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Review Article

Structural Characterization of Lignin and Its Degradation Products with Spectroscopic Methods

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Lignin is highly branched phenolic polymer and accounts 15–30% by weight of lignocellulosic biomass (LCBM). The acceptable molecular structure of lignin is composed with three main constituents linked by different linkages. However, the structure of lignin varies significantly according to the type of LCBM, and the composition of lignin strongly depends on the degradation process. Thus, the elucidation of structural features of lignin is important for the utilization of lignin in high efficient ways. Up to date, degradation of lignin with destructive methods is the main path for the analysis of molecular structure of lignin. Spectroscopic techniques can provide qualitative and quantitative information on functional groups and linkages of constituents in lignin as well as the degradation products. In this review, recent progresses on lignin degradation were presented and compared. Various spectroscopic methods, such as ultraviolet spectroscopy, Fourier-transformed infrared spectroscopy, Raman spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy, for the characterization of structural and compositional features of lignin were summarized. Various NMR techniques, such as ^{1}H, ^{13}C, ^{19}F, and ^{31}P, as well as 2D NMR, were highlighted for the comprehensive investigation of lignin structure. Quantitative ^{13}C NMR and various 2D NMR techniques provide both qualitative and quantitative results on the detailed lignin structure and composition produced from various processes which proved to be ideal methods in practice.

1. Introduction

The main components of lignocellulosic biomass (LCBM) are cellulose, hemicellulose, and lignin. Cellulose is a polymer of glucose, accounting for 30–50 wt% of dry LCBM; hemicellulose is a mixture of heteropolymers containing various polysaccharides, such as xylan, glucuronoxylan, and glucomannan, accounting for 20–35 wt%; the mainly remaining portion with 15–30 wt% is lignin, which is a multisubstituted phenolic polymer. Lignin is the most abundant aromatic biopolymer accounting for up to 30% of the organic carbon on Earth and thus can be treated as a potential renewable feedstock for energy supplement and aromatic chemicals production [1, 2]. The annual production of lignin is more than 70 million tons [3]. The most abundant industrial lignins are produced from kraft and sulfite pulping processes in the pulp and paper industries, so-called black liquor. However, only less than 2% of the lignin produced from pulping industries was value-addedly utilized, while the rest was abandoned or burned as a low-value fuel for energy supplement [4], leading to serious waste of precious aromatic resource and environmental pollution.

Lignin is an amorphous, irregular three-dimensional, and highly branched phenolic polymer. The functions of lignin in the plant cell wall are to cover structural support, transport water and nutrients, and issue protection to prevent chemical or biological attacks, and so forth. Though the chemical structure is extremely complex, it is generally accepted that lignin is formed via irregular biosynthesis process constructed from three basic phenylpropanoid monomers,

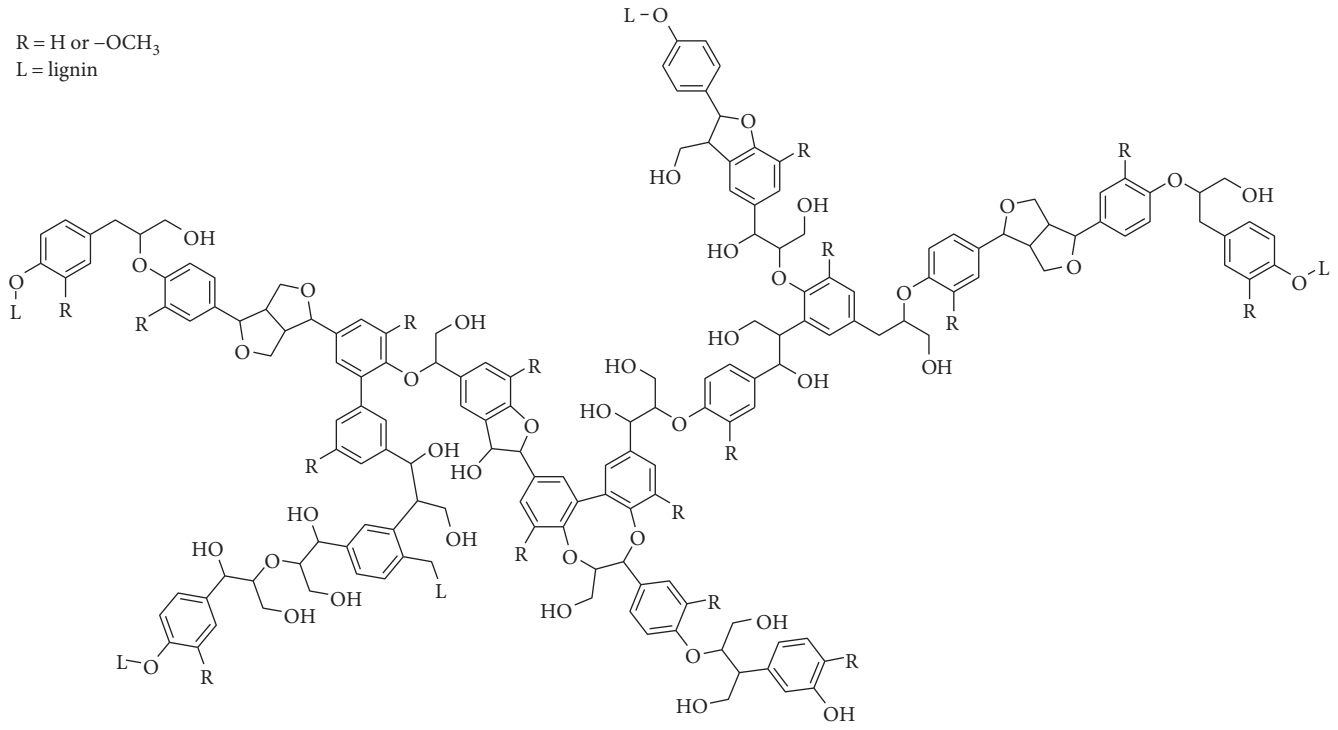

Figure 1

p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, derived from *p*-coumaryl, coniferyl, and sinapyl alcoholic precursors, respectively. Figure 1 shows a typical structural model of lignin. Gymnosperms contain almost entirely G unit in lignins; dicotyledonous angiosperms contain G and S units in lignins; and all the G, S, and H units can be found in monocotyledonous lignins [5]. Other units with relatively fewer contents were also identified in the lignin of LCBM, such as ferulates and coumarates [6]. Biosynthesis of lignin is a process that monomers undergo radical coupling reactions to form racemic, cross-linked, and phenolic polymer, by which lignin content and composition may vary significantly in different LCBMs [7]. Furthermore, the structure of lignin even varies among different tissues and ages of the same individual of LCBM [8].

