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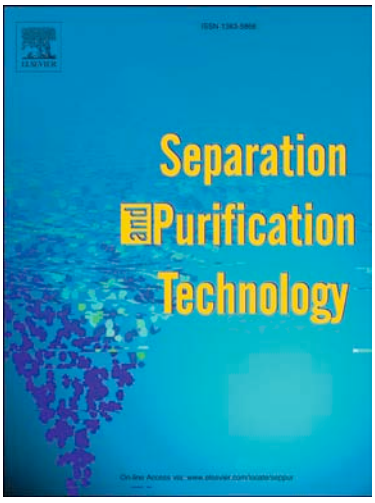

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PII: S1383-5866(23)01571-X
DOI: https://doi.org/10.1016/j.seppur.2023.124663
Reference: SEPPUR 124663

To appear in: *Separation and Purification Technology*

Received Date: 16 May 2023
Revised Date: 13 July 2023
Accepted Date: 24 July 2023

Please cite this article as: R. Pontes, M. Michelin, A. Romaní, A.M. Dias, J.A. Teixeira, J. Nunes, Lignin recovery from a mixture of SIX lignocellulosic biomasses within a biorefinery scheme based on a sequential process of autohydrolysis and organosolv, *Separation and Purification Technology* (2023), doi: https://doi.org/10.1016/j.seppur.2023.124663

Lignin recovery from a mixture of SIX lignocellulosic biomasses within a biorefinery scheme based on a sequential process of autohydrolysis and organosolv

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ABSTRACT

A mixture of six lignocellulosic biomasses could be a solution for sustainable biorefinery supply throughout the year compared with seasonal feedstock. In this study, a sequential process of autohydrolysis and organosolv was used for this mixture. To optimize lignin extraction, an experimental design was used to study the impacts of temperature, ethanol concentration, and time on lignin yield and related characteristics of the organosolv process. A highly pure organosolv lignin (OL) (ca. 90% Klason lignin) was recovered. FTIR analysis showed the main lignin structure was almost unchanged. NMR confirmed this and revealed the presence of guaiacyl-syringyl type with small amounts of p-hydroxyphenyl units (25% G, 70% S, and 5% H), predominantly presenting β-O-4 units (ca. 80%). Tg from 73 to 85 ºC was detected. OL showed radical scavenging activity as high as the commercial antioxidant BHT. Moreover, enzymatic susceptibility of delignified biomass was accessed, in which 50% glucose yield was achieved.

Keywords: Lignocellulosic feedstocks; Lignin; Antioxidant activity; Saccharification; Biorefinery

1. Introduction

Lignocellulosic biomass (LCB) has proven to be a promising resource for sustainable development towards a biobased society and is a key option for biorefineries [1], which are designed to process or fractionate biomass for integral valorization in order to maximize the outputs. Nevertheless, some factors such as feedstock type and availability, time of harvest, transportation costs, and storage have been identified as main drawbacks to the feasibility of biorefineries [2].

The combination of mixtures of biomasses from forest and marginal land could be a solution for the sustainable biorefinery supply when compared to single feedstock. The use of a mixture of biomasses, unlike a single raw material, overcomes the problems related to biomass availability, seasonality, price volatility, and storage. In addition, nearly half of Portugal's landmass (around 40% to 50%) comprises infertile soil unsuitable for profitable agriculture [3]. Typically, 60% to 70% of all fires occur in wooded and uncultivated regions, leading to an estimated annual loss of approximately 800 million Euros [3,4]. Until now, there has been no effective solution for utilizing this land and the forest wastes [3]. However, social and economic advantages could be gained using these resources to develop the bioeconomy [5].

Different lignocellulosic biorefinery concepts have been developed so far; however, their economic viability depends on the conversion of all fractions, namely, cellulose, hemicellulose, and lignin to value-added compounds, based on a cascade use concept [6]. The selective separation of these components enables their efficient utilization [7]. Thus, a pretreatment method is required to overcome the physical and chemical barriers present in lignocellulosic biomass by making it more accessible for conversion into a number of value-added products, thereby the pretreatment is a key factor for a viable biorefinery [8,9]. One potential strategy proposed as a first step is hemicellulose solubilization by

hydrothermal processing (or autohydrolysis) with hot and compressed water [10,11]. Under optimized conditions, this technology can produce a significant amount of soluble hemicellulose oligosaccharides without significantly dissolving the cellulose and the lignin that remain in the solid fraction [7,12].

