Valorization of Side-Streams from a SSF Biorefinery Plant: Wheat Straw Lignin Purification Study

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The lignocellulosic materials produced after each step of a biorefinery plant using simultaneous saccharification and fermentation (SSF) technology on wheat straw (*Triticum spp*.) for bioethanol production were characterized by spectroscopic and chromatographic techniques in order to investigate the macromolecular interactions between the lignin and polysaccharides. In order to valorize the lignin cakes, a purification step was set up and the extraction conditions (acid pretreatment, temperature, time, and NaOH concentration) were optimized by a chemiometric approach in terms of yield and purity. Residual carbohydrate impurities, free and/or chemically bonded to lignin (lignin carbohydrates complexes), were individuate as the most critical factor for a satisfactory lignin extraction. Finally, the lignin samples collected according to the optimized extraction conditions were chemically characterized and low molecular weight, high phenols concentration, and low carboxylic acids content were recognized as interesting features for industrial applications.

Keywords: Purification; Lignin; Wheat straw; Biorefinery; Ionic liquid

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INTRODUCTION

Due to the economic, environmental, and geo-political concerns generated by the dependence on fossil resources, the study of alternative sources from bio-based products is highly desirable (Cherubini 2010). Among different types of bioresources, lignocellulosic materials have emerged due to their renewability and large biomass stock (Menon and Rao 2012). The conversions of lignocellulosic biomass into biofuels and commodity chemicals *via* the biotechnological pathway have been recent trends (Loow *et al*. 2016a). To take advantage of all the various biomass components and to maximize their added value, lignocellulose must be fractionated into its three primary components, namely cellulose, hemicellulose, and lignin (Loow *et al*. 2015). This process is called biorefinery and involves an integrated conversion system for the production of fuels, power, heat, and value-added chemicals from biomasses (Menon and Rao 2012).

Many studies have focused on the extraction of fermentable sugar for bioethanol production from lignocellulosic materials (Lemons e Silva *et al*. 2015; Achinas and Euverink 2016). In fact, second generation bio-ethanol conversion has become a well-established technology (Sims *et al*. 2010). It comprises the following main steps: pretreatment (*i.e.*, steam explosion (SE)), saccharification by enzymatic hydrolysis of cellulose and hemicellulose, sugar fermentation, and recovery of ethanol (that needs to meet the fuel specification). During the last steps, there is a concomitant separation of solids containing lignin residue (lignin cake). On the basis of the biorefinery approach, the utilization and profitability of the lignin by-product is a prerequisite to the

commercial viability of ethanol production (Shevchenko *et al*. 1999). Lignin is one of the main energy sources in the bioethanol production process, and part of the lignin cake could be re-used in other industrial contexts. Its peculiar chemical nature makes lignin a potential candidate for the replacement of fossil resources in numerous applications (Ragauskas *et al*. 2014). Lignin or its byproducts could be used as a surfactant (Zhou *et al*. 2015), and in combination with other materials, it could act as an antimicrobial, antifungal, and antioxidant agent (Dong *et al*. 2011). It also absorbs UV radiation and exhibits flame-retardant properties (De Chirico *et al*. 2003). Furthermore, lignin can be used in epoxy resins (Lora and Glasser 2002), green composites (Bertini *et al*. 2012; Thakur *et al*. 2014), and as filler in tires (Frigerio *et al*. 2014; Barana *et al*. 2016a).

The major issue that precludes the complete utilization of lignin is that the SE process is not optimized to produce pure lignin (Cui *et al*. 2014; Frigerio *et al*. 2014), and the array of aromatic polymers obtained is contaminated by impurities such as ash and residual carbohydrates, which are often still covalently connected to lignin (Shevchenko *et al*. 1999). While lignin extraction from black liquor, for example in the pulp industry, has already been extensively explored (*e.g.* Lignoboost process in kraft mill permits to obtain high purity lignin) (Tomani *et al*. 2011; Ziesig *et al.* 2014), lignin extraction from biomass residue after SE process has only been investigated recently (Leskinen *et al*. 2015).

In this work, the final residues from a biorefinery plant using simultaneous saccharification and fermentation (SSF) technology on wheat straw were selected as the source of lignin. First, the lignocellulosic materials produced after each step of the biorefinery process (the wheat straw after SE, the lignin cake recovered after SSF, and the intact wheat straw) were chemically characterized. The 3D network that binds lignocellulosic components together (namely cellulose, hemicelluloses, and lignin) makes it practically impossible for these lignocellulosic components to be dissolved in its native form in conventional molecular solvents, which limits the applicability of wet analytical techniques. The analytical approach was thus based on the use of ionic liquids (ILs). ILs are non-derivatizing solvents entirely composed of ions, which are typically large organic cations and small inorganic anions (King *et al*. 2009). Among different ILs, imidazolium-based ILs containing chloride counteranions are widely recognized as a type of very effective solvent for lignocellulosic substrates, and thus, they are exploited for the homogeneous phase derivatization of the material (Zoia *et al*. 2011). Highly substituted lignocellulosic esters can be obtained under mild conditions by reacting lignocellulose dissolved in an IL with either acetyl chloride or benzoyl chloride in the presence of pyridine (Salanti *et al*. 2012). The extended derivation of these esters renders them soluble in molecular solvents that are traditionally employed for analytical purposes, which allows for several fundamental analyses to be conducted. For example, the chromatographic analysis of derivatized substrates is useful in the elucidation of the macromolecular interactions between the lignin and polysaccharides and their fate during the SSF process. The role of residual polysaccharide impurities, free and/or chemically bonded to lignin (lignin carbohydrate complexes, LCCs), is one of the most important factors affecting lignin purification.

In order to valorize the lignin cake, a purification system was set up. In this work, alkaline treatment was selected for the fractionation of the lignocellulosic materials, as NaOH is commonly used to remove lignin from lignocellulosic materials (Stoklosa and Hodge 2015; Loow *et al*. 2016b). The extraction conditions (acid pretreatment, temperature, time, and NaOH concentration) were optimized by a chemiometric approach

in terms of the lignin yield. Finally, the purified lignin samples obtained according to the best conditions were thoroughly characterized to elucidate the effect of the different treatments on the lignin structure.

EXPERIMENTAL

Reagents and Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. The wheat straw (WS) from *Triticum* spp. was supplied by a local factory located in Piedmont, Italy.

The solid residues of the wheat straw after steam explosion (WS/SE) and the solid residues after saccharification and fermentation (WS/SSF), in the form of lignin-rich cake, were provided by a biorefinery plant that produced bioethanol.