Typical lignin contents are 24–33% in softwoods, 19–28% in hardwoods, and 15–25% in grasses, respectively. Functional groups in lignin include methoxyl, carbonyl, carboxyl, and hydroxyl linking to aromatic or aliphatic moieties, with various amounts and proportions, leading to different compositions and structures of lignin [9]. Various linkages (see Figure 2) either in C-C or C-O type with different abundances formed in the coupling reactions involved in biosynthesis of lignin, including aryglycerol-β-ether dimer (β-O-4, 45–50%), biphenyl/dibenzodioxocin (5–5′, 18–25%), pino/resinol (β-5, 9–12%), diphenylethane (β-1, 7–10%), aryglycerol-α-ether dimer (α-O-4, 6–8%), phenylcoumaran (β-β', 0–3%), siaryl ether (4-O-5, 4–8%), and spirodienon.

It is difficult to draw accurate structural diagram for entire lignin by using up-to-date techniques in situ. Although relative new methods for imaging and analyzing chemical structure of lignin, such as confocal Raman scattering microscopy [10] and time-of-flight secondary ion mass spectrometry [11, 12], can provide chemical and spectral imaging of lignin for the distribution of componential units with high resolution and sensitive, these techniques are only available in several biological labs and have not been employed widely by chemical scientific groups. Up to now, the comprehensive elucidation of structural and compositional features of lignin relies on the processes for the degradation and isolation of lignin from LCBM and methods applied in the characterization of the corresponding products [2, 13]. However, in the degradation process, the original structural and compositional features of lignin may be sometimes ambiguous or even missed. Different degradation processes produce different types of lignins with various structures and compositions; furthermore, a specific analytical technique gives partial and/or limited information and is not able to provide a general picture for the entire lignin. The industrial applications of lignin are limited critically due to its complex nature and undefined chemical structure. For example, commercially purchased kraft lignins from softwoods may have different compositions as well as their structures [14]. Furthermore, the lignin-carbohydrate complex (LCC) increases the difficulty of structural analysis and isolation of lignin from LCBM [15]. The value-added utilization of lignin and its degradation products are one of the ultimate goals especially for biorefineries; therefore, the comprehensive understanding of the structure of lignin is crucial necessary, since it can provide theoretical direction on constructing and optimizing degradation processes, generating of valuable aromatic chemicals

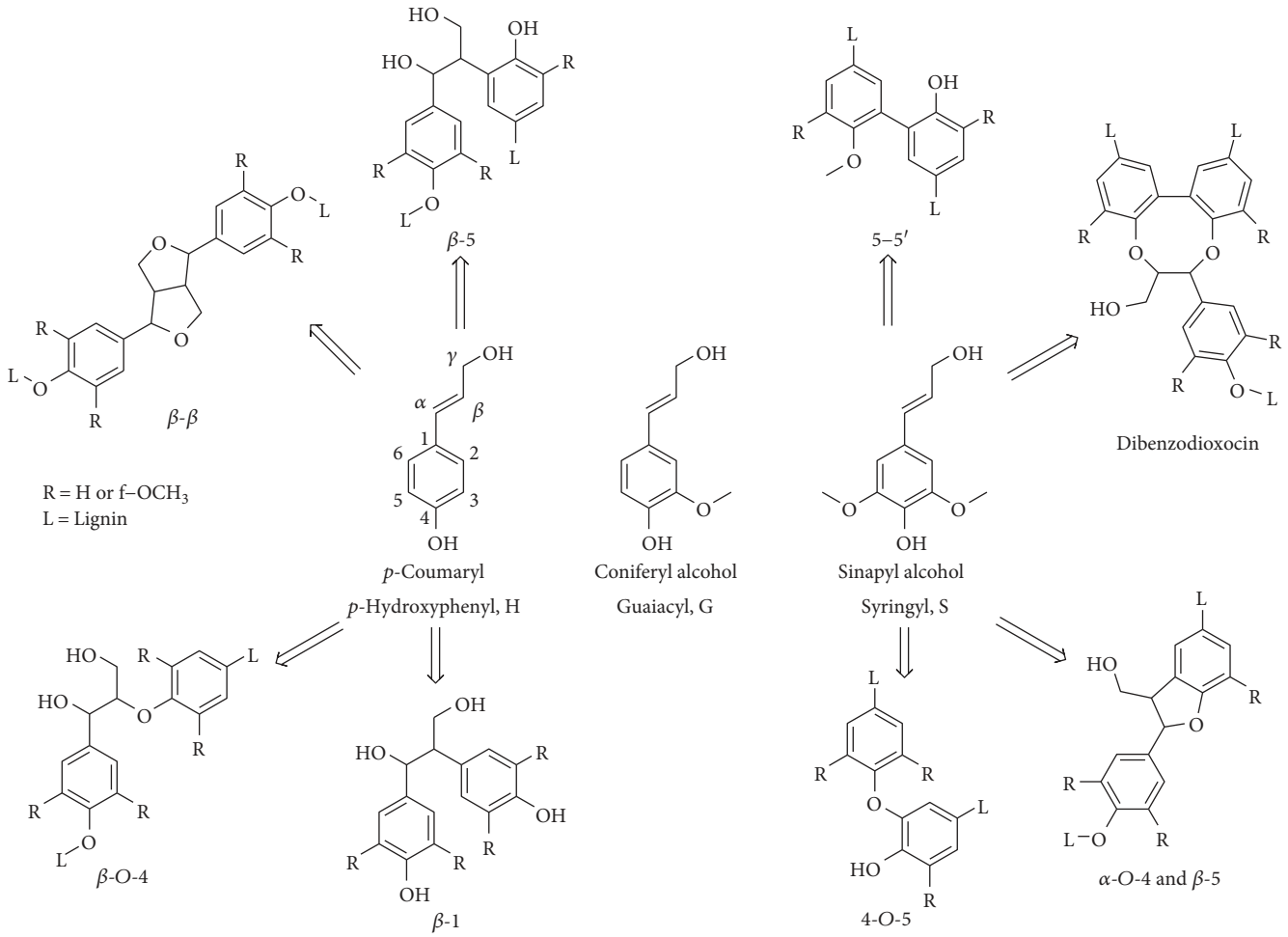

Figure 2

to act as low-molecular-mass feedstocks [16], estimating the economic viability, and so forth.

Traditionally, there are two ways to isolate lignin from other components in LCBM, so-called degradation processes: one is to extract cellulose and hemicellulose leaving most of the lignin as solid residue, and the other one is to extract lignin by using fractionation methods leaving the other components. For the former process, dilute sulfuric acid and hot water are often used to break down cellulose and hemicellulose releasing sugars and facilitating the further enzymatic hydrolysis, while leaving lignin as the main content in solid residue. For the later process, hydroxide solution, either with sodium, potassium or calcium, is used to remove lignin from LCBM samples. The degradation processes are designed to cleave the bonds between lignin and carbohydrates, leading to more or less extensive changes compared to native lignin structure. Consequently, the chemical compositional features of the resulting technical lignins, such as the relative abundance of S/G/H units, the status of side chains, and the contents of functional groups, are highly dependent on the methods and conditions used in degradation processes [17]. The most common linkages in lignin, namely, β-O-4 linkages, are relatively weak linkages and are the key target of most degradation pretreatments. Other linkages, such as β-5, β-1, β-β', 5–5′, and 4-O-5, are more complicated and difficult to be degraded. Toward the structural investigation, various lignins are produced via different degradation processes, such as milled wood lignin (MWL), acidic lignin, sulfite lignin, soda lignin, kraft lignin, organosolv lignin, cellulolytic enzyme lignin (CEL), enzymatic mild acidolysis lignin (EMAL), and lignin from thioacidolysis process [2]. In the recent years, extraction and depolymerization with ionic liquids (ILs) for the isolation or degradation of lignins were considered to be promising processes [18, 19].

