeably. $KL_{Acetone}$ has a higher content of total aromatic compounds (66.78%) and a lower carbohydrate content 2.86 mg/g than that of KL_{EtOH} and KL_{MeOH} (Table 3), which could be a reasonable explanation for the higher carbon percentage (66.26%, Table 1) in $KL_{Acetone}$. The increased carbohydrates in KL_{EtOH}, KL_{MeOH}, $KL_{Dioxane}$, and $KL_{Residue}$ would contribute negatively to their carbon content but positively to the oxygen content. However, KL_{EtOAc} has a higher carbon content (66.38%) but a lower total content of aromatic compounds (56.82%). Considering the following aspects: first, the molecular weight of these sequential fractions was increased gradually by the selected solvent sequences; second, in the alkyl region from HSQC spectra, the number and intensity of signals belong to extractives, presenting a downward trend, for example, hydrocarbon compounds of fatty acids and resin acids; third, from the detectable fragments of fatty acid and resin acids by Py-GC/MS, we presume that these hydrocarbon compounds may be tightly encapsulated by the lignin macromolecule with high molar mass, which would like "a barrier" protecting extractives from removal. Once such "a barrier" was destroyed by a suitable solvent, these hydrocarbon compounds could be released into the organic solvent system. There is also a possibility that some aliphatic hydrocarbon compounds may covalently bond with the lignin macromolecule, resulting in a higher carbon content but a lower aromatic content.

LGF-dh Lignin Fractionation. In general, the commonly referred softwood lignin should be a heterogenous aromatic polymer mainly consisting of the phenylpropane of coniferyl alcohol (G), paracoumaryl alcohol (H). However, as discussed in the first section, the KL from the pulping and papermaking

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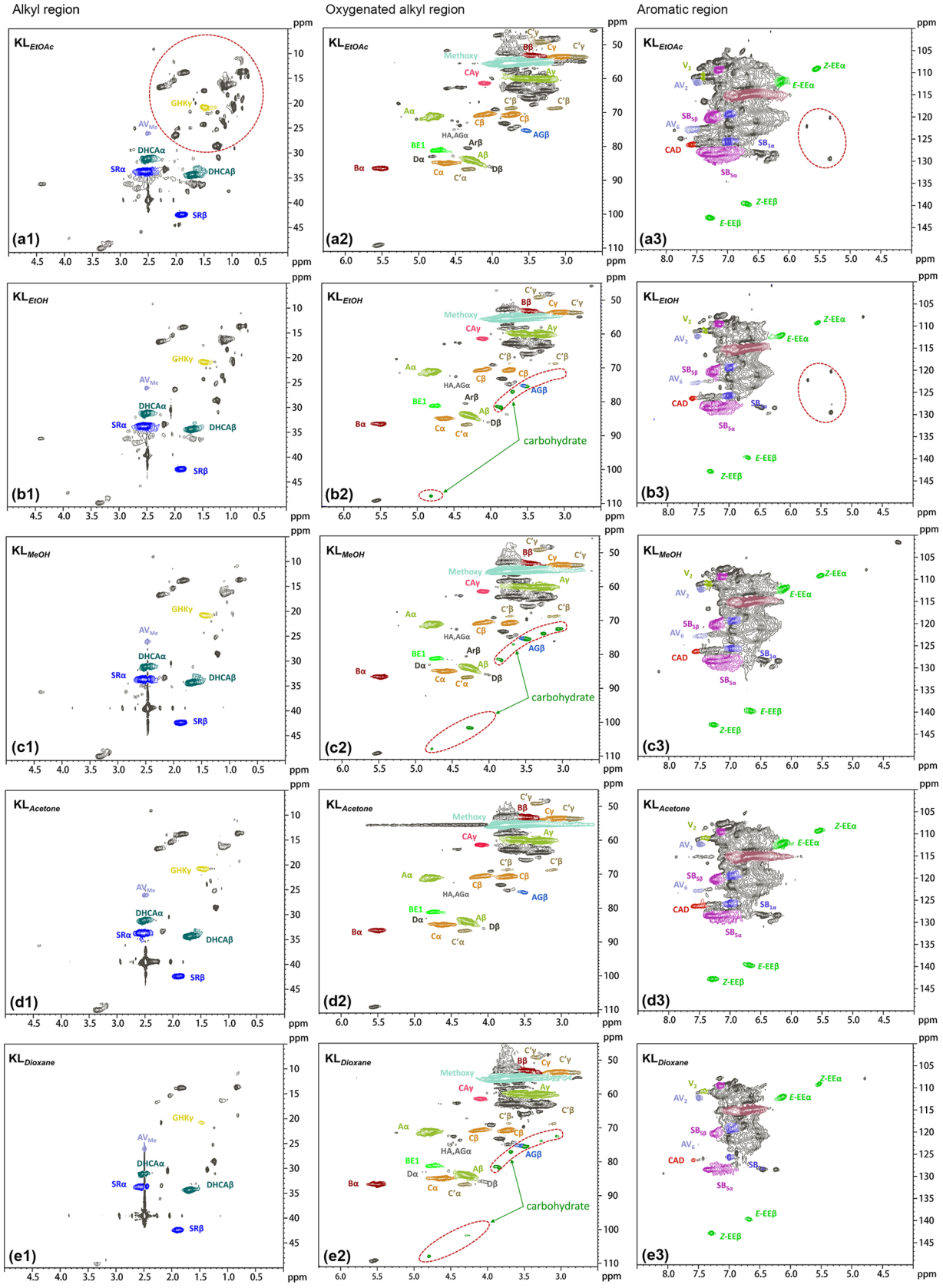