Methods

Material preparation

For the chemical characterization, lignocellulosic materials (WS, WS/SE, and WS/SSF) were oven-dried at 50 °C until a constant weight was reached. They were then milled in a planetary ball mill for 10 h at 300 rpm, using a 100 mL zirconium grinding bowl (zirconium dioxide, 95%) in the presence of 8 zirconium balls (10 mm in diameter each).

Lignin content

The amount of lignin (degree of purity) in the samples was calculated as the sum of the acid-insoluble (Klason lignin) and acid soluble lignin content, as measured according to the method reported by Yeh *et al*. (2005). The reported values were the average of 3 analyses ± 1.0% (P = 0.05, n = 3).

Ash content

The accurately weighed and dried samples (around 100 mg) were placed in tared, well-desiccated porcelain crucibles, and cooked in a muffle furnace at 550 °C for 3 h. The crucibles were then stored in a desiccator until room temperature was reached. The ash content was determined gravimetrically. The values reported in the text and in the tables were the average of 3 analyses ± 0.1% (P = 0.05, n = 3).

Holocellulose content

The holocellulose content of the different material was calculated by mass difference, according to Eq. 1.

$$Holocellulose\ (\%) = [100 - (Lignin\ content + Ash\ Content)] \quad (1)$$

Lignocellulosic benzoylation in ionic liquid

The benzoylation reaction was performed in 1-allyl-3-methylimidazolium chloride ([amim]Cl, 950 mg) with approximately 50 mg of the milled lignocellulosic materials (WS, WS/SE, and WS/SSF) and benzoyl chloride, as reported by Salanti *et al*. (2012). The benzoylated wood samples were solubilized in tetrahydrofuran (THF) at a concentration of approx.. 1 mg/mL for gel permeation chromatography (GPC) analyses.

Lignocellulosic acetylation in ionic liquid

The acetylation reaction was performed in 1-allyl-3-methylimidazolium chloride ([amim]Cl, 950 mg) with approximately 100 mg of the milled lignocellulosic materials (WS, WS/SE, and WS/SSF) and acetyl chloride, as reported by Salanti *et al*. (2012). The acetylated samples were solubilized in THF (1 mg/mL) for the GPC analysis and in dimethylsulfoxide deuterated (DMSO- d_6) for the nuclear magnetic resonance (NMR) analyses (50 mg/mL).

Lignin extraction

The dry lignin-rich cakes after SE and SSF (WS/SSF, 1.0 g) were treated in 10 mL of NaOH solution (0.01, 0.025, 0.05, 0.1, and 0.5 M) at 10% weight-volume concentration (w/v %) for different times (30 and 60 min) and at different temperatures (50, 75, and 100 °C). For a series of experiments, an acidic pretreatment (0.01 M HCl, 30 min, 100 °C) at 10% consistency was performed before the alkaline extraction. After the acidic treatment, the pH was increased to neutral by 5 M NaOH, and then the necessary amount of NaOH was added. All the experiments are reported in Table 1. At different times, the alkaline suspension was centrifuged to separate the liquid from the solid fraction. The solid fraction was washed with water and centrifuged 3 times (4000 rpm, 5 min), dried, and weighed (Residues %). The liquid fraction was acidified with HCl (5 M) until a pH of 1 was reached and stored at 4 °C overnight to allow the lignin to precipitate. The precipitated material was then collected by centrifugation (4000 rpm, 15 min), washed with deionized water, and air-dried (Lignin extracted). The yield was calculated according to Eq. 2,

$$Lignin\ yield\ (\%) = \frac{Lignin\ extracted\ (g)}{0.568\ (g)} * 100 \quad (2)$$

where 0.568 (g) is the lignin amount (calculated by Klason method) in 1 g of the starting material (WS/SSF). The purity of the extracted lignin was always higher than 95%. The losses (%) that occurred during the extraction were calculated by Eq. 3.

$$Losses\ (\%) = [100 - (Residues\ + Lignin\ extracted\ )] \quad (3)$$

The reported values were the average of 2 analyses ± 1.0% (P = 0.05, n = 2).

Chemiometric analyses

The data were evaluated by multivariate regression in order to quantitatively estimate the effects of the factors on the yield. In particular, the Partial Least Square (PLS) regression was calibrated with the set of experiments that had reasonable variation levels for the following factors: [HCl] (0.00 M to 0.01 M), temperature (75 to 100 °C), [NaOH] (0.01 to 0.50 M), and time (30 to 60 min). The software used was MODDE 6.0 (2001) released by UMETRICS (Malmo, Sweden).

Lignin acetylation

The extracted lignins (50 mg) were acetylated in a pyridine-acetic anhydride solution (1:1 v/v, 2 mL) and kept overnight at 40 °C. After stripping with ethanol, toluene, and chloroform (25 mL × 3 for each solvent), the samples were dried under a vacuum. The acetylated lignin was solubilized in THF for the GPC analysis and in DMSO-d_6 for the two-dimensional heteronuclear single quantum coherence (2D-HSQC) NMR analysis.

ATR-FTIR

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was used for the qualitative characterization of samples. The analyses were performed with a Nicolet iS10 spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with an iTR Smart device (total scan 32, range 4000 to 800 cm^{-1}, resolution 1 cm^{-1}).

^{31}P-NMR analyses

The accurately weighted lignin samples (30 mg) were dissolved in a pyridine-deuterated chloroform solution (1.6:1 v/v mL, 800 µL) containing 1 mg/mL of chromium(III) acetylacetonate, [$Cr(acac)_3$]. Next, 100 µL of an e-HNDI solution (121.5 mM, $CDCl_3$/pyridine 4.5:0.5) was added, along with 100 µL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane as the derivatizing agent to quantitate the amount of different labile hydroxyl groups (aliphatics, phenolics, and acidic). The ^{31}P-NMR spectra were recorded for 800 µL samples on a Bruker Avance 500 MHz instrument (Billerica, MA USA). The ^{31}P-NMR data reported in this paper was the average of 3 experiments. The maximum standard deviation was 2×10^{-2} mmol/g, while the maximum standard error was 1×10^{-2} mmol/g.