For the structural and compositional elucidation of complex samples, various instrumental methods were used. For example, the chromatographic techniques coupled to mass spectrometers and high-resolution mass spectrometric techniques were used extensively in the analysis of the bio-oil, biomass, and lignin samples [20–24]. These methods concentrate on the detection of individual species basing on the chromatographic separation and high molecular resolution. However, on the other hand, spectroscopic methods, such as ultraviolet spectroscopy (UV), Fourier-transformed infrared spectroscopy (FTIR), and nuclear magnetic resonance

(NMR), concern about the analysis of the whole structure and direct detection of moieties in samples over degradation techniques [25, 26]. Detailed spectrometric information related to structural features, including functional groups, bond types, and chemical state of atoms, can be obtained. Furthermore, both of the qualitative and quantitative analyses can be carried out simultaneously.

In this review, we focused on the recent development and interesting findings on the structural investigation of lignin with spectroscopic methods over various degradation processes. Structural and compositional characters of lignin samples produced from different degradation processes were presented and compared, and developments of spectroscopic methodologies on the qualitative and quantitative elucidation of lignin structure were also summarized. The degradation processes and instrumental methods involved in the detailed and comprehensive understanding of the lignin structure were prospected.

2. Degradation Processes of Lignin

Various physical/chemical methods were carried out for the degradation and isolation of lignin. Optimization or modification of these methods was conducted on various LCBMs due to the difference in the structure of lignin. In order to facilitate further structural and/or compositional analyses or to produce high purity lignin, modified or multistep processes were usually carried out.

2.1. Milled Wood Lignin. MWL is produced via the extraction of milled sample particles from LCBM with a neutral organic solvent (e.g., 1,4-dioxane) under mild conditions to remove other components. In the extraction process, only minor changes may occur with respect to the milled sample; hence, the obtained lignin has similar property with the milled sample. Nevertheless, MWL is not considered to be a representative of the original lignin in the LCBM due to its relative low yield (based on Klason lignin).

2.2. Cellulolytic Enzyme Lignin. In order to improve the yield, CEL was developed from the extraction of enzymatically hydrolyzed MWL residue. Typically, the residual carbohydrate contents in CEL account 10–15 wt% of initial MWL sample. The structure of CEL is similar to MWL, and it is more representative of total lignin in LCBM than in MWL. CEL has commonly been used for the structural analysis of lignin in the cell wall of plants. In a recent study, cellulolytic enzyme hydrolysis was carried out prior to water/dioxane extraction of MWL to remove carbohydrates. The lignin was obtained with high yield and purity [27]. Enzymatic lignin degradation has several advantages such as mild conditions and potentially fewer inhibitors for microbes. However, the degradation of lignin in LCBM still gave a very low yield of fragmented and soluble lignin, which may due to the limitations on efficient electron transfer [28] in the process.

2.3. Sulfite, Soda, and Kraft Lignins. Sulfite, soda, and kraft lignins are by far the main technical lignins produced via industrial processes. Among them, sulfite and kraft methods are sulfur-involving processes, accounting more than 90% of the chemical pulp production worldwide [29], and soda method is sulfur-free process. In the sulfite process, water-soluble lignosulfonates are formed. Further purification is needed to remove unexpected carbohydrate impurities. This process produces the largest amount of technical lignin. However, the obtained lignin contains considerable amount of sulfur. In the soda process, lignin is dissolved in hydroxide solution and following steps including precipitation, maturation, and filtration. In the kraft process, LCBM particles are emerged in an aqueous solution containing NaOH and Na_2S. Lignins are depolymerized as water-/alkali-soluble fragments with approximately 70–75% of the hydroxyl groups become sulfonated. Industrially, kraft lignin, produced chemically from the lignin degradation in aqueous alkali, is the major constituent of black liquor (90–95%). Neither kraft lignin nor sulfite lignin is suitable for investigating the original native structures of lignins, because significant structural changes occur especially the cleavage of α-O-4 and β-O-4 linkages under the conditions of these processes. Additionally, undesirable impurities such as sulfurous compounds or carbohydrates are present in derived lignin for these fractionation processes. Currently, almost all the produced technical lignins are only high yield industrial by-products and recovered as low-value fuel. This dilemma may rely on the progress in structural characterization of lignins from various LCBMs and the further upgrading of the technical lignins targeting value-added chemical production.

2.4. Organosolv Lignin. In the organosolv process, high purity lignin and cellulose are produced at the same time with various solvents; however, no technical lignins are commercially available from this process up to now. Organosolv process typically results in more than 50% lignin removal from LCBM through cleavage of lignin-carbohydrate bonds and β-O-4 linkages. The separation of organosolv lignin can be achieved either by removing of the solvent or by precipitation with water followed by distillation. Most organosolv lignin is easily soluble in basic solutions and polar solvents, that is, ethanol or ethanol/water mixture, but will be insoluble in acidic aqueous solutions. Organosolv lignin is sulfur-free, high purity, and rich in functionality including phenolics, exhibits a narrow polydispersity, and has limited carbohydrate contamination.

The extraction conditions affect the structure of organosolv lignin, that is, severity factor (H-factor). The molecular weight of the ethanol organosolv lignin decreased within a 36–56% range with respect to the MWL with the increase of the severity. Moreover, an obvious decrease in the content of aliphatic hydroxyl groups and an increase of syringyl phenolic units and condensed phenolic structures with the increase in severity of the organosolv treatment were also observed [30]. An integrated process of hot water extraction followed by high-boiling-solvent cooking with 1,4-butanediol can fractionate bagasse vigorously into cellulose, hemicelluloses, and lignin. The organosolv lignin formed exhibited a chemical structure similar to EMAL with more newly formed phenolic OH groups [31].

2.5. Acidic Lignin. Traditionally, in the acidolysis process, lignin is extracted from LCBM sample with 1,4-dioxane containing hydrochloric acid under room temperature. The obtained lignin with high purity is considered to be a representative of the original lignin. However, a limitation of this process is that the same conditions used to hydrolyze polysaccharides also degrade the liberated monosaccharides, leading to overestimate monosaccharide degradation and introducing bias between polysaccharides of different liability. Modifications were introduced to reduce these errors [32]. A modified acidolysis process was carried out by Gong et al. [33]. The acetic acid lignin from bamboo shoot shell had a higher yield of lignin (74 wt%) and lower content of associated carbohydrates (2.96 wt%) than MWL (5.16 wt%). Additionally, acetic acid lignin possessed a molecular weight 2789 Da and a narrow polydispersity index (i.e., $M_w/M_n = 1.54$). Higher phenolic hydroxyl group content and S/G ratio were also obtained in this lignin compared to MWL [33]. Enzymatic mild acidolysis lignin (EMAL) is obtained from acidolysis of CEL with dilute acid, such as hydrochloric acid. The remaining carbohydrates linking to lignin can be removed further in the acidolysis producing lignin with higher purity [34].