Figure 3. HSQC spectra of KL_{EtOAc}, KL_{EtOH}, KL_{MeOH}, $KL_{Acetone}$, and $KL_{Dioxane}$ in contour mode. (a1), (b1), (c1), (d1), and (e1) in the alkyl region, (a2), (b2), (c2), (d2), and (e2) in the oxygenated alkyl region, and (a3), (b3), (c3), (d3), and (e3) in the aromatic region.

industry could contain a rich amount of nonlignin polymer without precise purification. The lignin polymer with low heterogeneity, low PDI, and nonimpurities is important for lignin application in specific areas, such as biomedicine and

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Table 5. Summary of the Peak Area Percentage (%) of Different Types of Compounds from Chromatograms of Py-GC/MS[a,b,c,d]

	KL_{EtOAc} (%)	KL_{EtOH} (%)	KL_{MeOH} (%)	$KL_{Acetone}$ (%)	$KL_{Dioxane}$ (%)
H-units	0.98	1.71	0.61	1.56	2.30
G-units	57.00	57.15	60.67	55.78	43.26
methylated lignin units	1.30	ND	ND	1.20	0.73
other aromatic compounds	3.85	8.05	5.17	8.24	14.08
sulfur compounds	2.58	1.54	1.49	0.24	0.30
carbohydrate compounds	0.71	0.92	1.08	0.63	0.89
fatty acid and resin acids	0.53	1.09	0.50	1.13	1.41
total aromatic compounds	63.13	66.91	66.45	66.78	60.37

[a]The detailed percentage of each compound assigned to, for example, H-units are listed in the Supporting Information. [b]ND, nondetected. [c]Peak area percentage (%) of was calculated based on the ratio of the peak area of each identified compounds to the sum of the total peak area. [d]The total aromatic compounds (peak area, %) = H-units + G-units + methylated lignin units + other aromatic compounds.

tissue engineering materials. Therefore, it is extremely necessary to develop a simple and feasible method to obtain low heterogenous lignin fractions.

The detailed characterization of each lignin fraction clearly indicates the highly heterogeneous structure and complex composition of softwood KL. Taken together, the sequential fractions showed difference in impurity content (non-lignin-relevant substance), molar mass distribution, elemental content, carbohydrate content, as well as the content of lignin-relevant total aromatic fragments. Among these different fractions, the one sequentially isolated using anhydrous acetone would be low heterogeneity lignin with a narrow molar mass distribution, a minimal amount of carbohydrate residues, and a high content of aromatic basic units. However, this time-consuming, tedious, and expensive sequential fractionation is not suitable for such a low heterogeneous lignin fractionation. Therefore, the following work was to develop a parsimonious method for such a lignin fractionation.