GPC analyses

The GPC analyses were performed on an Agilent HP 1100 series liquid chromatography system (Santa Clara, CA, USA) equipped with a UV-Vis detector set at 220 or 260 nm. The injection port was a Rheodyne loop valve (Landsberg am Lech Germany) equipped with a 20 µL loop. The GP-column system was composed of a sequence of an Agilent PL gel 5 µm, 500 Å, and an Agilent PL gel 5 µm, 10^4 Å (Santa Clara, CA, USA). The solvent used was tetrahydrofuran (99.8%, Fluka, St. Louis, MO, USA). The PL Polymer Standards of Polystyrene from Polymer Laboratories (Shropshire, UK) were used for calibration. The evaluation of the number-average molecular weight (M_n) and the weight-average molecular weight (M_w) of the extracted lignin samples was performed. The peak molecular weight M_p is defined as the molecular weight of the species with maximum absorbance. Moreover, the ratio $I = M_w/M_n$, defined as Poly Dispersity Index was also calculated. The M_n, M_w, and M_p values reported are the average of three analyses (M_w: 1000 g mol^{-1}; M_n, M_p: 100 g mol^{-1}, P = 0.05, n = 3). The acetylated lignin, and acetylated and benzoylated samples were dissolved in THF (1 mg/mL) and analyzed at a flow rate of 1 mL/min.

2D-HSQC-NMR analyses

The 2D-HSQC spectra were run in DMSO-d_6 on the acetylated samples. The inverse detected 1H-13C correlation spectra were measured on a Bruker Avance 500 MHz instrument (Billerica, MA, USA) at 308 K. The spectral width was set at 5 kHz in F2 and 25 kHz in F1. Altogether, 128 transients in 256 time increments were collected. The polarization transfer delay was set at the assumed coupling of 140 Hz, and a relaxation delay of 2 s was used. The spectra were processed using Π/2 shifted squared sinebell functions in both dimensions before FT. The assignment of the predominant signals was based on the chemical shift data of the lignin model compounds and milled wood lignin (MWL), as reported in the literature (Barana *et al*. 2016b).

RESULTS AND DISCUSSION

Characterization of the Lignocellulosic Materials

The lignocellulosic materials recovered during the main steps of the biorefinery process (Fig. 1), namely WS, WS/SE, and WS/SSF, were chemically characterized by means of the compositional analysis, FTIR, 2D-NMR-HSQC, and GPC. The compositional analysis was based on the measure of ash and lignin content, and the holocellulose content was calculated by the difference. As shown in Fig. 1, the WS contained 16.6% lignin, 9.4% ash, and 74.0% holocellulose (cellulose plus hemicellulose). The extractives were not taken into account in this simple approach. After SE, there was a limited loss of material (around 10%), and the compositional analysis indicated the presence of 26.3% lignin, 9.0% ash, and 64.7% holocellulose. The enrichment in lignin and the complementary decrease of holocellulose were rationalized in terms of hemicellulose solubilization during the SE step (Shevchenko *et al*. 1999). After SSF, the recovered material (mass loss around 75%) was composed of 56.8% lignin, 17.1% ash, and 26.1% holocellulose. The SE step enhanced the enzymatic digestibility of the polysaccharide fraction that was then hydrolyzed to fermentable sugars. The resulting material was highly enriched in lignin and ash.

The FTIR analyses were consistent with the compositional analyses. There was little difference observed for the spectra of WS and WS/SE (decrease in the shoulder at 1700 cm^{-1} due to the hemicellulose lixiviation), while the WS/SSF sample spectrum showed an increase in absorbance of the typical lignin bands at 1505, 1453, and 1426 cm^{-1} (Fig. 1).

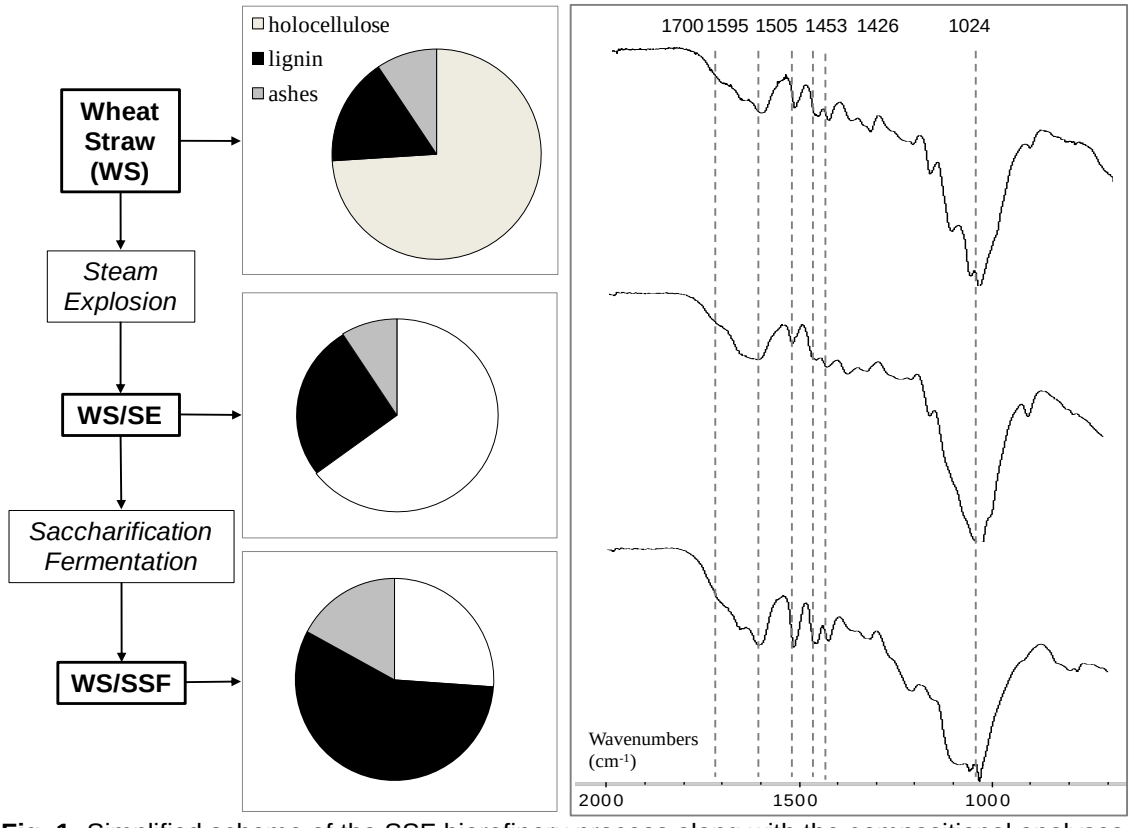

Fig. 1. Simplified scheme of the SSF biorefinery process along with the compositional analyses and the FTIR spectra for the WS, WS/SE, and WS/SSF samples.