2.6. Thioacidic Lignin. Modified acidolysis processes were carried out to produce lignin with high yield and purity. Thioacidolysis process, in which ethanethiol is used instead of water, produced more lignin and less complex monomer mixtures. In this process, thioethylated H, G, and S monomers by the cleavage of β-O-4 ether linkages are produced. Traditional thioacidolysis methods require several steps before down streaming analysis or further treatments. Hence, higher-throughput quantitative method is needed for screening various types of LCBMs [35].

2.7. Ionic Liquid Degradation Lignin. IL provides an alternative path for lignin removal to classic organosolv pretreatment for enhancing subsequent enzymatic hydrolysis and isolation. Some ILs, such as 1-ethyl-3-methylimidazolium acetate, can extract lignin from poplar and birch with most structural features retained [36]. Some acidic ILs, such as 1-*H*-3-methylimidazolium chloride, will hydrolyze ether linkages [37] and further degrade lignin. The following are some recent progresses concentrated on the lignin degradation and isolation by ILs.

The ILs containing 1-butyl-3-methylimidazolium (bmim), 1-ethyl-3-methylimidazolium (emim), and 1-allyl-3-methylimidazolium (amim) cations either with acetate or chloride as the anions are commonly used in the lignin dissolution [38]. ILs have the capability to disrupt various linkages between the components in the LCBM by the formation of several types of interactions such as hydrogen bond, dipole-dipole, and van der Waals interactions [39]. Pyridinium formate (PyFor) showed a high capacity for the dissolution of kraft lignin (70 *w*/*w*%) at a relatively lower temperature (75°C) [40].

Cholinium ILs are novel bio-ILs used in the lignin valorization, in which different chemical reactions take place during the lignin dissolution from imidazolium ILs [41]. In the dissolution of kraft lignin in cholinium ILs, significant changes in the structure and thermal properties of kraft lignin occurred via depolymerization, dehydration, and demethoxylation followed by recondensation. Thermal properties of kraft lignin were altered, that is, increased the maximal decomposition temperature (T_m) and glass transition temperature (T_g); and the molecular weights were reduced after regeneration from cholinium ILs [41].

Other ILs, such as 1-ethyl-3-methylimidazolium xylenesulfonate [emim][ABS] and 1-butyl-3-methylimidazolium methylsulfate [bmim][$MeSO_4$], could promote depolymerization of organosolv lignin and Klason lignin under the oxidative conditions using a Cu/EDTA complex in the presence of a monomeric phenol (4-tert-butyl-2,6-dimethylphenol) [42].

An acidic IL, called 1-(4-sulfobutyl)-3-methyl imidazolium hydrosulfate ([$C_4H_8SO_3Hmim$]HSO_4), was proven to be an efficient catalyst for direct liquefaction of bagasse lignin, where more than 65% degree of liquefaction and 13.5% yield of phenolic monomer without any char formation [43].

A switchable ionic liquid (SIL), synthesized from 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), monoethanol amine (MEA), and CO_2, named CO_2-switched [DBU][MEASIL], was demonstrated to have high ability to extract the interlinked polysaccharide impurities from the sodium lignosulfonate while the linkages and aromatic subunits remain unaffected during the dissolution-recovery cycle. This SIL can be used as an affordable solvent medium to obtain carbohydrate-free lignin from an impure lignin source [44].

Future developments on the IL degradation of lignin will focus on selective lignin extraction/degradation and functionalization as well as minimization of process costs for recovery and recycling of ILs.

2.8. Multistep Processes. Multistep processes were used to enhance the removal of lignin. A two-step process was carried out in which anhydrous ammonia pretreatment was followed by mild NaOH extraction on corn stover to solubilize and fractionate lignin [45]. Lignin removal of more than 65% with over 84% carbohydrate retention was achieved. Furthermore, a significant reduction in the weight-average molecular weight (M_w) of extracted lignin was also achieved. Synergistic effects were found in the combination of pretreatments to enhance the isolation or conversion of lignins [28, 46]. In the sequential fractionation of *Tamarix spp.*, MWL, organosolv lignin, and alkaline lignin were conducted with dioxane, alkaline organosolv, and alkaline solutions, respectively. The results indicated that the alkaline organosolv extraction released a higher yield of lignin (17.7%) than dioxane and alkaline solution extractions. Small amounts of carbohydrates (0.79%) were detected in the organosolv lignin fraction, suggesting a significant cleavage of α-ether bonds between lignin and carbohydrates in the alkaline organosolv fractionation process [47].

2.9. Comparison of the Processes. Alkaline lignins were found to have higher carbohydrate content (up to 30 wt%) with higher molecular weights around 3000 Da; on the other hand, organosolv lignins had considerable high purity (better than

93 wt%) with molecular weights in the range of 600–1600 Da [48]. The structure and composition of alkali lignin, CEL, and MWL from valonea of *Quercus variabilis* Blume were compared by Yang et al. [49]. The isolation processes of alkali lignin and CEL caused some damages to the structure of lignin. The β-O-4 linkages were largely cleaved during the CEL process since the relative content of β-O-4 linkages in CEL was much lower than those in alkali lignin and MWL. High S/G ratio for alkali lignin was observed, indicating that the S-units were easily released under the alkali conditions.

Yang et al. [50] compared four lignins produced from valonea of *Quercus variabilis*, namely, ethanol lignin, alkali lignin, MWL, and enzyme hydrolysis lignin (EHL). The results showed that the four lignins contained GSH-type with little differences. The MWL contained the least functional groups with the poorest thermostability and the highest antioxidant activity. The EHL had the highest molecular weight (i.e., $M_w = 1429$ g/mol; $M_n = 746.18$ g/mol). In a comparison of pretreatments on hardwood (red oak), softwood (loblolly pine), and herbaceous biomass (corn stover) for lignin valorization through pyrolysis, organosolv lignins contained fewer volatiles in comparison to the corresponding MWLs for all the tested samples [51]. Red oak lignin was affected mostly by the organosolv process, since the greatest decrease in volatile content and increase in carbon content were observed. Corn stover lignin had the highest potential for volatilization because it retained highly branched polymer structure enriched in tricin, ferulate, and coumarate groups.

Clearly, different degradation processes or pretreatments have significant influence on the compositional and structural features of lignin. The selectivity and efficiency of these processes are the main consideration. To elucidate the original structure of lignin, relatively undestroyed and effective degradation methods are feasible, such as IL extraction and organosolv process. To produce value-added chemical from lignin, more aggressive methods aiming at the cleavage of the weak linkages in lignin (i.e., β-O-4 linkages) and the interunits between lignin and polysaccharides can be used in the degradation process. Of course, biological conversion with suitable selectivity might be another orientation for degradation of lignin [52].

3. Spectroscopic Methods

Structural investigation of lignin with spectroscopic techniques has been considered to be promising high-throughput and routine methods, which can provide detailed qualitative and quantitative information on structural features including functional groups, types of chemical bonds, and states of atoms.