To enhance fractionation, it is advisable to remove lignin prior to cellulose hydrolysis into glucose, followed by sugar fermentation. This approach helps to avoid issues caused by the non-specific attachment of cellulolytic enzymes to lignin and the precipitation of soluble lignin. Also, enzymatic hydrolysis efficiency depends on the crystallinity of pretreated biomass. Thus, it is crucial to decrease cellulose crystallinity, increase cellulose surface accessibility by the removal of lignin and/or hemicellulose, and promote the swelling of the biomass, since these issues can make cellulose fibers more resistant to enzyme digestion, leading to a decrease in fermentation yield [7,13–15].

Among the different processes able to break lignin bonds from cellulose, the organosolv delignification process stands out, which is highly optimized for this purpose [10,16]. The organosolv is regarded as the most cost-effective and eco-friendly process, which operates through the use of organic solvents to cleave bonds in the lignin macromolecule, such as α-aryl ether and aryl glycerol-β-aryl ether [17–19]. To date, different combinations of solvents (e.g., methanol, ethanol, acetone, and/or organic acids) and many treatment conditions have been studied for autohydrolyzed biomass [7,12,17,20–22]. Nevertheless, ethanol is the most used due to its low cost and easy recovery, with promising results [12,13].

The products commonly obtained by this process include sulfur-free lignin fragments, which are useful for producing high-value lignin-based products due to their purity and low molecular weight [12,23]. In addition to its present use as a solid fuel, lignin has the potential for various applications, including but not limited to cosmetic [24], resins [25],

adhesives [26], oxygenated aromatic compounds, encapsulants, pharmaceutical, intermediates, fillers, and carbon fibers could be developed [2]. In addition, the potential of organosolv lignin as an antioxidant has been reported [19,27,28]. It is a highly efficient radical scavenger that can effectively prevent or slow down oxidation processes and cellulose depolymerization. Its addition to synthetic polymer systems can delay photo- and thermal oxidation. Furthermore, the use of lignin as a significant element in dietary fiber can impede the action of enzymes that generate free radicals and restrict the growth and survival of cancer cells [27,29].

In order to find new lignin valorization routes, it is essential to perform an extensive characterization of this fraction since lignin demonstrates different properties according to the lignocellulosic biomass, pretreatment, and isolation process [30]. The primary objective of this study was to optimize the extraction of lignin from a mixture of six lignocellulosic biomasses obtained from forest and marginal lands. This was achieved through a sequential process of autohydrolysis and organosolv. The purpose of using a mixture of biomasses was to ensure a constant supply of resources all year round for a biorefinery, as a secure supply and sustainable exploration are critical factors in the industrialization of such systems. Notably, this was the first instance of the organosolv process employed to pretreat complex mixtures of lignocellulosic biomass, particularly for raw material obtained from marginal lands residues without economic activity or significant uses. The effect of operational conditions, such as temperature, time, and ethanol concentration, was assessed through experimental design. Moreover, the study also examined the physicochemical and antioxidant properties of the extracted lignins for further valorization. The research also encompassed an assessment of the enzymatic hydrolysis of the recovered cellulosic fraction.

2. Materials and methods

2.1. Materials

The chemicals 2,2 diphenyl-1-picrylhydrazyl (DPPH) with Quality Level 100 and butylated hydroxytoluene (BHT) with a nominal purity of 99 % was supplied by Sigma-Aldrich; dimethylsulfoxide-d6 with a purity of 99,8%D was obtained from EurisoTop; ethanol 96% and sulphuric acid 72% were obtained from Panreac Química SLU (Spain); acetic acid glacial from Fisher Scientific; sodium hydroxide from Supelco. Cellic Ctec2 was obtained from Novozymes (Bagsvaerd, Denmark), and Whatman® filter paper grade 1 (Whatman International Ltd, England) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All standards for HPLC and other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich (St.Louis, MO, USA).

