Physical–chemical characteristics of lignins separated from biomasses for second-generation ethanol

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ABSTRACT

Lignin was extracted by two extraction methods from two biomasses for energy (Miscanthus and Giant Reed) and a lignocellulosic material resulting from a microbial treatment of giant reed. One method of extraction involved the use of $\text{H}_2\text{SO}_4$ (SA), providing a highly aromatic water-insoluble material, while a second method employed $\text{H}_2\text{O}_2$ at alkaline pH (Ox), resulting in a water-soluble lignin. Extraction yields were related to the total Klason lignin measured for the three materials. We compared the physical–chemical features of the isolated lignins, by employing solid-state nuclear magnetic resonance spectroscopy ($^{13}$C-CPMAS spectra and derived $T_1$ relaxation times), thermogravimetric analyses, infrared spectrometry and high performance size exclusion chromatography (HPSEC). We found that lignin separated by the Ox method owned a more mobile molecular conformation, and was largely more water-soluble and fragmented than the lignin obtained by the SA treatment. In line with $T_1$H-NMR and thermogravimetric results, the HPSEC of Ox lignins showed nominal molecular weights less than 3 kDa, indicating well depolymerized materials. Such low-molecular weight and fragmented lignin obtained from biomasses for energy may become useful for application of recycled products in agriculture and in green chemistry reactions, thereby promoting an increase in the economic sustainability of biorefineries.

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1. Introduction

Plant biomasses rich in lignocellulose represent the largest renewable source of hexose and pentose sugars for potential conversion in either ethanol for fuels or various chemicals for industry [1]. First-generation ethanol has been massively obtained from food crops such as wheat and maize [2,3], with consequent reduction of food for animal and human nutrition [1] and overexploitation of valuable productive soil [4]. In order to correct such an unsustainable approach, a second-generation ethanol is now planned to be obtained from non-food biomasses rich in lignocellulose [5,6], and easily grown on marginal soils [7]. Examples of such biomasses are the perennial rhizomatous herbaceous plants, such as miscanthus (Miscanthus × Giganteus, Greff et Deuter) in cool and humid areas, and giant reed (Arundo donax, L.) in warm and dry zones. Both these grasses exhibit high productivity and favorable energy balance even at low nutrient and energy inputs [8–10]. Since no sowing or tillage is required, cultivation of these
biomasses appears to be more ecologically sustainable than annual food crops, thereby contributing to reduce soil erosion risk [13] and improve soil carbon storage and biodiversity.

Different methods have been devised to obtain cellulose from biomasses rich in lignocellulose and, thus, ethanol and other chemicals [12]. Once cellulose is separated, the lignin-rich residue is usually burnt or discarded [1], disregarding a more profitable exploitation of precious aromatic photosynthates. One technique separates cellulose from the bulk of biomass lignocellulose at acidic pH by strong inorganic acids such as sulfuric (H₂SO₄) and hydrochloric (HCl) acids [13]. While this method enables extraction of easily fermentable sugars, it also produces furfural and hydroxymethylfurfural, which are well-known inhibitors of microbial growth and may impair full fermentation of carbohydrates [14]. Even though this harsh acidic cellulose extraction and degradation employs hazardous and rather expensive chemicals, it is still of practical interest and widely applied [15].

A method of lignin separation from cellulose, originally developed in the paper-making industry as an alternative to environmentally hazardous chlorine-based reagents, consists in treating biomasses with alkaline solutions [16]. Strong bases, such as NaOH or Ca(OH)₂, are both environmentally compatible and efficient agents for delignification of cellulose and solubilization of separated lignin Ref. [17]. Furthermore, lignin hydrolysis is significantly improved by adding hydrogen peroxide (H₂O₂) to the reaction mixture [18]. In fact, this alkaline oxidative solution easily disrupts cell walls, efficiently dissolves hemicellulose and lignin by hydrolyzing uranic and acetic acid esters, and yields poorly crystalline cellulose [19]. Moreover, α-aryl ether linkages in lignin phenolic units are readily cleaved and the conversion of the resulting phenolic units into, first, quinone ether linkages in lignin phenolic units are readily cleaved and, then, fragmented hydroxymethylfurural, which are well-known inhibitors of microbial growth and may impair full fermentation of carbohydrates [14]. Even though this harsh acidic cellulose extraction and degradation employs hazardous and rather expensive chemicals, it is still of practical interest and widely applied [15].