3.1. UV Spectroscopy. The content of acid-soluble lignin, the purity, and the components of isolated lignin, can be determined by using UV spectroscopy [53]. National Renewable Energy Laboratory (NREL) proposed an accurate method for the determination of lignin, by which the absorbance of lignin was recorded at the recommended wavelength [54]. According to the intrinsic structure of lignin, several absorption maxima attributed to different functional groups were observed, as shown in Table 1 [25, 30, 50, 53–56]. The determination of phenolic hydroxyl groups can be achieved basing on the difference in absorption at 292 and 370 nm between phenolic units in neutral and alkaline solutions [30, 50, 55, 56]. Attributed to the symmetrical syringyl unit, the maximum absorbance of lignins produced from different processes exhibited a blue shift. Furthermore, an additional absorbance at approximately 370 nm due to the presence of conjugated phenolic hydroxyl groups was also observed [50] (see Figure 3).

Table 1: UV spectroscopic absorptions of typical structures in lignin [25, 30, 50, 53–56].

Absorption maxima/nm	Electronic transition style	Chromophores and structures
200	π-π^*	Conjugated bonds/aromatic ring
240	n-π^*	Free -OH
282	π-π^*	Conjugated bonds/aromatic ring
320	π-π^*	Aromatic ring conjugated bond with C = C
320	n-π^*	C = O groups conjugated to aromatic ring
325	n-π^*	Etherified ferulic acid

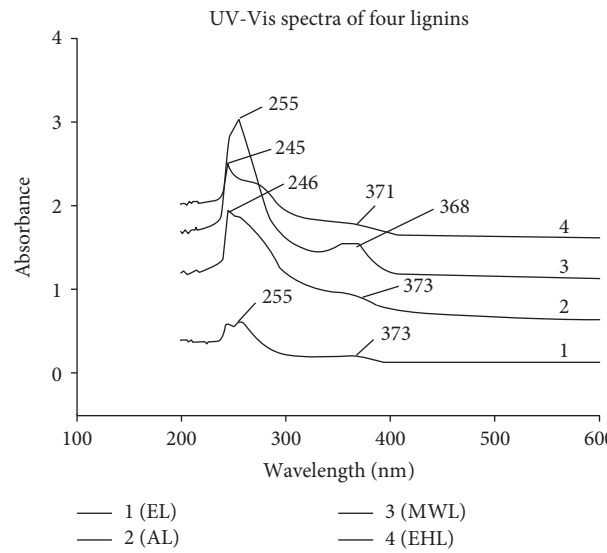

Figure 3

Basing on the Lambert-Beer's Law, UV spectroscopy can be used for the semiquantitative determination of the purity of lignin and its degradation products by using extinction coefficient (EC) [57, 58]. Because of the cross-linking structures of lignin with carbohydrates, cellulose, and hemicelluloses, the isolation of pure lignin is extremely difficult. The low value of EC represents the high nonlignin substance content in the isolated lignin.

3.2. FTIR Spectroscopy. FTIR spectroscopy is the most widely used technique in the functional group determination basing

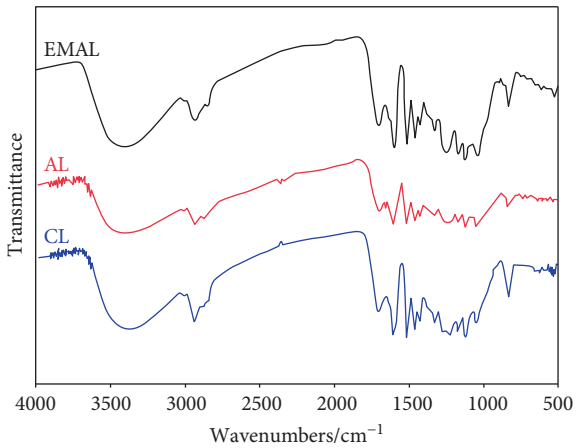

Figure 4

on the substances with chromophores. It can be treated as a nondestructive, noninvasive, highly sensitive, and rapid technique. Typical functional groups contained in lignin, such as hydroxyl, carbonyl, methoxyl, carboxyl, and aromatic and aliphatic C-H, can be assigned well in the FTIR spectrum. Figure 4 shows FTIR spectra for detection of different lignins, namely, EMAL, autocooking lignin (AL), and citric acid-catalyzed cooking lignin (CL) [31]. For the assignments of signals in FTIR spectra, Table 2 lists the typical wavelength assigned for possible functional groups and structures in lignin [25, 29, 38].

Attenuated total reflectance- (ATR-) FTIR could be used for the evaluation of kraft lignin in acylation with different acyl chlorides [59] and lignin structural changes during the cooking process with solid alkali and active oxygen [60]. FTIR spectroscopy could also characterize changes in the chemical structure of wood polymers in relation to the tree growth location and conditions [61]. Untreated solid samples (Norway spruce, *P. abies L. Karst.*) from three provenances in Europe were selected. Principal component analysis (PCA) and cluster analysis (CA) were used for evaluation of spectral data obtained by FTIR spectroscopy. The results showed that the samples belonging to the same wood species differ due to the origin. FTIR analysis was able to correctly discriminate samples originating from three different provenances in Europe.

It is known that functional properties of oxyethylated lignins (OELs) and the resulting substances are strongly affected by the degree of oxyethylation (DOE) of phenolic hydroxyl groups (OH_{phen}). Passauer et al. [62] found the strong linear correlations between OH_{phen} contents of lignin/OEL and FTIR vibrations attributed to phenolic and aliphatic acetoxy groups. With appropriate calibration, FTIR spectroscopy combined with sample preacetylation is considered to be a promising tool for rapid and accurate determination of the DOE of OELs with qualitative and quantitative results.

3.3. Raman Spectroscopy. Raman spectroscopy, as the sister spectroscopic technique of FTIR, can provide complementary information on the structural features even for the samples containing water. Furthermore, more absorption bands were detected with Raman spectroscopy than FTIR [63]. Generally, the assignments of the absorption bands in Raman spectra are similar with FTIR spectra.

Raman spectroscopy is suitable for the investigation of the chemical structure of lignin, because it can provide in situ determination on the cell wall of plants even with no sample preparation. However, when analyzing a lignin sample in solutions with various solvents, one should consider the environmental effects of the solvents [64]. Confocal Raman microscopy was used to investigate the structural changes of lignocellulosic cell walls during the dilute acid pretreatments. According to the intensity of the Raman images, the ratio of lignin/cellulose [I(1600 cm^{-1})/I(900 cm^{-1})] was low for oxalic acid-pretreated biomass compared to sulfuric acid-pretreated biomass [65].

3.4. NMR Spectroscopy. NMR spectroscopy provides more precise and comprehensive information on qualitative and quantitative assays for the frequencies of linkages and the composition of H/G/S units in the lignin analysis. The first discovery of dibenzodioxocine and spirodienone structures in lignin was carried out by Ralph et al. [66] and Zhang et al. [67], respectively. ^{1}H, ^{13}C, ^{19}F, and ^{31}P as well as various 2D NMR spectroscopic techniques can be used in the structural and compositional analyses of lignin. Among them, ^{1}H and ^{13}C NMR tend to be the regular tools for the analysis of lignin; and solid-state ^{13}C NMR and 2D heteronuclear single-quantum coherence (HSQC) NMR can provide accurate quantitative results on the functional groups and side chain moieties.