2.2 Lignocellulosic biomass

A mixture of six lignocellulosic biomasses from the forest (52.5%, including 24.6% of pine, 27.9% of eucalyptus) and marginal lands (47.5%, including 13.8% broom, 12.5% mimosa, 10.7% carqueja, 10.4% rockrose) sources was used as raw material. These species were identified as the most important sources of forest fire cases in Portugal [3]. The criteria for the mixture of biomasses was based on territory distribution, species of high fire risk in the summer months in Portugal, and total sugar content, previously studied [4]. The studied biomass mixture was named M2-3 (mixture 2nd and 3rd quarters) and resulted from forest management practices (including branches and twigs with barks and leaves). Particles size from 40 to 60 mesh was used. After processing, the raw material was stored at room temperature for further use.

2.3 Biomass processing: autohydrolysis and organosolv

An autohydrolysis pretreatment at 206 ºC (T_{MAX}) under a non-isothermal regime was used to process the M2-3 biomass mixture. The pretreatment was conducted using a 2 L stainless steel reactor (Parr Instruments Company in Moline, Illinois, USA), and the reactor was equipped with a Parr PID temperature controller of model 4848. A mixture of M2-3 was prepared with a liquid-to-solid ratio (LSR) of 8 g of water per g of oven-dry raw material. The reaction media in the reactor was stirred at 150 rpm and heated using an external jacket. The heating followed a standard temperature-time profile to reach the desired maximum temperature. After reaching the desired temperature, the reactor was rapidly cooled down by circulating water through an internal loop [4]. The resulting solid fraction of this process was named AM2-3.

The AM2-3 biomass was subsequently pretreated by the organosolv process using ethanol/water mixtures to isolate the lignin fraction, according to the design of experiments (DOE) described in the next section. For that, a 2 L stainless steel reactor (Parr Instruments Company, Moline, Illinois, USA) equipped with a Parr PID temperature controller (model 4848) was used, with an LSR of 8 g/g (w/w).

After the reaction, the solid phase was vacuum filtrated using Whatman® Nº1 and washed twice with an ethanol/water mixture (at the same treatment concentration). The recovered liquid fraction (black liquor) and the washing ethanol/water mixture were combined to recover the lignin by precipitation. For that, three volumes of water acidified with acetic acid to pH 2.0 were added. The precipitated lignins were centrifuged, washed, and freeze-dried.

The delignified solids were washed with 1% (w/w) NaOH (10 g per g of solids) at room temperature plus washes until pH 7 (approx. 1 L of DI water per g of solids) to remove adsorbed lignin from solids as described elsewhere [31]. Washed delignified solids were air-dried at 45 ºC.

The lignin yield was calculated by Eq (1).

$$Y_{lignin} \qquad \frac{m_{lignin}}{KL_{AM2-}} \tag{1}$$

where $m_{recovered}$ is the mass of precipitated lignin (g), $KL_{AM2\text{-}3}$ is the quantity of the Klason lignin in the AM2-3 biomass (g).

The delignification extent was determined by Eq. (2).

$$DE \qquad \frac{KL_{AM2-} - KL_{delignified\ AM2-}\ Y_{delignified\ AM2-}}{KL_{AM2-}} \tag{2}$$

where $KL_{AM2\text{-}3}$ is the quantity of Klason lignin in the AM2-3 biomass (%), $KL_{delignified\ AM2\text{-}3}$ is the quantity of the Klason lignin in the solids after organosolv process (%), and $Y_{delignified\ AM2\text{-}3}$ is the yield of solid residue (%).

2.3.1 Experimental design for organosolv delignification process

A design of experiments (DOE) was used to study the effect of temperature, time, and ethanol concentration on lignin removal for its valorization and cellulose preservation for further enzymatic hydrolysis. For that, a 2^3 design was used, with two central points. Three independent variables were evaluated: temperature (T, ºC) [160 (-1), 180 (0), 200

(+1)], time (t, min) [20 (-1), 40 (0), 60 (+1)] and ethanol/water mixture (E, % v/v) [30 (-1), 55 (0), 80 (1)]. The scheme of the experimental design is summarized in **Figure 1**.