The objective of this work was to compare the physical–chemical characteristics of lignin separated from three different plant sources rich in lignocellulose and used for second-generation ethanol: miscanthus, giant reed, and a microbially pre-treated giant reed. The isolated lignin was characterized by Attenuated Total Reflectance Infrared Fourier Transform spectroscopy (ATR-IR), solid state NMR spectroscopy (¹³C-CPMAS spectra and derived T¹/H relaxation times), thermogravimetric analysis (TGA), and high performance size exclusion chromatography (HPSEC).

2. Materials and methods

2.1. Biomasses

Miscanthus (Miscanthus × Giganteus, Greef et Deuter) sample (MG) was provided by the Institute of Biological, Environmental & Rural Sciences (IBERS) of the University of Birmingham, in collaboration with Phytatec Ltd (UK). MG biomasses were grown in open field trials in Aberystwyth, Wales (UK) and harvested in February 2007. Giant reed (Arundo donax, L) sample (AD) was cropped at the experimental farm of the University of Naples Federico II (Bellizzi, IT) and harvested in January 2009. A third lignocellulose material (RL) was provided by Mossi & Ghisolfi (Alessandria, IT), and obtained by applying on giant reed (harvested in January 2008 from a field near Torino, IT) the PROESA® technology, consisting in a microbial enzyme treatment of biomass to release cellulose [23]. MG and AD stems, as well as the fibrous RL material, were oven-dried at 333 K for 5 d, and then ground with a blender. All biomasses with <1 mm equivalent diameter were subjected to lignin separation and extraction in four replications.

2.2. Sulfuric acid hydrolysis

Lignocellulose samples were treated with H₂SO₄ (SA) to remove cellulose, as proposed earlier [24]. Briefly, 1 g of each biomass was treated with 30 mL of a 12 mol L⁻¹ H₂SO₄ solution for 16 h at room temperature and under stirring. Then, the H₂SO₄ concentration was reduced to 1 mol L⁻¹ by adding distilled water and the suspension was kept under continuous stirring for 3 h at 378 K. The mixture was then centrifuged at 15,400 RCF for 30 min and the supernatant containing the lignin separated from biomasses (SA-MG, SA-AD, SA-RL) was dialyzed against deionized water, freeze-dried and stored in dried conditions before further analysis.

2.3. Alkaline oxidative hydrolysis

An aliquot (5.0 g) of each lignocellulose material was added with 150 mL of a 2% H₂O₂ (v v⁻¹) aqueous solution brought to pH 11.5 with a 4 mol L⁻¹ NaOH solution. After stirring at 323 K overnight, the mixture was centrifuged (15,400 RCF × 20 min) and the supernatant, containing lignin and hemicellulose, was separated from cellulose by paper filtration. The pH of filtrate was lowered to 5.5 with 5% HCl, and added with 3 volumes of ethanol to flocculate the hemicellulose, that was further separated by filtration. The ethanol in the filtrate was removed by roto-evaporation and the aqueous solution was then added with 5% HCl to lower pH to 1.5–2.0 to precipitate the lignin. The lignin was separated by centrifugation (15,400 RCF × 15 min), dialyzed (1 kDa cut-off dialysis tubes) against deionized water, freeze-dried, and stored in dried conditions for further analyses. The lignins obtained from different biomasses were labeled as Ox-MG, Ox-AD and Ox-RL.

2.4. Klason lignin determination

The content of lignin in raw biomasses was determined by using the Klason assay as by the ASTM Standard method E 1721 [25]. Briefly, 0.3 g of each sample were treated with 3.0 mL of 72% H₂SO₄ at 303 K for 2 h. The hydrolyzed material was then transferred into a glass bottle and the acid concentration diluted until 4%. The suspensions were autoclaved in the
sealed glass bottles for 1 h at 394 K, then filtered through a crucible and dried at 323 K for 2 h to reach constant weight. Finally, the crucibles containing the acid-insoluble lignin and the acid-insoluble ash were placed in a muffle furnace and ignited at 848 ± 298 K for 3 h, applying a heating rate of 283 K min⁻¹. After cooling in a dessicator, the weight of crucibles with only acid-insoluble ash was recorded to the nearest 10⁻³ g. Klasson lignin (also called the acid-insoluble lignin) was determined as it follows:

\[
\% \text{lignin} = \frac{W_2 - W_3 \times 100}{W_1} \quad (1)
\]

where \(W_1\) was the initial sample weight (dried at 303 K); \(W_2\) the weight of crucible + acid-insoluble lignin + acid-insoluble ash; \(W_3\) the weight of crucible + acid-insoluble ash. All analyses were conducted in duplicate.