Compared with the spectroscopic methods mentioned above, NMR spectroscopic methods possess much higher resolution and enable a larger amount of information to be obtained. One-dimensional (1D) NMR methods, including ^{1}H, ^{13}C, ^{19}F, and ^{31}P NMR, and two-dimensional (2D) NMR methods, such as 2D HSQC NMR, were applied for the analysis of lignin samples with both solid and liquid states. The distribution of functional groups and amount of linkages and H/G/S units as well as other components in lignin can be qualitatively and quantitatively determined. The chemical shifts of functional groups in the spectra have been established.

^{1}H NMR is the method routinely used in the structural investigation of lignin, because of the simple preparation of samples and fast scanning speed. Almost all the compositional investigations of lignins use ^{1}H NMR for the detection of the chemical environment of proton. In the spectra, the signal observed around 7.5 ppm can be assigned to aromatic protons of H units and the other two chemical shifts around 7.0 ppm and 6.5 ppm are attributed to aromatic protons in G and S units, respectively [68, 69]. The chemical shifts in the range of 6.3–4.0 ppm are assigned to aliphatic protons in the linkages of β-O-4, β-β, and β-5. The signals in the range of 4.0–3.5 ppm are attributed to protons in methoxyl groups. The chemical shifts around 3.10 ppm may be attributed to the protons in anhydroxylose units [31, 70]. Typical peeks are assigned to functional groups in lignin, as shown in Table 3 [25, 31].

Table 2: Assignments of signals in FTIR spectrum to functional groups in lignin [25, 29, 38].

Wavenumbers/cm^{-1}	Assignments	Functional groups and structures in lignin
3400–3600	ν (O-H)	Free -OH
3100–3400	ν (O-H)	Associated -OH
2820–2960	ν (C-H)	$-CH_2$, $-CH_3$
2920	ν (C-H)	Carboxylic -OH
2650–2890	ν (C-H)	Methyl group in methoxyl
1771	ν (C = O)	Aromatic
1700–1750	ν (C = O)	Unconjugated ketones, carbonyls, and ester groups
1722	ν (C = O)	Aliphatic
1650–1680	ν (C = O)	Conjugated *p*-substituent carbonyl and carboxyl
1500–1600, 1420–1430	ν (aromatic skeletal)	Benzene ring
1450–1470, 1360–1370	ν (C-H)	$-CH_2$, $-CH_3$
1325–1330, 1230–1235	ν (C-O)	Syringyl ring
1270–1275	ν (C-O)	Guaiacyl ring
1215	ν (C-O)	Ether
1140–1145	ν (C-H)	Guaiacyl
1130	ν (C-H)	Syringyl
1085–1090	ν (C-O)	Secondary alcohol and aliphatic ether
1025–1035	ν (C-O, C-H)	Aromatic ring and primary alcohol
750–860	ν (C-H)	Aromatic ring

Table 3: Assignments of signals in ^{1}H NMR spectrum to typical functional groups in lignin (in CD_3Cl) [25, 31].

Chemical shift/ppm	Assignments
9.7–9.9	Cinamaldehydes and benzaldehydes
6.7–7.1	Aromatic-H in guaiacyl
6.2–6.7	Aromatic-H in syringyl
5.8–6.2	Benzylic OH in β-O-4 and β-1
4.9–5.1	Carbohydrates
3.3-4.0	Methoxyl
3.0–3.1	H_β in β-1
2.2–2.4	Phenolic OH
1.6–2.2	Aliphatic OH

^{13}C NMR can be carried out to overcome the overlapping resonances of some structures in ^{1}H NMR spectra, providing qualitative and quantitative results with nondestructive detection of solid or solution samples. Although with a higher resolution, it is recommended that relative pure lignin sample is necessary in the ^{13}C NMR analysis, since the unexpected overlapping of spectra was due to the complexity of sample. Typical ^{13}C NMR spectra are shown in Figure 5 [31], and the assignments of signals are presented in Table 4 [31, 38, 49, 71]. By using the data from quantitative ^{13}C NMR, basic parameters which summarizes the main structural characteristics of lignins can be obtained, such as content of β-O-4 structures, degree of condensation, and unit ratio of S/G/H. Radar plots include these parameters and allow a direct classification of different lignins by comparison of the key descriptors [71]. Solid-state ^{13}C NMR analysis is a nondestructive method and not limited by sample insolubility. The cross-polarization/magic angle spinning (CP/MAS) method extensively used NMR technique for elucidating the structure of lignin. The detections take a very short time with high resolution; however, the quantitative analysis of CP/MAS is not sufficient enough [72]. Solid-state ^{13}C NMR is considered to be an advanced method for structural investigation of LCBM at atomic level; however, by using this technique, the structure remains largely unexplored due to the complexity of lignin and the severe spectral crowding of the responding signals [73]. A sensitive hyperpolarization solid-state NMR technique by combining high-field dynamic nuclear polarization (DNP) and MAS was used to improve the resolution of the determination [74]. Furthermore, this technique can provide 2D homonuclear ^{13}C-^{13}C correlation solid-state NMR spectra at natural isotopic abundance, yielding, and an atomic level structural investigation [75, 76]. Most of current lignin content analytical techniques require solo or sequential degradation or dissociation steps, which are time-consuming. By using the solid-state ^{13}C CP/MAS NMR technique with an internal standard (sodium-3-trimethylsilylpropionate, TMSP), a simple yet reliable method was established to analyze content of lignin in various LCBMs without destroying their native structures [77].

Constant et al. [78] carried out the quantification and classification of carbonyls in industrial humins and lignins by ^{19}F NMR. The carbonyl groups were transformed to corresponding hydrazone with 4-(trifluoromethyl)phenylhydrazine before quantification by ^{19}F NMR. By using model compound library, the carbonyl functional groups in Indulin Kraft and Alcell lignins were quantified and classified for the first time.

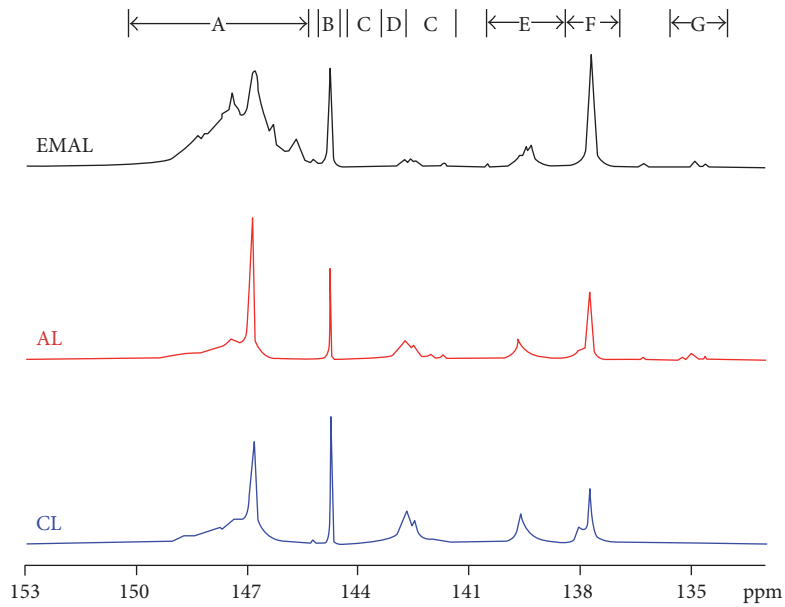

Figure 5

Table 4: Assignments of signals in ^{13}C NMR spectrum to functional groups in lignin [31, 38, 49, 71].