For fractionation of lignin, green organic solvents are widely advocated concerning the issue of safety, environment, and energy consumption.[17,45] In the following study, we observed the solubility of each lignin fraction in green cosolvent systems of EtOH/water, MeOH/water, and acetone/water, which are widely acceptable solvents systems for lignin fractionations.[15] As shown in Figure 4a–c, 70~80% EtOH/water cosolvent was not

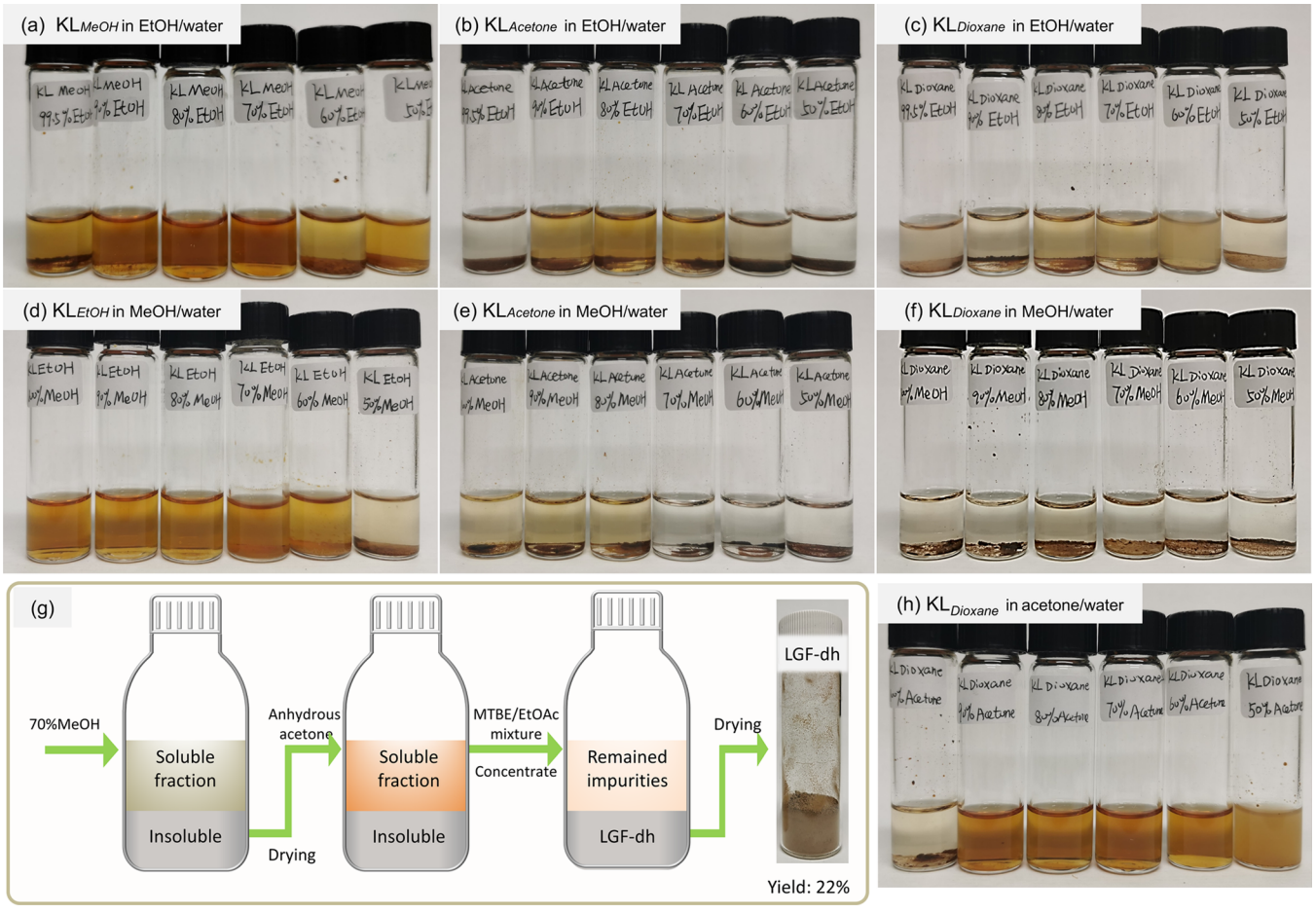