2.5 Solubility assay

The solubility of the separated lignin was evaluated by placing 100 mg of sample in 100 mL of distilled water and titrating the suspension to pH 7 with 0.1 mol L⁻¹ NaOH. The solid residue remaining after titration was separated by centrifugation (15,400 RCF × 10 min), dried in an oven (313 K), and weighed. The amount of soluble material was obtained by subtracting the weight of residue from that of initial lignin.

2.6 High performance size exclusion chromatography (HPSEC)

The HPSEC system was composed by a Shimadzu LC-10-AD pump equipped with a Rheodyne rotary injector and 100-μL sample loop and two detectors in series: a UV/VIS detector (Perkin–Elmer LC295, set at 280 nm) and a Refractive Index (RI) detector (Fisons Instruments, Refractometer IV). Since the UV-detector only responds to presence of chromophores, a RI detector was also used to evaluate full mass of the lignin solubilized from biomasses. The chromatographic column was a Biosep SEC s2000 300 × 7.80 mm (Phenomenex, USA), proceeded by a Biosep S-2000 35 × 7.80 pre-column (Phenomenex, USA) and a 2 mm inlet filter. The elution flow rate was set to 0.6 mL min⁻¹ for an eluting solution made of 0.1 mol L⁻¹ NaH₂PO₄ solution, buffered at pH 6.5 with 0.1 mol L⁻¹ NaOH, added with NaN₃ up to 0.3 g L⁻¹, and filtered through 0.45 μm Millipore filter. A Unipoint Gilson Software was used to record and elaborate chromatograms. The column calibration was obtained with the following sodium polystyrene sulfonates of known molecular masses: 123,000, 16,900, 6780 and 1200 Da. Ferulic acid (194 Da) and catechol (110 Da) were used as low molecular weight standards. The calibration curves provided the following relations between molecular weight (MW) and retention time (RT): log MW = −0.14 * RT + 6.339, for the UV detector, and, log MW = −0.138 * RT + 6.338, for the RI detector. The coefficient of determination (R²) for both curves was 0.97. Weight-averaged (Mw) and number-averaged (Mn) molecular weights, and polydispersity (P = Mw/Mn) were calculated as described elsewhere [26].

2.7 Thermal analysis

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves were obtained by air combustion of approximately 10 mg of each sample in a simultaneous thermal analyzer (STA 6000-Perkin Elmer). The initial and final temperatures were 303 K and 973 K, respectively, with an increasing temperature rate of 283 K min⁻¹.

2.8 Attenuated total reflectance infrared fourier transform spectroscopy (ATR-IR)

The ATR-IR spectra were recorded with a Perkin Elmer 1720-X FT-IR spectrometer, equipped with a Perkin–Elmer Attenuated Total Reflectance accessory, by accumulating up to 8 scans with a resolution of 4 cm⁻¹.

2.9 Solid-state nuclear magnetic resonance (NMR) spectroscopy

¹³C NMR spectra of separated lignin were recorded with a Cross Polarization Magic Angle Spinning (CPMAS) solid-state probe mounted on a Bruker AVANCE 300 magnet, by using a rotating speed of 13 ± 1 kHz, a recycle time of 2 s, an acquisition time of 33 ms, 4000 scans, and a contact time of 1 ms. A ¹H ramp sequence was used to account for possible inhomogeneity of the Hartmann-Hahn condition. The free induction decays (FID) were transformed by applying a 4k zero filling and an exponential filter function with a line broadening of 100 Hz. Spectra processing was conducted by the 4.9.5.9 Mestre-C software. Proton spin–lattice relaxation

<table>
<thead>
<tr>
<th>Table 1 – Mass fraction (%) of lignin separated by sulfuric acid (SA), alkaline-H₂O₂ (Ox) and Klasson methods, respect to the initial weight of lignocellulose biomasses subjected to extraction.</th>
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<tbody>
<tr>
<td>Biomass*</td>
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<td>RL</td>
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<td>AD</td>
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<td>MG</td>
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* RL = lignin from lignocellulose residue of a microbially treated giant reed; MG = lignin from miscanthus biomass; AD = lignin from giant reed biomass.