In particular, all spectra showed the broad and strong band of the hydroxyl groups stretching centered around 3300 cm^{-1} and the characteristic peaks related to C-H stretching at 2900 cm^{-1} (not reported in Figure 1 for clarity reason). After SSF, the intensity of several of the peaks increased. One such peak was around 1650 cm^{-1} and was associated with C=O groups of carbonyl and carboxylic groups. Other peaks clearly visible were at 1505, 1453, and 1423 cm^{-1} and they were associated with aromatic skeletal vibrations. These peaks were related to the presence of lignin. A strong band related to the C-O stretching of aliphatic alcohols and ether was found at 1024 cm^{-1}. It appeared in all spectra and it reflected the presence of polysaccharides (Barana *et al*. 2016a). In the same region it is possible to find the Si-O stretching (around 1000 cm^{-1}) related to the presence of silicate as inorganic fraction (Barana *et al*., 2016b). The ashes content was found relatively high in all the samples, with enrichment in the WS/SSF one that could explain the increased broadness of the peak at around 1000 cm^{-1} (Fig. 1).

The ball-milled WS, WS/SE, and WS/SSF samples were dissolved in 1-allyl-3-methylimidazolium chloride [amim]Cl and then reacted with benzoyl chloride or acetyl chloride in the presence of pyridine. This system, developed by Kilpeläinen *et al*. (2007), has been extensively utilized on lignocellulosic materials characterization (Zoia *et al.* 2011; Salanti *et al.* 2012) and for archaeological wood chemical characterization (Zoia *et al*. 2015). The per-acetylated materials developed enhanced solubility in DMSO-d_6, which allowed for 2D-HSQC-NMR analysis to be performed. The HSQC spectra are reported in Fig. 2. The WS and WS/SE samples were qualitatively similar (side chain region), and the main cross-peaks detected were related to the cellulose backbone. Other than the methoxy groups cross-peak at 4.75/56.0 ppm (MeO-) arising from lignin, the spectra of the WS and WS/SE samples were dominated by 6 cross-peaks arising from the anhydroglucan unit of cellulose acetate (indicated by the capital letter C in Fig. 2).

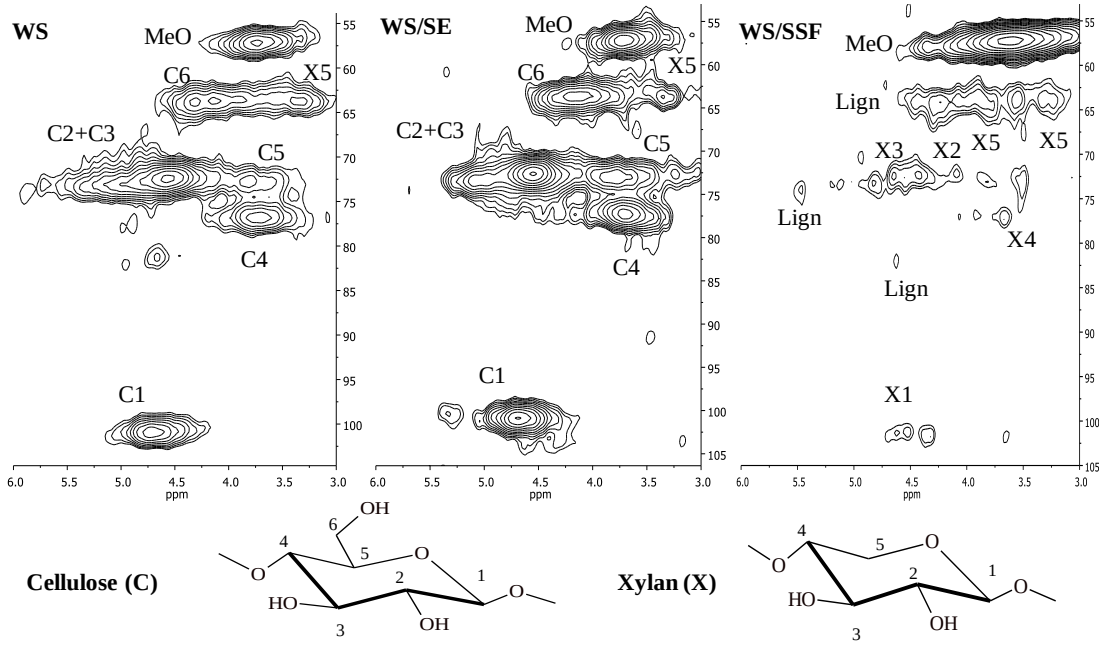

Fig. 2. 2D-HSQC-NMR spectra (side chain region) for the WS, WS/SE, and WS/SSF acetylated samples in DMSO-d_6. The structures of cellulose and xylan backbone are shown at the bottom of the figure. Lign refers to the lignin cross-peaks.

After SE, the only significant change seems to have been related to the decrease in the cross-peak at 3.25/64 ppm, which was associated with the C-H bond at the 5-position (axial) of the xylose units in xylans. This was in agreement with the well documented partial loss of hemicelluloses by solubilization during the SE process and with the compositional and FTIR analyses. The HSQC spectrum of the WS/SSF sample showed changes, and the peaks pertaining to cellulose seemed to have been replaced by xylan cross-peaks (X). Moreover, cross-peaks related to lignin (in particular to the β-O-4 structure, Fig. 3) were clearly visible. The data were consistent with the loss of hydrolysable polysaccharides that occurred after enzymatic treatment and fermentation.