The dependent variables (analyzed responses) were lignin yield, delignification extent, and cellulose content from solids. STATISTICA® software, version 12 (StatSoft, Inc., 2014) (www.statsoft.com) was used to analyze the experimental results, with a 95% significance level.

2.4 Chemical composition of the lignocellulosic fractions

The aliquots of lignin and delignified solids were chemically analyzed according to the following National Renewable Energy Laboratory (NREL) methods: moisture (NREL/TP-510-42621) [32] and structural carbohydrates and lignin (NREL/TP-510-42618) [33].

Sugars (glucose, xylose, arabinose), acetic acid, hydroxymethylfurfural (HMF), and furfural released from quantitative acid hydrolysis with 72% w/w sulphuric acid were analyzed by high-performance liquid chromatography (HPLC) using the columns Aminex HPX-87H and 0.005 M H_2SO_4 as mobile phase, at a flow rate of 0.6 mL/min, 60 ºC and refractive index detector; and used to calculate the contents of cellulose and hemicellulose [4,34]. The solid phase from the quantitative acid hydrolysis was considered Klason lignin. Analyses were performed in triplicate.

2.5 Lignin characterization

2.5.1 Fourier transform infrared spectroscopy (FT-IR)

The organosolv lignins were analyzed by an FTIR spectrometer (IRAffinity-1S Shimadzu) operating in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} recorded over 20 scans.

2.5.2 NMR Analysis

The 2D HSQC NMR spectra were recorded on a Bruker AVIII 400 MHz spectrometer, according to Yuan et al. [35]. Thus, the HSQC experiments were recorded at 25 ºC using 90 mg of lignin dissolved in 0.5 mL of DMSO-d6, and the DMSO peak was used as an internal reference point (δC/δH 39.5/2.49). Assignment of the HSQC correlation peaks was performed by comparing their chemical shifts with the literature data [36,37]. Then, the HSQC correlation peaks of interest were submitted to a semi-quantitative analysis [35] of the volume integrals (uncorrected) using the software of the equipment. In the aliphatic oxygenated region, the Cβ–Hβ correlations were used to estimate the relative abundances of side chains involved in the various interunit linkages. In the aromatic/unsaturated region, the relative abundance from G units was estimated based on the C2–H2 correlations, while the C2–H2 and C6-H6 correlations were selected to estimate relative abundances from H and S lignin units.

2.5.3 Thermal analyses

2.5.3.1 Thermo gravimetric analysis (TGA)

TGA of organosolv lignins were carried out in Perkin Elmer TGA 4000 equipment. The run included temperature from 20 ºC to 800 ºC, a heating rate of 10 ºC/min under a nitrogen atmosphere, and 10 mg of lignin. Analyses were performed in duplicate.

2.5.3.2 Differential scanning calorimetric (DSC)

DSC analysis of organosolv lignins was carried out in Perkin Elmer DSC 6000 equipment to determine the glass transition temperature (Tg). The run included heating from 0 ºC to 120 ºC and then cooled until 0 ºC for a new heating up to 200 ºC. A constant heating rate of 20 ºC/min under a nitrogen atmosphere was used. Analysis was performed in triplicate using 4 mg of lignin samples placed in a hermetically sealed DSC pan, the top of each sealed pan was punctured to allow volatiles to escape. Glass transition (Tg) was calculated by using the half-height technique in the transition region. Analyses were performed in triplicate.

2.5.4. DPPH free radical scavenging activity

The antioxidant activity of lignins was determined according to the slightly modified method of Blois [38], as described in Michelin et al. [22]. Commercial antioxidants like 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and butylated hydroxytoluene (BHT) were used as references. RSA and IC_{50} were also determined according to Michelin et al. [22]. The assay was performed in duplicate.