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<th>Table 2 – Average percentage (%) of solubility in water at pH 7 and its standard error (in parentheses), as well as T₁H-NMR values (ms), for lignins separated by sulfuric acid (SA) and alkaline oxidation (Ox) methods from different biomasses.</th>
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<tr>
<td>Biomass*</td>
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<td>Treatment</td>
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<td>Solubility</td>
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<td>T₁H</td>
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</table>

* RL = lignin from lignocellulose residue of giant reed; MG = lignin from miscanthus biomass; AD = lignin from giant reed biomass.
times ($T_1r(H)$) were measured by CPMAS-NMR and calculated by using the following equation:

$$I(t) = I_0 \exp(-t_{\text{SL}}/T_1r(H))$$

(2)

where $t_{\text{SL}}$ is the variable spin lock time, $I_0$ is the signal area at $t_0$, spin lock time, and $I(t)$ is the signal area in absence of any relaxation. The $t_{\text{SL}}$ values were comprised between 10 and 10,000 ms.

2.10. Statistical analysis

Statistical analysis (four replicates per each extraction procedure) was carried out by applying the Kruskal–Wallis one-way analysis of variance test [27] at $P \leq 5\%$. According to this test, there was no significant difference between at least one pair of solubility median, since the probability associated to the calculated H-statistics was 0.44.

3. Results and discussion

3.1. Gravimetric analyses

The gravimetric results for the isolated lignins are reported in Table 1. The percent of Klason lignin in MG (26.5%) was comparable to that reported in literature [28,29], whereas the percent yield obtained from AD (28.72%) was slightly greater than usually reported [30–32]. This difference may be explained with the delayed harvesting season for the AD of this work, that may have affected the relative composition of plant biomasses. In fact, delay in harvest is reported to reduce moisture content in the biomass [33]. Since the bio-synthesis of lignin in giant reed is closely dependent upon the stage of plant maturation, the AD harvested here at the winter onset may have contained a large lignin content in plants [34]. Finally, the acid-insoluble lignin released by RL residues in greater amount than by AD plants (Table 1), is the consequence of the microbial enzyme treatment undergone by RL to

<table>
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<th>Biomass</th>
<th>UV-detector</th>
<th>RI-detector</th>
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<tr>
<td></td>
<td>Interval $^b$</td>
<td>$M_w$</td>
</tr>
<tr>
<td>Ox-RL</td>
<td>A</td>
<td>2302</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>64</td>
</tr>
<tr>
<td>Ox-AD</td>
<td>A</td>
<td>1541</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>70</td>
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<tr>
<td>Ox-MG</td>
<td>A</td>
<td>1319</td>
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<tr>
<td></td>
<td>B</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>74</td>
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</table>

$^a$ Ox-RL = lignin from lignocellulose residue of giant reed; Ox-AD = lignin from giant reed biomass; Ox-MG = lignin from miscanthus biomass.

$^b$ Interval of elution volumes in HPSEC chromatograms: $A = 10–17$ mL; $B = 17.9–18.2$ mL; $C = 18.4–18.6$ mL.

$^c$ ND = Not detectable in chromatogram.
solubilize cellulose, that has, in turn, increased the lignin concentration in the substrate.

The sulfuric-acid method extracted more lignin than that determined by the Klason method (Table 1). An explanation may reside in the condensation of cell wall carbohydrates with lignin molecules and the incorporation of sulfate into the lignin macrostructure, which can both occur during strong acidic hydrolysis [35]. Conversely, a lower lignin recovery was the result of the alkaline oxidative extraction (Table 1). The gravimetric data showed that the Ox-lignin was 5.1, 6.0 and 11.6% of initial biomass weight, and 19.1, 20.9 and 32.1% of lignin measured by the Klason method for Ox-MG, Ox-AD and Ox-RL, respectively. A possible explanation of the poor extraction capacity of the Ox method is the oxidative degradation of lignin components induced by H2O2 at alkaline pH, such as ring opening of aromatic structures and consequent production of linear organic acids [36]. These low molecular-weight organic acids may be still soluble at the pH 2.0 used to precipitate lignin after separation from cellulose and hemicellulose, thereby lowering the overall lignin yield of the Ox method. Another possible reason for the low lignin recovery by this method may be the failure in disrupting lignin-hemicellulose bonds during alkaline oxidation, and the consequent loss of some lignin components remaining in the hemicellulose fraction.