Chemical shift/ppm	Assignments
167–178	Unconjugated -COOH
162–168	Conjugated -COOH
140–155	C_3, C_4 aromatic ether or hydroxyl
127–140	C_1, aromatic C-C
123–127	C_5, aromatic C-C
117–123	C_6, aromatic C-H
114–117	C_5, aromatic C-H
106–114	C_2, aromatic C-H
78–90	Aliphatic C_β-O
67–78	Aliphatic C_α-O
54–57.5	Methoxyl

^{31}P NMR has also been widely used to quantitatively determine the amount of aliphatic and phenolic hydroxyl groups as well as carboxyl groups in lignin after phosphitylation with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) [29, 79, 80]. The high phenolic OH content reflecting the presence of condensed aromatic units, such as 5–5 units, was found by ^{31}P NMR in a biolignin produced by acetic acid/formic acid/water hydrolysis from wheat straw [81]. The ^{31}P NMR analysis of the insoluble fraction of kraft lignin provided an accurate and quantitative way to illustrate the effects of the laccase-HBT (1-hydroxybenzotriazole) system on lignin chemical bond cleavage [82]. Typical ^{31}P NMR spectra and signal assignments are shown in Figure 6 and Table 5, respectively [29, 82].

Solid-/solution-state ^{13}C NMR spectroscopes are powerful in lignin structural elucidation either in their solid or solution state. However, solid-state ^{13}C NMR spectroscopy is only suitable for the analysis of lignin samples that have restricted solubility and can observe some structural features of lignin due to its low resolution; and lignin is subjected to acetylation by anhydride/pyridine solution before the solution-state ^{13}C NMR spectrum collection [83] since dissolving lignin is difficult.

Various 2D NMR methods were carried out to overcome the overlapping of resonances in 1D NMR with higher resolution and providing more reliability to the assignments of the signals, especially in the determination of lignin [44, 84–93]. 2D NMR methods, such as heteronuclear multiple-quantum coherence (HMQC) spectroscopy, heteronuclear correlation (HETCOR) spectroscopy, homonuclear Hartmann-Hahn (HOHAHA) spectroscopy, total correlation spectroscopy (TOCSY), rotating frame Overhauser experiment spectroscopy (ROESY), heteronuclear single-quantum coherence (HSQC) spectroscopy, and heteronuclear multiple bond coherence (HMBC) spectroscopy, have been employed in lignin structure characterization [44, 84–86]. Among these, 2D HSQC NMR is the most extensively used due to its versatility in illustrating structural features and structural transformations of isolated lignin fractions. Figure 7 presents a typical investigation of lignin with accurate assignments of different structures [71]. 2D HSQC NMR is able to clearly characterize the structures of lignin and polysaccharides in cell walls and the linkages among the lignin without isolating each component [87]. Structural changes of lignin and the other components in LCBM in chemical reaction can be easily monitored by this method. The relationship between the degree of acetylation and the introduction positions of acetyl groups during the acetylation of ground pulp was investigated with 2D HSQC NMR [88]. Acetylation was found to occur firstly on the primary hydroxyl groups of polysaccharides and lignin, followed by the secondary hydroxyl groups of polysaccharides, and finally the hydroxyl groups at the α-position in lignin [88].

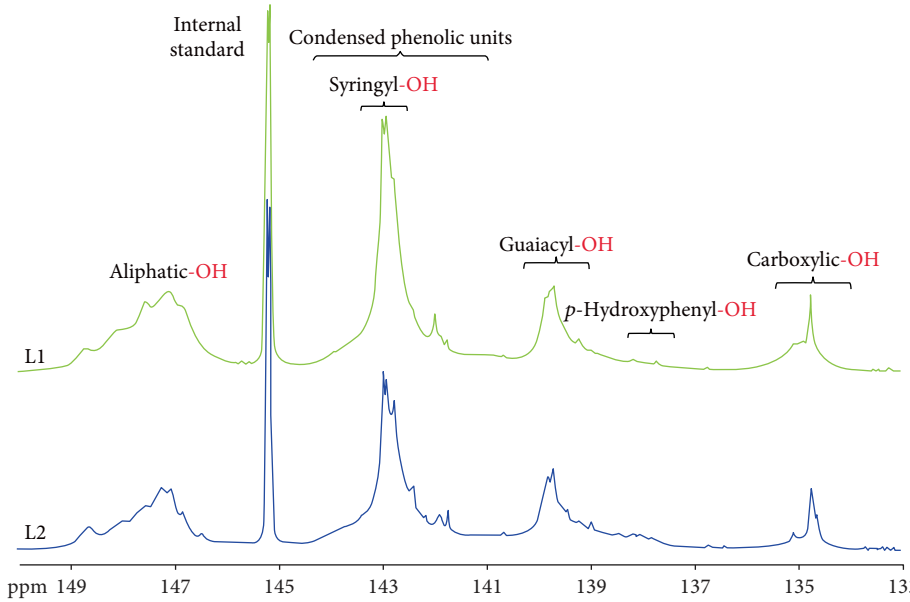

Figure 6

Table 5: Assignments of signals in ^{31}P NMR spectrum to hydroxyl groups in lignin [29, 82].

Chemical shift/ppm	Structural assignments
145.5–150.0	Aliphatic -OH
136.5–144.7	Phenols
140.0–144.5	C_5 substituted
143.5	β-5
142.7	Syringyl
142.3	4-O-5
141.2	5–5
139.0–140.0	Guaiacyl
138.2–139.0	Catechol
137.3–138.2	*p*-Hydroxyphenyl
133.6–136.6	Carboxylic acid -OH

Thioacidolysis was usually used as pretreatment before 2D HSQC NMR analysis in the characterization of the structures of the lignin monomers and oligomers [89]. Changes in the interunit linkage types during solvolysis were investigated. Lignin oligomers ranging from monomers to tetramers were released through considerable cleavage of the β-O-4 linkages [89]. In a study of various lignins derived from brewer's spent grain, 2D HSQC NMR revealed the substructures including β-O-4′ alkyl-aryl ethers (77–79%), β-5′ phenylcoumarans (11–13%), β-β′ resinols (5-6%), and 5–5′ dibenzodioxocins (3–5%); while 2D HMBC NMR and derivatization followed by reductive cleavage analyses showed that *p*-coumarates were acylating at the γ-position of lignin side chains and were mostly occurred in condensed structures [90]. By using high-resolution 2D HSQC NMR, the chemical structures both on low and high molecular weight fractions of bio-oil derived from kraft lignin were determined. In the degradation of kraft lignin to bio-oil, cleavages of both aliphatic carbon-oxygen (C-O) and to some extent carbon-carbon (C-C) bonds as well as repolymerization were observed simultaneously [91].