2.6 Enzymatic hydrolysis of delignified biomass

The delignified solids recovered after the organosolv process at the hardest condition (200 ºC, 60 min, and 80% ethanol concentration) were subjected to enzymatic hydrolysis (EH). Hydrolysis was conducted in 100 mL Erlenmeyer flasks with the Cellic CTec2 enzyme (Novozymes, Bagsvaerd, Denmark) at 50 ºC and pH 4.8 (0.05 N sodium citrate buffer)

in orbital agitation at 150 rpm for until 72 h. The enzyme activity was 120 FPU/mL (measured as described by Ghose) [39]. The percentage of solids in the EH assays was 5% and 10% (w/v), with an enzyme-to-substrate ratio (ESR) of 20 FPU/g of the substrate (or delignified solid). The samples were centrifugated and analyzed by HPLC as described in the 2.3 section. To determine the enzymatic susceptibility of delignified biomass, glucose yield (GY) was calculated according to Gomes et al. [40]. The assay was performed in duplicate.

2.7. Statistical analyses

Analyses were performed in duplicate or triplicate, as mentioned in each section. The values reported are the mean ± standard deviation and were calculated using Excel 2021.

3. Results and discussion

3.1 Lignin extraction by organosolv process

Firstly, this biomass mixture (M2-3) was treated by autohydrolysis (AM2-3) to recover the hemicellulose-derived compounds, such as xylooligosaccharides and xylose, in the liquid phase, and cellulose and lignin in the solid phase, as the previous report Pontes et al. [4]. The mixture AM2-3 was chemically analyzed, and its composition was as follows: 41.87 ± 2.36% cellulose, 6.30 ± 0.35% xylan, 50.62 ± 1.07% Klason lignin, and the remaining minor compounds were ashes and soluble lignin. The autohydrolysis treatment was highly selective for hemicellulose solubilization. In fact, 68.60% of hemicellulose was recovered in the liquid phase, mainly composed of xylooligosaccharides and xylose, achieving 14.33 g/L and 2.11 g/L, respectively. The operational condition of

autohydrolysis was selected taking into account the high hemicellulose solubilization and higher preservation of cellulose and lignin in the solid fraction, 84.65% and 97.01%, respectively, as described and optimized in previous work [4]. To achieve an integral valorization of biomass, the organosolv process after the autohydrolysis pretreatment has been proposed as a suitable fractionation process to recover the cellulose and lignin in separate streams for further valorization within a biorefinery context.

In this sense, results of the cellulose content of delignified biomass, % of delignification, lignin yield, and purity obtained from the organosolv process were displayed in **Figure 1**, showing the effects of operational conditions evaluated in this work (time, temperature, and ethanol concentration) on the delignification. The most suitable results according to delignification (49.4%) and lignin yield (48.3%) were obtained on the hardest pretreatment condition (200 ºC, 60 min, 80% EtOH), corresponding to high ethanol concentration (80%), temperature (200 ºC) and time (60 min).

Although these results showed a solid fraction with less lignin content, the delignification was no higher than 50%. Amendola et al. [41] also reported a low delignification rate; that is, from an autohydrolyzed grape stalks residue containing 56% lignin and 27.9% cellulose, only 6.7% solids were solubilized in the organosolv process, lignin precipitation corresponded to 28% of the total solids of the liquor and 2.3% of grape stalks lignin. Vargas et al. [42] also showed a high fraction of lignin (24–29%) remaining in the cellulosic fraction after the organosolv process of an autohydrolyzed barley straw, a less recalcitrant biomass. Other authors have reported the presence of residual lignin in the cellulosic fraction after sequential autohydrolysis-organosolv pretreatment, independently of the biomass nature or pretreatment hardness [7,20,22,43]. According to Obama et al.[44] this fact can be explained by the autohydrolysis pretreatment, where

lignin was deconstructed to enable the break of lignin-carbohydrate bonds during the organosolv process; however, the formation of small fragments of lignin is associated with repolymerization reactions (C-C linkages), leading to negative effects on the delignification, resulting in a lignin fraction difficult to extract even under harsh conditions[44].