Figure 4. Solubility of sequential solvent-fractionated lignin samples in different cosolvent systems at a concentration of 5 mg/mL. KL_{MeOH} (a), $KL_{Acetone}$ (b), and $KL_{Dioxane}$ (c) in different EtOH/water cosolvent systems; KL_{EtOH} (d), $KL_{Acetone}$ (e), and $KL_{Dioxane}$ (f) in different MeOH/water cosolvent systems. (h) Schematic description of the simplified refining approach for the lignin fraction with decreased heterogeneity. $KL_{Dioxane}$ (h) in different acetone/water cosolvent systems.

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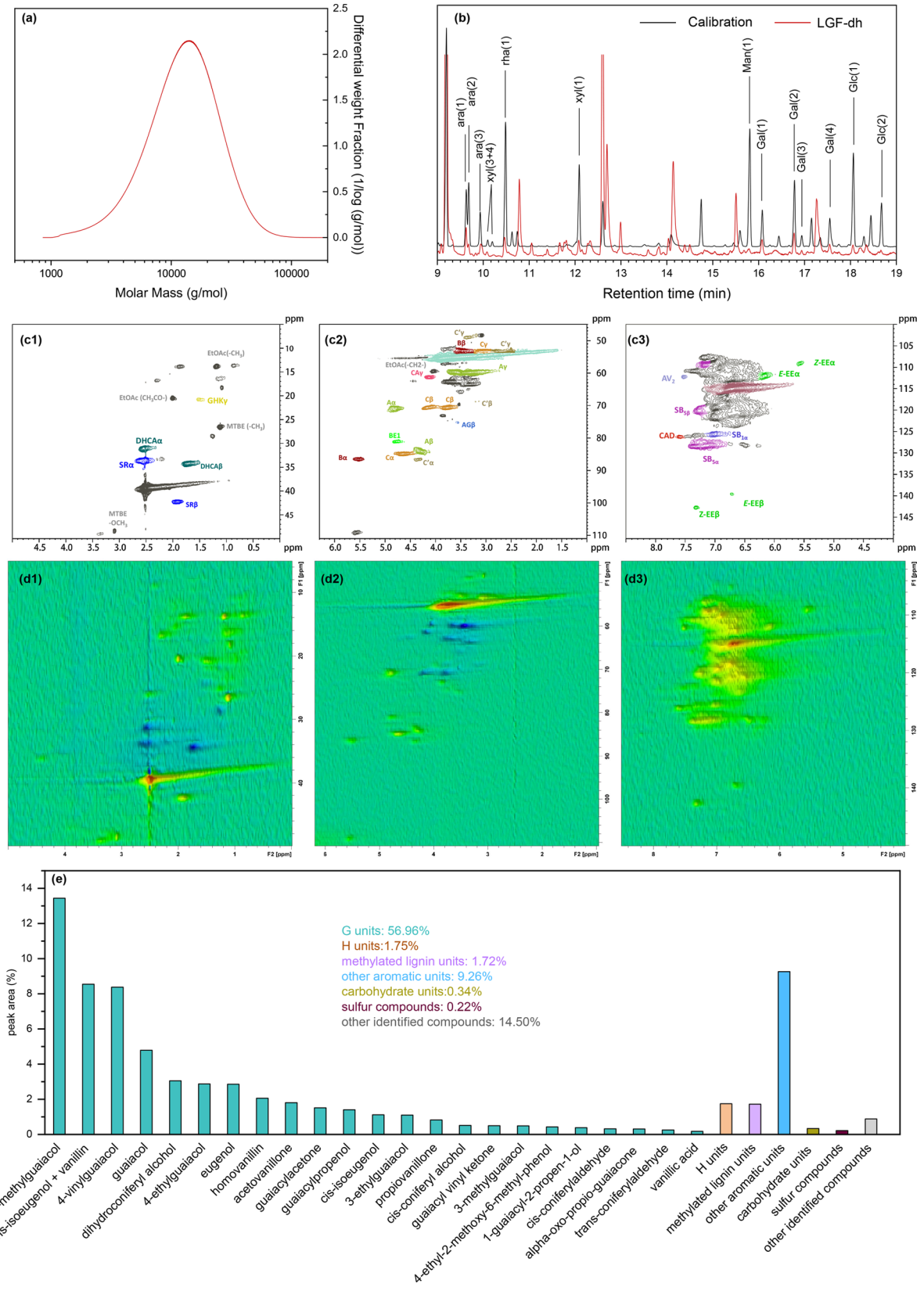