The extent of lignin fragmentation and oxidation reached during extraction from biomasses by the SA and Ox-methods was assessed by a solubility test (Table 2). While more than 85% of Ox-lignins were easily dissolved, only about 5% of the SA lignins were soluble in water. The lower solubility by the SA method is attributed to either poor lignin depolymerization or lignin-carbohydrate condensation caused by the strong acid treatment [35]. Conversely, the presence of the hydroperoxide anion in the Ox method is known to promote lignin oxidation, with a considerable yield of oxidized aromatic compounds [36]. In fact, the large oxidation of lignin extracted by the alkaline H2O2 solution can be attributed to both saponification and Dakin-like reactions [36]. The first reaction is the alkaline hydrolysis of ester groups, while the second reaction consists in the oxidation of quinone methide (a well-known reaction intermediate formed in alkaline conditions) by the hydroperoxide anion. Both these reactions favor extensive oxidation of aromatic constituents in the lignocellulose material and depolymerization of lignin.

The solubility of Ox-lignins may be of interest when these extracts are employed as plant growth stimulators. In fact, water-soluble lignins were shown to increase growth and yield of beans (Phaseolus vulgaris, L) [37] when obtained by the soda-pulping process, while water-soluble lignosulphonates acted as hormones (auxin- and gibberellin-like) towards maize [38]. Moreover, aromatic-rich lignins may become useful substrates for green chemistry reactions to produce new materials.

3.2. High performance size exclusion chromatography

The readily soluble lignin obtained by the Ox method was characterized for their molecular size by HPSEC. The size-chromatograms detected by UV detector are reported in Fig. 1, while the resulting nominal weight-averaged ($M_w$) and

![Fig. 3 – TGA curves for SA (I) and Ox (II) lignins. RL: lignin from lignocellulose residue of giant reed; AD = lignin from giant reed biomass; MG = lignin from miscanthus biomass.](image1)

![Fig. 4 – DTG curves for SA (I) and Ox (II) lignins. RL: lignin from lignocellulose residue of giant reed; AD = lignin from giant reed biomass; MG = lignin from miscanthus biomass.](image2)
18.5 mL. The Ox-RL showed the largest nominal sharp and intense peak at 18 mL, and a small last signal at a broad and poorly intense region between 10 and 17 mL, a to progressively smaller-size signals along the elution profile: chromatograms with three different absorbance regions due of their similar third peak seemed of comparable hydrodynamic size, because Conversely, all lignin materials appearing with the second and great degree of heterogeneity among molecules (Table 3). values resulted well above 1 for all Ox lignins, indicating a the first elution region, followed by Ox-AD and Ox-MG, while P values resulted well above 1 for all Ox lignins, indicating a great degree of heterogeneity among molecules (Table 3). Conversely, all lignin materials appearing with the second and third peak seemed of comparable hydrodynamic size, because of their similar M_w and M_n values, and high homogeneous polydispersity (P = 1).

The RI-detected chromatograms for the three soluble lignins separated by the Ox method are reported in Fig. 2, and showed a much less signal sensitivity, as it is customary for RI detection [39]. No differences were noted in the chromatograms among lignins, nor the values calculated from chromatograms showed any diversity, thereby indicating a substantial similar conformations for all these soluble lignins (Table 3).

3.3. Thermal analyses

The TGA curves for both the SA and Ox lignins are shown in Fig. 3. The SA-AD and SA-RL had very similar thermal behavior. Conversely, SA-MG displayed a greater rate of weight loss up to approximately 673 K while its further thermal decay became comparable to that of the other two SA lignins. As for the lignins separated from biomasses by the Ox method, they showed similar decomposition behavior up to 623 K, whereas Ox-MG seemed to be degraded more slowly than Ox-AD and Ox-RL after that temperature.

The DTG (Differential Thermo Gravimetry) profiles, obtained from the derivatization of TGA curves (Fig. 4), revealed differences among lignins. The peak around 473 K for SA-MG was attributed to decomposition of lignin lateral chain [40], whereas the ones around 533 K in the DTG curves of Ox-AD and Ox-MG had been explained with degradation of both lignin lateral chains and hemicellulose [40–42]. Furthermore, signals around 593 K may be ascribed to cleavage of C–C bonds between lignin monomers [43] and evaporation of phenols [44]. Finally, the peaks around 673–773 K may be related to aromatic rings decomposition [45].