Lignin samples recovered by precipitation showed a high purity of about 90% (**Figure 1**) with no detection of carbohydrates. Regarding glucan preservation, the cellulose content on solid residues was an average of 60% for all conditions, being 68% for the optimal condition (**Figure 1**). Vallejos et al. [45] have reported a higher delignification and glucan preservation when compared to the results obtained in this work. However, a less recalcitrant biomass (sugarcane bagasse) was used, as well as an autohydrolyzed biomass with much lower lignin content (26%).

In the current work, a complex mixture of biomass was used, including hardwood, softwood, and bush from the forest and marginal land resources, which could have hindered delignification. This is the first report using the organosolv process in complex mixtures of autohydrolyzed biomasses. The purpose of using multi-supply raw biomass is to provide enough feedstocks for biorefineries throughout the year, increasing the sustainability of the value chain in terms of biomass (having no pressure on the same feedstock) and also avoiding the risk of forest fire, commonly related to these biomasses [4]. However, more than one step of delignification will probably be required to improve the lignin removal, such as the extended delignification reported by Wen et al. [46], as well as a more severe process.

According to the Pareto diagrams (**Figure 2**), in which EtOH corresponds to ethanol concentration, T for temperature and t means time, temperature and ethanol concentration

were the factors with the most significant effect on the response of lignin yield (**Figure 2A**) and cellulose content (**Figure 2C**), indicating that the lignin extraction process is favored at higher temperature and ethanol concentration for a confidence level of 95 % (*p*-value of 0.05; **Table S1**). Therefore, the best agreement for lignin removal and cellulose preservation was verified at high temperature and ethanol concentration. Delignification was positively influenced by ethanol concentration (**Figure 2B**).

In order to evaluate as graphical way, the influence of independent variables on dependent variables, response surface methodology was used (**Figure 3**). For evidence of this consistency, the plot of predicted versus observed data demonstrated a good correlation between the predicted responses and those observed for lignin yield (R^2= 0.95), delignification (R^2= 0.92) and cellulose content (R^2= 0.96).

3.2 Organosolv lignin characterization

3.2.1 Chemical structure characterization

3.2.1.1 FTIR spectral analysis

The influence of the organosolv process conditions in the chemical structure of the OL was investigated by FTIR. The band assignments of FTIR spectra are presented in **Table 1**. Characteristic lignin bands for O-H stretching (3412-3460 cm^{-1}), C-H stretching (3000-2842 cm^{-1}), and aromatic skeletal vibrations (1593-1605 cm^{-1} and 1505-1515 cm^{-1}) were observed for all fractions [47]. The high purity of these lignin fractions is also verified by the absence of the cellulose band (1150 cm^{-1}) [48].

Furthermore, the spectra and the intensity of the bands were very similar for the different fractions of lignin from organosolv, which confirmed that the structure of lignin did not change with the different conditions of organosolv pretreatment (**Fig. S1**).

3.2.1.2 NMR spectral analysis

Additional information on the structure of the isolated lignins was obtained by 2D-NMR, analyzing the samples OL_2 and OL_{10} according to methods previously described in section 2.4.2. The two samples were easily dissolved in DMSO-d_6, and the two-dimensional heteronuclear single quantum correlation (HSQC) solution NMR spectra were obtained. The qualitative analysis of the HSQC (1 bond ^{13}C-^{1}H correlation) spectra was performed by comparing the signals observed in two regions, the oxygenated side-chain (δ_C/δ_H 50−90/3.0−5.5), and the aromatic/unsaturated (δ_C/δ_H 100−135/6.5−8.0), with reported data [36,37] enabling to identify the aromatic components and the types of the interunit bonding patterns of these lignin fractions (**Figure 4; Table S2**).

In addition, the semi-quantitative analysis of the volume integration of contours in the HSQC spectra enabled to estimate the relative abundances of the main lignin interunit linkages and end-groups, as well as molar abundances of the different lignin components (H, G, and S), and the molar S/G ratios of both lignins (**Table 2**).