Figure 5. Characterization of LGF-dh. (a) Molar mass distribution detected by SEC-MALS; (b) carbohydrate moieties determined by GC-FID; HSQC results in contour mode (c) and oblique mode (d), alkyl region (c1) (d1), oxygenated alkyl region (c2) (d2), and aromatic region (c3) (d3); and (e) content of major compounds identified by Py-GC/MS.

only powerful to dissolve the fraction of KL_{MeOH}, but also a suitable system to dissolve high molecular weight lignin fractions of $KL_{Acetone}$ and $KL_{Dioxane}$ at a certain content. However, the MeOH/water cosolvent system presented good solubility to

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KL_{EtOH}, but it almost had no effect on the dissolution of fraction of $KL_{Acetone}$ and $KL_{Dioxane}$, Figure 4e–g. It is elaborate here that wood extractives and KL_{EtOAc} dissolved well in these cosolvent systems in our preliminary experiments; herein, they were not discussed.

In the light of the fact that the 70% MeOH/water cosolvent system was less sensitive to $KL_{Acetone}$ and $KL_{Dioxane}$, a simplified extraction approach was proposed. As shown in Figure 4g, we directly used this cosolvent system to extract the raw lignin so that extractives and low molecular weight lignin polymers with/without carbohydrate content could be removed. Then, anhydrous acetone was used to separate the low heterogeneity lignin fraction from the precipitation that was collected after MeOH/water pretreatment. An important step was that the insoluble fraction collected after the MeOH/water system should be dried to remove water, because trace amounts of water in acetone could effectively increase the solubility of $KL_{Dioxane}$, Figure 4h. Finally, an MTBE/EtOAc mixture was employed to eliminate some stubborn impurities, for example, elemental sulfur.

The obtained LGF-dh was characterized by SEC-MALS, GC-FID, HSQC, and Py-GC/MS, as shown in Figure 5. LGF-dh had an average molar mass of M_n = 6129 g/mol and M_w = 9404 g/mol with PDI = 1.53 (Figure 5a). The detectable carbohydrate content in LGF-dh was 2.89 mg/g in total, including 1.18 mg/g Ara, 0.53 mg/g Gal, 0.39 mg/g Xyl, 0.64 mg/g GlcA, and negligible amounts of Glc and Man (0.09 and 0.06 mg/g), respectively (Figure 5b). Moreover, in the HSQC spectra, Figure 5c1, c2, c3 and d1, d2, d3, the LGF-dh obtained by the simplified method gave no obvious signals that were attributed to extractives and non-lignin-relevant substances compared to the sequential solvent fractions that were presented in Figure 2. Moreover, the Py-GC/MS results showed that there were no glaring fragment signals belonging to fatty acid and resin acid in this LGF-dh, Supporting Information S17. In our experiment, the yield by 70% MeOH/water extraction and the following further purification by MTBE/EtOAc mixture was 51 and 3.5%, respectively. The final yield of LGF-dh was 22%, which was higher than that obtained by carefully sequential-extracted fractions. These results indicated that the cosolvent system and the mixture solvent could effectively remove wood extractives and low molar mass lignin fractions. As shown in Figure 5e, the aromatic related fragments of LGF-dh were evaluated by Py-GC/MS. The total aromatic unit content was up to 69.67% with 1.75% H-units, 56.96% G-units, 1.72% methylated lignin units, and 9.26% other aromatic units. Moreover, the elemental analysis shows that this LGF-dh was composed of 66.43% C, 5.94% H, 1.03% S, and 26.59% O. The elemental composition of C, H, and O in LGF-dh was close to the carefully purified softwood KL in previous report,[46] as well as to Klason lignin, that is, 66.67% C, 5.49% H, and 27.84% O.[47] In terms of the content of functional hydroxyl groups, total phenolic −OH was 2.63 mmol/g, including 1.32 mmol/g C_5-substituted −OH, 1.24 mmol/g guaiacyl −OH, and 0.07 mmol/g *p*-hydroxylphenyl −OH, and the aliphatic −OH and carboxylic −OH was 1.75 and 0.12 mmol/g, respectively.