The combination of quantitative ^{13}C NMR and 2D HSQC NMR has been proven to be a powerful way in structural elucidation of complex samples since it takes advantage of the spectral dispersion afforded by the 2D spectrum to serve as an internal standard to measure the integral values obtained from the quantitative ^{13}C spectrum [92]. This method can overcome the severe overlap of signals and reduce errors in signal quantification due to differential line widths, quantitative abundance of S/G/H units, hydroxycinnamates, and tricin units, as well as various types of side chain substructures by selecting the proper internal standard reference signals [93]. Other combinations of NMR techniques were also reported; for instance, the existence of low energy dipole-dipole interactions and the absence of covalent bond between lignin and chitosan could be revealed clearly by solid-state ^{1}H-^{13}C CP/MAS NMR [94].

4. Conclusions

The comprehensive understanding of the lignin structure relies greatly on the developments of analytical strategies used, which is extremely important for the value-added utilization of biomass. Although significant progresses have been made in the degradation and isolation of the lignin from other components in LCBM, only a fraction of lignin can be identified and analyzed. Structure and composition of lignins from different LCBMs vary significantly according to both issue and age. Furthermore, the analytical results are strongly dependent on the degradation processes and instrumental equipment used.

For the structural investigation of lignin, undestroyed, selective, and efficient isolation methods should be built to

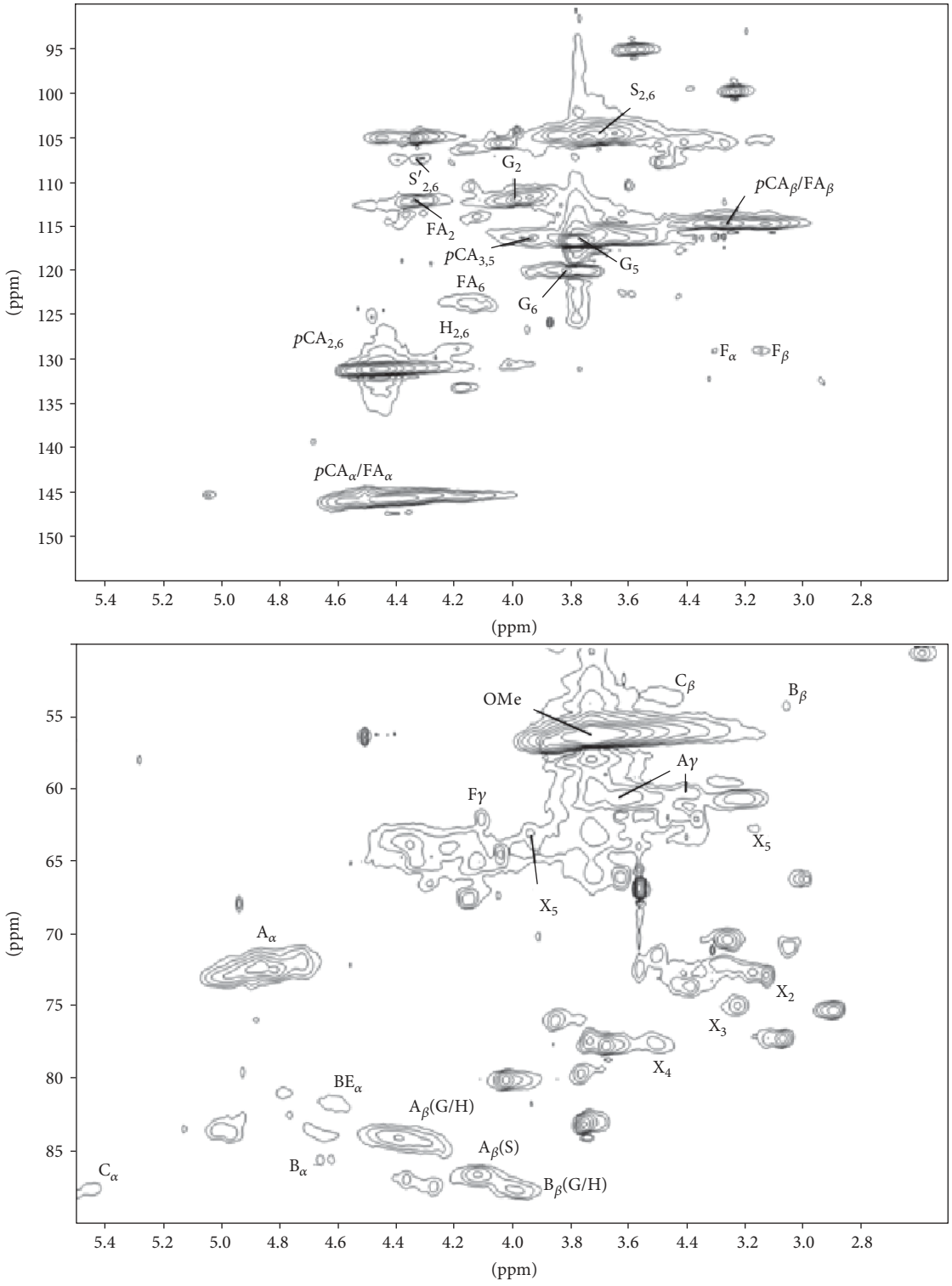

Figure 7

preserve the initial structure of lignin and obtain as much sample to be analyzed. Among the wet-chemistry techniques used, IL extraction and organosolv process are the promising methods. They are treated as environmentally friendly methods since relatively mild conditions used and the reagents can be recycled. Biological degradation might be another possible pathway for the oriented isolation of lignin since the outstanding selectivity and rate of conversion.

Various spectroscopic methods are routinely used for the investigation of lignin structures. These methods can provide both qualitative and quantitative information on functional groups and linkages in lignin as well as degradation products

of lignin. Among these spectroscopic techniques, UV spectroscopy is less likely to be used since it can provide relatively less information on the structural features of lignin. Generally, FTIR spectroscopy is much more frequently used than Raman spectroscopy. FTIR, ^{1}H NMR, and ^{13}C NMR are commonly used in most of the investigations for the characterization of structure of lignins. Recently, ^{31}P NMR is more adopted in this area. Significant progresses for structural elucidation of lignin rely on the application of quantitative ^{13}C NMR and various 2D NMRs. They are robust techniques by providing detailed qualitative and quantitative results with high resolution and precision and can be treated as ideal methods. Rapid, accurate, and nondestructive spectroscopic techniques can be combined to overcome their individual intrinsic limitations for better elucidation of lignin structure. The data collected from these methods contributes to the understanding of LCBM structure and facilitates the design of effective processes to obtain lignin-based value-added chemicals.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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