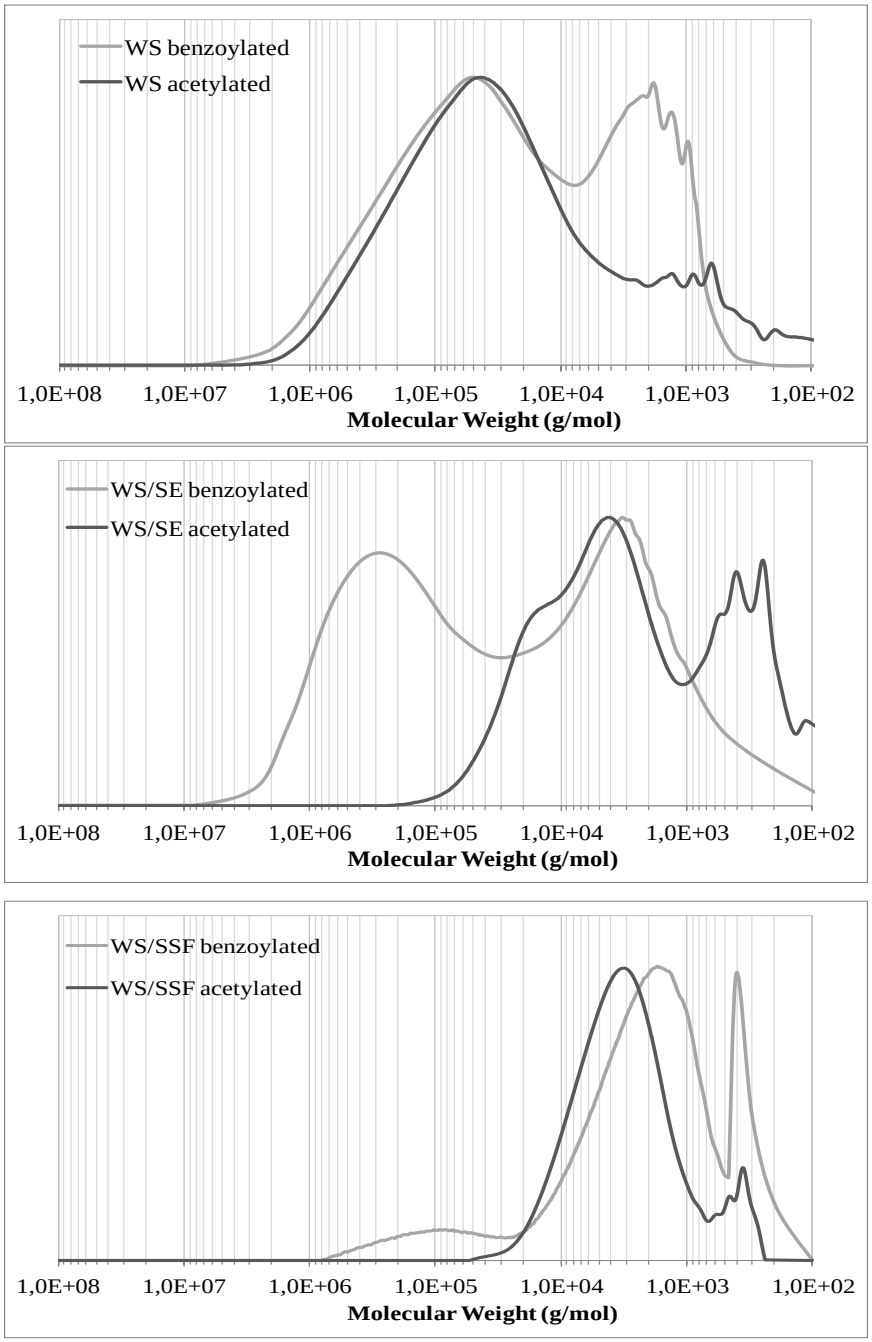

Fig. 3. GPC chromatograms of benzoylated and acetylated WS, WS/SE, and WS/SSF samples

The best information in terms of the macromolecular interactions between the lignin and polysaccharides was obtained by the GPC analyses. The benzoylated and acetylated samples were analyzed by GPC-UV at 240 and 280 nm, respectively, to maximize their analytical response. The resulting chromatograms are reported in Fig. 3. As previously outlined (Salanti *et al*. 2012), after benzoylation, polysaccharides and lignin have a similar instrumental response. Therefore, the chromatograms of the benzoylated samples were supposed to report the molecular weight distribution of the whole wall cell material (cellulose, hemicelluloses, lignin, and LCCs). In contrast, the acetylated sample chromatograms almost exclusively accounted for the molecular weight distribution of those lignocellulosic fractions that naturally contain aromatic groups (LCCs and free lignin) due to the higher instrumental response observed at 280 nm for lignin compared to the free acetylated polysaccharides.

The chromatograms of the acetylated and benzoylated materials were compared. The differences between the benzoylated and acetylated profiles provided information about the presence of polysaccharides, and whether they were connected to lignin or not, *i.e.*, the presence of LCCs. As reported by Salanti *et al*. (2012), the chromatograms of the WS indicated the presence of high molecular weight polysaccharides that were free and/or bonded to lignin (cellulose and LCCs) and the presence of lower molecular weight polysaccharides not connected to lignin (free hemicelluloses). After SE, unlike the compositional and FTIR analyses, the GPC analysis showed a dramatic change in the chemical structure of the WS/SE. The benzoylated chromatogram showed a bimodal molecular weight distribution like the WS, but in this case, the first peak of high molecular weights was not overlaid with the acetylated one, which indicated that this peak was related only to cellulose. Instead, the second peak overlapped with the acetylated chromatogram, which indicated that it was related to either lignin or LCCs. The peak of free hemicelluloses (present in the WS benzoylated sample) disappeared. Therefore, it seems reasonable to infer that after SE, a certain amount of cellulose was released and hemicelluloses were partially solubilized, while the LCCs were broken down. In fact, the acetylated peak in the WS/SE sample was greatly shifted at lower molecular weight with respect to the WS acetylated sample. Moreover, a fraction of low molecular weight lignin seems to have been generated (10^3 to 10^2 g/mol). After SSF, both the benzoylated and acetylated chromatograms were shifted at lower molecular weights with respect to the WS/SE sample as a consequence of the polysaccharides hydrolysis. The cellulose almost completely disappeared, though a weak shoulder was still present in benzoylated WS/SSF, and also, the structures of the LCCs were slightly degraded. Comprehensively, the set of GPC analyses conveyed a lot of information about the chemical composition of the WS/SSF cake. It was composed, for the most part, of lignin, which was probably highly connected to the partially or totally degraded hemicelluloses, and there was a small fraction of residual cellulose. Thus, the presence of residual LCCs affected the extraction and purification of lignin.

Optimization of Lignin Extraction Condition

To extract high yields of high purity lignin from the WS/SSF lignin cake, an alkaline process was performed. The conditions explored included: NaOH concentration (0.01, 0.1, 0.25, and 0.5 M), reaction temperature (50, 75, and 100 °C), and time of treatment (30 and 60 min). All experiments were run at 10% consistency (w/v %). Moreover, an acidic pretreatment was applied for entries 6 to 11 as described in the Experimental section. The acid pretreatment increases the release of lignin from the

LCCs network, and it enhances enzymatic cellulose digestibility (Salanti *et al*. 2013) and increases lignocellulosic deconstruction (Barana *et al*. 2016b).