In short, an effective method for low heterogeneity lignin fractions was proposed based on the preliminary study of the chemical characteristic of sequentially extracted lignin fractions.

CONCLUSIONS

In this study, based on a detailed characteristic examination of sequentially extracted technical softwood lignin fractions by MTBE, EtOAc, ethanol, methanol, acetone, and dioxane, we proposed a feasible and practicable sequential method to isolate lignin with reduced heterogeneity. In short, a three-step process was used by the 70% MeOH/water cosystem to first remove extractives and low molar mass lignin followed by anhydrous acetone extraction, and finally, a purification process by the MTBE/EtOAc mixture. The lignin fraction with decreased heterogeneity has a moderate molar mass (M_n = 6129 g/mol, M_w = 9404 g/mol with PDI = 1.53), a low amount of remaining carbohydrates (2.82 mg/g), a high content of total aromatic units (69.67%), and a low sulfur percentage (1.03%). However, one should keep in mind that the characteristics of different technical lignins, such as wood extractive content, carbohydrate content, molar mass distribution, and the content of each molar mass can vary dramatically depending on the delignification processes and the delignification stage of lignin separation. It is necessary to have a comprehensive understanding about the compositions as well as the chemical characteristics of technical lignin for their fractionation and valorization.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c04725.

GC/MS results of water-removed substances, MTBE-extracted substances, cyclohexasulfur and cyclooctasulfur isolated from softwood KL; the HSQC results and the main lignin structure assignment; GC-FID and GC–MS results of acid methanolysis of sugar moieties; and the Py/GC–MS results of sequential lignin fractions of KL_{EtOAc}, KL_{EtOH}, KL_{MeOH}, $KL_{Acetone}$, and $KL_{Dioxane}$, and the lignin fraction with deceased heterogeneity (PDF)

AUTHOR INFORMATION

Corresponding Authors

Hongbo Zhang − *Pharmaceutical Sciences Laboratory and Turku Bioscience Center, Åbo Akademi University, Turku, Turku FI-20500, Finland;* orcid.org/0000-0002-1071-4416; Email: hongbo.zhang@abo.fi

Chunlin Xu − *Laboratory of Natural Materials Technology, Åbo Akademi University, Turku, Turku FI-20500, Finland;* orcid.org/0000-0003-1860-9669; Email: chunlin.xu@abo.fi

Authors

Rui Liu − *Laboratory of Natural Materials Technology, Åbo Akademi University, Turku, Turku FI-20500, Finland*

Annika Smeds − *Laboratory of Natural Materials Technology, Åbo Akademi University, Turku, Turku FI-20500, Finland*

Luyao Wang − *Laboratory of Natural Materials Technology, Åbo Akademi University, Turku, Turku FI-20500, Finland*

Andrey Pranovich − *Laboratory of Natural Materials Technology, Åbo Akademi University, Turku, Turku FI-20500, Finland*

Jarl Hemming − *Laboratory of Natural Materials Technology, Åbo Akademi University, Turku, Turku FI-20500, Finland*

Stefan Willför − *Laboratory of Natural Materials Technology, Åbo Akademi University, Turku, Turku FI-20500, Finland*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssuschemeng.1c04725

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Notes

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