Differential Scanning Calorimetry (DSC) further contributes to distinguish SA from Ox lignins (Fig. 5) The exothermic peaks around 593 K may be related to the energy released from cleavage of intermolecular lignin bonds, while the peak around 723–753 K may be attributed to both lignin and char volatilization [46]. The peak around 573 K in DSC curves for lignins separated from biomasses by the Ox method revealed the presence of hemicellulose [46], thus confirming the assignment of the peak at 533 K in the DTG curves. According to these results, lignin extracted by the Ox method was not completely pure, since it carried along some residual carbohydrates, whereas sugars of plant cell wall were completely removed from lignin residues by the SA method.

3.4. ATR-IR spectra

The infrared spectra of lignins extracted by both SA and Ox methods (Fig. 6) showed significant absorption around 3300 cm⁻¹ for O–H stretchings, small peaks at about 2900 and 2800 for alkyl groups, and an intense signal at 1460 cm⁻¹ to be assigned to both alkyl skeletal and aromatic C–C bonds [47]. The signal around 1730 cm⁻¹ may be related to the C=O stretching vibrations of either unconjugated ketone or carbonyl or ester groups [48], whereas the peak around 1370 cm⁻¹ may be attributed to the O–H in-plane deformation of alcohols and phenols, and the one at 1210 cm⁻¹ to the C–O vibrations of primary alcohols. Vibrations for aromatic molecules can be related to absorptions at 1600, 1590, 1500, 1330, 1260, 1120, 1030 and 800 cm⁻¹ [49]. More specifically, the peaks around 1590 cm⁻¹ were attributed to the aromatic skeleton vibration plus C–O stretchings, whereas absorption at 1328 cm⁻¹ can be due to vibration of syringyl with guaiacyl rings mutually condensed through position 5 [47]. The signals around 1260 and 820 cm⁻¹ are related to the guaiacyl ring breathing and the out of plane C–H vibration in guaiacyl units (C2, C5 and C6), respectively [50], while the one at 1123 cm⁻¹ may be assigned to the syringyl ring breathing vibration. Furthermore, the peak at 1030 cm⁻¹ was attributed to the
aromatic C–H in-plane deformation plus C–O deformation in primary alcohols plus unconjugated C–H stretching [51]. Ether linkages in lignins are revealed by the absorption at 1151 cm$^{-1}$. Finally, the peak around 1108 cm$^{-1}$ in lignin spectra obtained by the SA method may account for the incorporation of sulfates into lignin molecular structures [52]. While spectra of lignins separated by the Ox methods revealed similar absorption bands as by SA, they showed a better resolution, probably because of the greater molecular homogeneity of lignins obtained by alkaline oxidation.

3.5. $^{13}$C-CPMAS-NMR spectra

The solid-state NMR spectra of the lignins of this study (Fig. 7) showed resonances similar to those reported in literature [53–55]. While the signal at 170 ppm describes carboxyl groups, the peak at 152 ppm indicates the C3 and C5 ether bonds between two syringyl units, and that at 148 ppm refers to the C3 and C4 ether linkage between guaiacyl units. The resonance around 133 ppm can be assigned to the C1 position in both syringyl and guaiacyl monomers, whereas the peak at about 27 ppm may be attributed to carbons in lignin lateral chains. Moreover, signals at 112 and 116 ppm are due to C2 and C5 position in guaiacyl units, respectively, and that at 104 ppm to residual carbohydrates in the lignin structure. The resonances at 82 and 72 ppm are typical of alcoholic carbons, and may be assigned to hydroxylated C-α and C-β in α-O-4/β-O-4 linkages in guaiacyl and syringyl units, respectively. Finally, the signal at 56 ppm is due to carbon in methoxy groups, whilst signals around 39, 32 and 29 ppm are associated to alkyl carbons.

These solid-state spectra also revealed the absence of signals for carbohydrates in lignins separated by the SA method, thus confirming the results reached by thermal analyses and infrared spectrometry. Again, the Ox-lignins provided spectra with a larger resolution than for SA-lignins, thus suggesting a greater homogeneity for the former separate.