The outputs were lignin yield, residues after extraction, and losses (calculated by the difference). For all experiments, the extracted lignin was characterized by high purity (≥ 95%). The experimental conditions and the data are displayed in Table 1. The collected data was evaluated by multivariate regression to quantitatively estimate the effect of the different factors on the yield variation (Fig. 4). In particular, the PLS regression was calibrated with the set of experiments that had reasonable variation levels. The output data of the calculations are shown in Fig. 4.

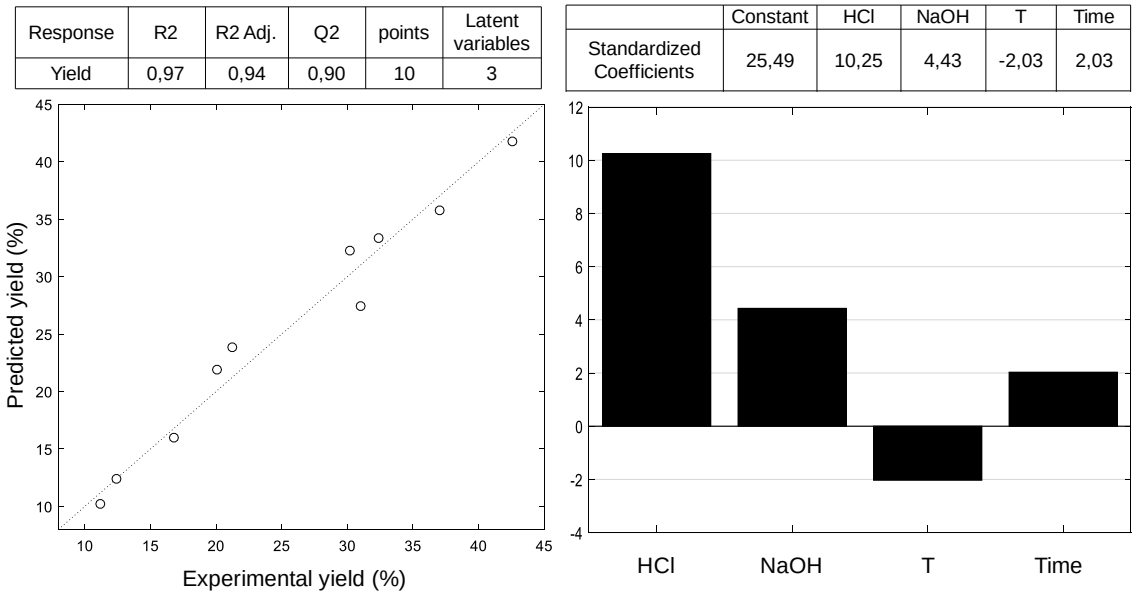

Fig. 4. Output data of the PLS model (left) and standard coefficients of the factors affecting the yield variation (right)

Table 1. Experimental Variables Studied in the Lignin Purification Step and Outputs (Lignin Yield, Residues, and Losses (%))

	Acidic Pretreatment			Alkaline Extraction			Lignin Yield (%)	Residue (%)	Losses (%)
Entry	[HCl] (M)	Temperature (°C)	Time (min)	[NaOH] (M)	Temperature (°C)	Time (min)			
1	-	-	-	0.25	50	60	12	85	3
2	-	-	-	0.01	75	60	11	87	2
3	-	-	-	0.10	75	60	12	80	8
4	-	-	-	0.25	75	60	17	72	11
5	-	-	-	0.50	75	60	20	60	20
6	0.01	100	30	0.10	75	60	30	64	5
7	0.01	100	30	0.25	75	60	37	55	8
8	0.01	100	30	0.50	75	60	42	52	6
9	0.01	100	30	0.10	100	30	21	58	21
10	0.01	100	30	0.25	100	30	31	50	19
11	0.01	100	30	0.50	100	30	32	48	19

The calibrated PLS model had an acceptable performance ($R^2 = 0.97$, $R^2_{adj} = 0.94$) and was additionally validated by a leave-one-out procedure, giving a Q2 in cross validation equal to 0.90, which indicated a stable regression relationship between the analyzed factors and the yield. Thus, the standardized regression coefficients were evaluated to understand how the factors affected the modeled response. Each coefficient represented the variation in the response when a factor changed from 0 to 1, in coded units (where 0 and 1 represent the average and maximum factor values in the experimental design, respectively), while the other factors were kept at their averages. By default, the coefficient plot was for the centered and scaled data, and this scaling made the coefficients comparable.

The acidic pretreatment played the most important role in determining the yield of extraction, followed by the NaOH concentration and time. Interestingly, the temperature had a negative coefficient. The best results were obtained at 75 °C. This result was explained by looking at the losses (%). At higher temperatures, the material loss was higher, perhaps because the lignin was partially degraded and more difficult to precipitate. The optimum conditions for the extraction of lignin resulted in a 42% yield of lignin. The acidic pretreatment seemed to boost the lignin isolation yield. This result was interpreted by taking into account that several lignin-polysaccharide linkages could have been cleaved during the acidic treatment, which allowed for a better dissolution of lignin in the alkaline medium.

Lignin Characterization

The lignin specimens that obtained the best yields (entry 5 and entry 8 in Table 1 were NaOH and HCl+NaOH, respectively) were fully characterized to investigate the effect of the SE process and the extraction procedure on its structure. A chemical characterization was also important because the lignin chemical features determine its industrial application.

Both of the lignin specimens were recovered with high purity after NaOH and HCl+NaOH extraction (98.0% and 99.0%, respectively). In this regard this application has the pivotal role in obtaining lignin with high purity (Cui *et al*. 2014; Frigerio *et al*. 2014). A deep chemical characterization was achieved by the GPC, FTIR, 2D-HSQC, and ^{31}P-NMR analyses. The selected lignin specimens were compared with the starting material (WS/SSF) and a reference of acidolytic WS lignin.

The FTIR spectra showed that the lignin from entry 5 (NaOH), entry 8 (HCl+NaOH), and the acidolytic reference were quite similar, while the WS/SSF spectrum was dominated by a peak at 1024 cm^{-1} that was associated with C-O stretching vibration of carbohydrates (Fig. 5, top). The increasing intensity of the typical lignin peaks at 1505, 1453, and 1423 cm^{-1} associated with aromatic skeletal vibrations reflected the purification caused by the extraction procedure. It is noteworthy that the peak at 1700 cm^{-1} in NaOH and HCl+ NaOH has relatively less intensity respect to the reference lignin, probably indicating a lower amount of carboxylic acid and/or ester bonds.

The GPC analyses (Fig. 5, bottom) showed that the acidolytic lignin had the higher molecular weight. The molecular weight distribution of the WS/SSF sample was shifted to lower molecular weights. Taking into consideration the presence of the residual carbohydrates bonded to lignin in this sample, it was evident that the SE process heavily modified the lignin structure.

After extraction with NaOH and HCl+NaOH, a further decrease in the molecular weight was observed. This was a consequence of the extraction process and its ability to

break down lignin-carbohydrate bonds, which released lignin with high purity, but decreased its apparent molecular weight. The two molecular weight distributions were practically identical, which indicated that the differences in the extraction conditions did not seem to affect the molecular weight of the extracted lignin, but did affect the lignin extraction yield.

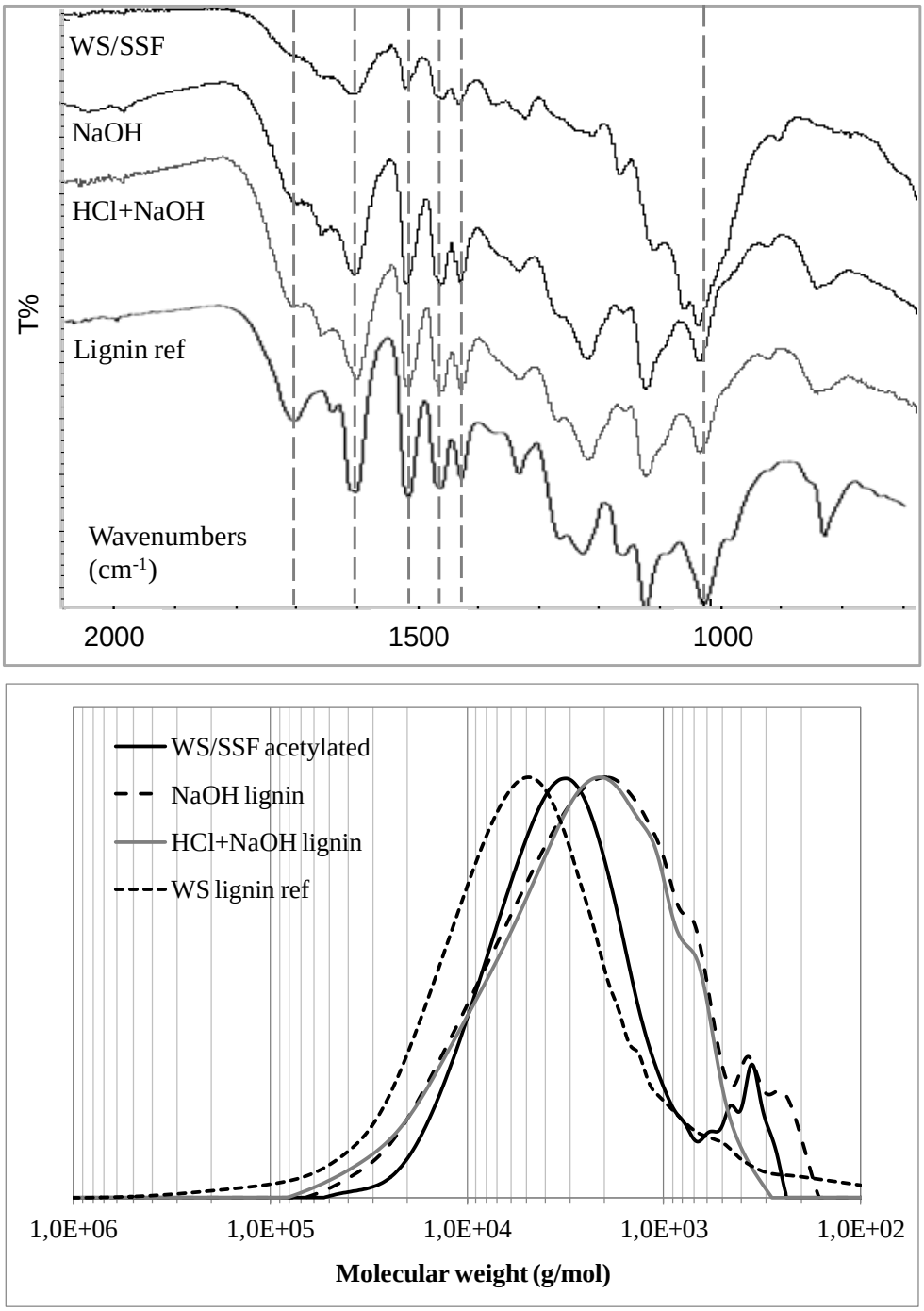

Fig. 5. FTIR spectra (top) and GPC chromatograms (bottom) for the WS/SSF sample, lignin entry 5 (NaOH), lignin entry 8 (HCl+NaOH), and acidolytic WS lignin reference. For the GPC analyses, the samples were acetylated.