3.6. Relaxation times ($T_1\rho H$) as measured by solid-state NMR spectroscopy

An evaluation of the molecular rigidity of the lignins separated from the different biomasses by two methods of extraction, was achieved by calculating the proton relaxation time in the rotating frame ($T_1\rho H$) from CPMAS-NMR spectral data (Table 1). The $T_1\rho H$ parameter is used to characterize polymers dynamics within the 10–100 kHz range, the anisotropic motions of which are associated to the collective...
movements of a large number of monomer units [56,57]. Moreover, the T1_H values also correlate to the spin diffusion rate, that is the transfer of magnetization over many proton nuclei. Hence, spin diffusion is, in turn, inversely related to the proton–proton intermolecular distance and, by this, to the degree of molecular motions [58]. The T1_H values reflect the strength of 1H–1H dipolar interactions and indicate whether the various protons belong to common spin reservoir. Thus, the observed T1_H is an average relaxation time for all protons in a solid sample and a result of spin diffusion [58]. T1_H experiments provide structural information on complex molecules, because T1_H values depend on whether two protons of a solid material are sufficiently rigid and close in space to be “intimately” mixed [59]. This means that this relaxation time becomes an indication of the rigidity of a molecular system [60].

The T1_H values were larger for all SA lignins than for all Ox lignins, thereby suggesting a reduced mobility for lignins separated by the SA method in respect to the more structurally mobile water-soluble lignin obtained by the Ox method (Table 1). Moreover, the greater flexibility revealed by the Ox lignins appears to go along with a larger proton density around carbon atoms, that favors a faster relaxation in the rotating frame.

Various authors claimed a negative correlation between the T1_H values and mobility of plant cell walls. Fenwick et al. [61] studied the rigidity of tomato cell walls and found that it was positively correlated to T1_H values, which increased the more rigid the plant cell wall became. The same authors [62] also studied growing and non-growing celery collenchyma by solid-state NMR relaxometry in vivo and reported a shorter T1_H for growing, more mobile tissues, whereas non-growing, less mobile cells exhibited longer T1_H values. Gil et al. [63] studied the mobility of cell wall components before and after removal of suberin, and found that the desuberization caused an increase in cell wall flexibility, thus justifying the T1_H decrease. Furthermore, a catalyzed oligomerization of humic molecules was found to increase the T1_H values in comparison to the loosely associated humic superstructures [64]. These studies corroborate the indication that the lignins separated by the Ox method were depolymerized, as compared to SA lignins, thus also confirming the validity of the alkaline oxidation as an extraction method for obtaining a fragmented and readily water-soluble lignin.

4. Conclusions

We characterized the physical–chemical properties of lignin extracted from three different biomasses for energy (miscanthus, giant reed and a lignocellulosic residue from a microbially treated giant reed) by either hydrolysis in concentrated H2SO4 or alkaline oxidation with H2O2. Thermal analyses and both ATR-IR spectrometry and solid-state NMR spectroscopy indicated that lignin isolated by sulfuric acid contained a lower amount of residual cell wall carbohydrates than the Ox-lignins. Moreover, these techniques enabled to
confirm the larger degree of oxidation of Ox-lignins with respect to lignins separated by SA hydrolysis.

The greater yield of the SA lignins had to be accounted to both condensation of cellulose carbohydrates and incorporation of sulfate into the lignin matrix, whereas the smaller lignin amount separated by the alkaline oxidation of biomass had to be ascribed to the oxidative degradation process occurring during extraction. The larger oxidation degree of the Ox-lignins was confirmed by the far greater solubility of these lignins as compared to those isolated by the SA method. The depolymerization of the complex lignin structure that accompanied the oxidation of the Ox lignins was also suggested by the relaxation measurements obtained with CPMAS-NMR spectroscopy. These T1H values were shorter for the Ox lignins than for those separated by the SA method, thereby indicating a larger mobility of lignin and, hence, a greater degree of depolymerization than for the SA lignins. These spectroscopic results were well in line with results by HPSEC chromatograms, which suggested small hydrodynamic radii for lignins extracted by the Ox method.

The availability of aromatic-rich and water-soluble lignin residues from biorefineries may become useful as substrates for green chemistry applications and as plant-growth promoting agrochemicals. Both these uses would increase the ecological and economic liability of biorefineries.

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