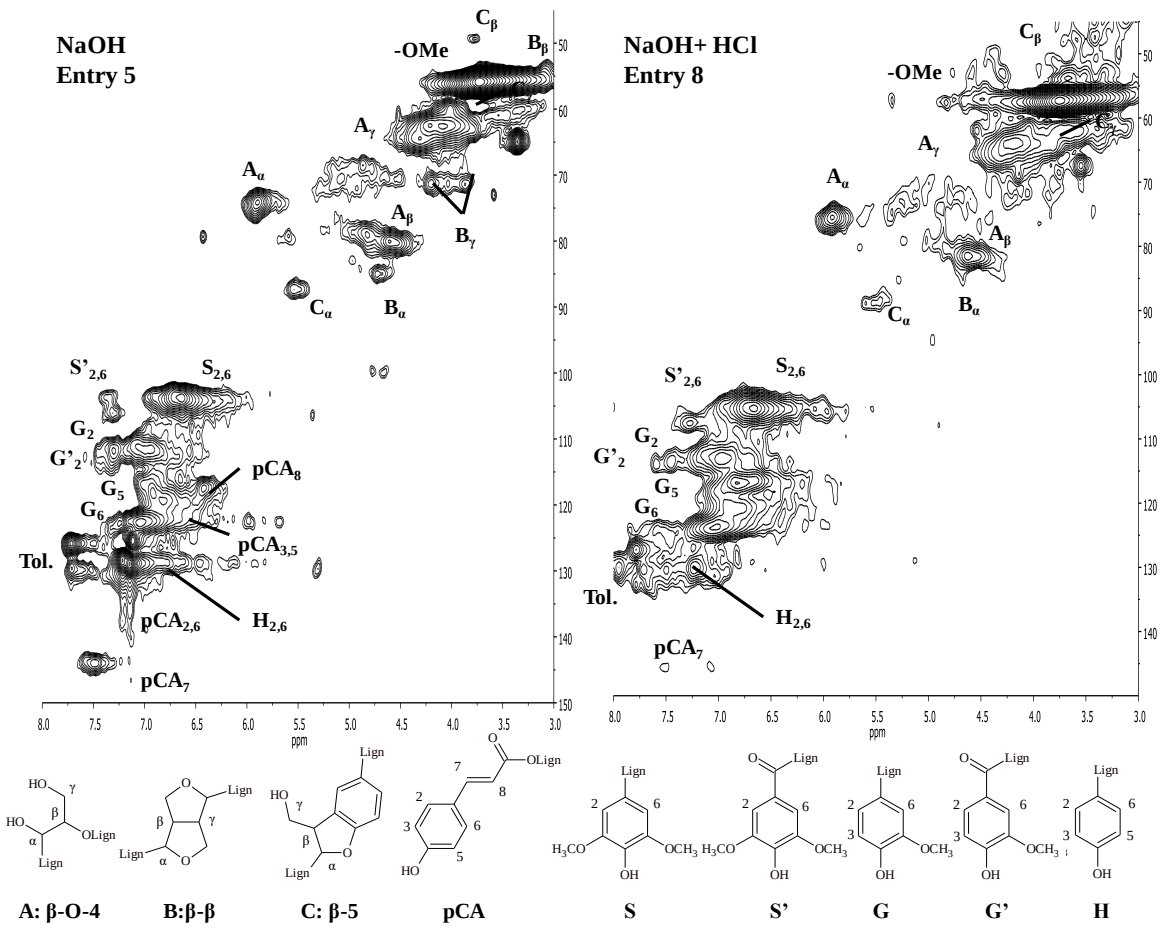

Fig. 6. 2D-HSQC spectra for the acetylated lignin sample entry 5 NaOH on the left and entry 8 HCl+NaOH on the right. The main lignin structures considered in the interpretation are shown at the bottom of the figure.

To identify possible differences in the molecular structure of the two lignin specimens (entry 5 and entry 8), the NMR analyses were performed. Figure 6 reports the acquired 2D-HSQC spectra for the acetylated samples, and Table 2 displays the quantification of the labile hydroxyl group through ^{31}P-NMR, along with the molecular weight indexes calculated by the GPC and HSQC qualitative description of the intermonomeric bonds and aromatic units.

In terms of the intermonomeric bonds and aromatic units, both of the lignin specimens were characterized by the presence of β-O-4, β-β, and β-5 structures and syringyl (S), guaiacyl (G), and *p*-hydroxycumaryl (H) units, respectively, in comparable amounts. However, the lignin extracted by applying the combined acidic-alkaline treatment seemed to have lost the *p*-coumarate units (*p*CA). Compared with the acidolytic lignin reference, both samples appeared to lose the ferulate unit. This last cleavage could have already occurred at the SE step, while the cleavage of the *p*CA units was directly associated with the acidic pretreatment. This is in agreement with the FT-IR analyses that indicated a decreasing of ester bonds. It is worth noting that the cleavage of the *p*CA units, which are involved in the formation of LCCs, could be another explanation for the boost in the lignin extraction yield achieved by the combined purification process.

Table 2. Compositional Analyses, 2D-HSQC-NMR Qualitative Estimation of Intermonomeric Bond and Aromatic Units, and ^{31}P-NMR Quantification for Lignin Sample Entry 5 (NaOH) and Entry 8 (HCl+NaOH)

	Lignin NaOH	Lignin HCl+NaOH	Lignin Reference*
Purity	98.0	99.1	99.0
Yield	20	42	na
GPC			
M_n (g/mol)	4360	4950	10200
M_w (g/mol)	11900	14500	57500
M_p (g/mol)	2280	2120	4900
I	2.74	2.92	5.6
2D-HSQC-NMR Side-chains Region			
β-O-4 (A)	+++	+++	+ + +
β-β (B)	++	+	+
β-5 (C)	++	++	+
2D-HSQC-NMR Aromatic Region			
S	+++	+++	+ + +
S', α-ketone	+	+	+ +
G	+++	+++	+ + +
G', α-ketone	+	+	+
Ferulate	-	-	+
H	+++	+++	+ + +
***p*-Coumarate (*p*CA)**	+	-	+ + +
^{31}P-NMR			
Aliphatic -OH, tot (mmol/g)	1.84	1.84	3.42
Cond. PhOH (L) + S-OH (D) (mmol/g)	0.79	0.80	0.29
G-OH (F) (mmol/g)	0.74	0.70	0.67
P-OH (H) (mmol/g)	0.24	0.24	0.43
COOH (mmol/g)	0.51	0.34	0.29

*The lignin reference data is from Salanti *et al*. (2012).

Moreover, the ^{31}P-NMR quantitative analysis showed that both of the SE lignins were characterized by a high value of condensed and S unit structures, compared with the lignin reference. This seems to have been due to the condensation reactions promoted during the SE step, which probably involved the reaction of aldehydes and activated aromatic rings of lignin (Zoia and Argyropoulos 2010). Finally the HCl+NaOH lignin was characterized by a lower amount of carboxylic acid (0.34 mmol g^{-1}) respect the only NaOH one (0.54 mmol g^{-1}).

CONCLUSIONS

1. The lignin-rich side-stream obtained after the SE and SSF of WS in a biorefinery plant was composed mainly of lignin (approximately 56%) extensively connected to residual polysaccharides (27%). A significant amount of ash was present (17%). The heterogeneous composition dramatically reduced the applicability of the material, and a purification step was required.
2. A combination of acid pretreatment and mild alkaline extraction gave the best results in terms of lignin purity (99.0%) and yield (42%).
3. The lignin isolated with the conditions reported in Table 1, entry 8, had interesting features for industrial applications. Its purity was around 99%, and it was characterized by low molecular weight, high phenols concentration, and low carboxylic acids content.
4. Future studies could investigate the environmental performance in the case of the total recovery of lignin in the biorefinery plant and the use of the residue after lignin purification as an energy source for the process.

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