In the aromatic region (**Table 2; Fig. S2**), by integrating the contours of the 2D 13C–1H correlation (HSQC) spectra was obtained the composition in the *p*-hydroxyphenyl/guaiacyl/syringyl (H/G/S) distributions of the two lignins. Integrals from the aromatic C–H correlation signals in like environments were selected (i.e., G_2 and $S_{2/6}$). As the contour for syringyl units contains two symmetrical C–H correlations ($S_{2,6}$) versus

one in guaiacyl units (G_2), a factor of ½ was used to adjust the volume of the $S_{2,6}$ contours. The same adjustment was made for the $H_{2/6}$ correlations in the H units. Based on the obtained results, both lignins show a guaiacyl-syringyl type with small amounts of *p*-hydroxyphenyl units (L_2, 3.1 (H) /24.9 (G) / 72.0 (S); OL_{10}, 5.6 (H) /25.9 (G) / 68.5 (S)). Comparing the two lignins, only slight differences were observed between them. OL_{10} presents only 3.5% less syringyl (S) units, 1.0% more guaiacyl (G) units, and 2.5% more *p*-hydroxyphenyl (H) units than L_2. Both samples showed similar S/G ratios, but a significant H/G ratio reduction in OL_{10} sample was observed. The increased proportion of the H units in OL_{10} might be associated with the harsher extraction conditions used in the isolation of OL_{10} as higher cross-coupling and/or molecular weight are expected for the lignin polymer chain containing H units.

The analysis of the aliphatic-oxygenated region of spectra (**Table 2; Fig. S3**) revealed that OL_2 and OL_{10} present basically the same interunit linkages, without significant differences in their distribution in the two lignin samples. β-Ether (β–O–4) units A (**Figure 4**) are the major interunit structures of both lignins (L2, 78.6%; OL10, 79.1%), followed by almost equivalent amounts of the resinol units C (β–β; L_2, 8.0%; OL_{10}, 10.1%) and phenylcoumaran units B (β–5; OL_2, 11.3%; OL_{10}, 9.2%). The presence of the minor dibenzodioxocins (5–5/ 4–O–β) units D and spirodienones (β-1) SD substructures were not detected.

The reduced amount of phenylcoumaran (β–5) units B is consistent with the higher levels of the syringyl (S) units, as the formation of B interunits requires the coupling of a monolignol with guaiacyl (G) or *p*-hydroxyphenyl (H) units. So, relatively fewer phenylcoumaran units B exist when compared to the major β-ether (β –O–4) units A in these lignins. Likewise, dibenzodioxocins D were not detected because they also involve

coupling between a monolignol and 5–5-coupled (biphenyl) units that cannot be formed from S units. Finally, and as expected, another consequence of the syringyl predominance in both samples is the higher β-ether (β–O–4) A levels, which is easily explained by the simple fact that for the addition of a monolignol to a syringyl unit, a single pathway involving a β-O–4-coupling is essentially available. Ovejoro-Pérez et al. also reported lignin from eucalyptus wood mainly containing syringyl units over guaiacyl units and only traces of *p*-hydroxyphenyl units, being the β-O-4′ the main interunit linkages, reaching up to an 80% of total interunit linkages. However, the pretreatment of eucalyptus biomass with ionic liquid resulted in a β-O-4′ linkages degradation and an enrichment of resinol substructures as severity conditions increased, while phenylcoumaran structures were only verified at the mildest conditions [49].

3.2.2 Thermal analyses

3.2.2.1 Thermal stability

Thermal analysis includes some techniques that establish a connection between temperature and the physical properties of materials. The thermal stability of the lignin samples (OL_1-OL_{10}) was studied by TGA. **Table 3** presents the starting degradation temperature (T_{onset}), the maximum degradation temperature (T_{max}) and the char residue at 800 ºC for the lignin fractions. The values of T_{onset} vary from 234.30 – 262.45 ºC for the fractions obtained from organosolv. The lowest temperature corresponded to the less severe condition (experiment 1), and the higher temperature to the second most severe condition (experiment 9). These results were in agreement with Ovejero-Pérez et al. [49], in which the highest thermal stability of the extracted lignins was obtained under the most severe conditions due to their content in resinol structures.

Sebio-Punal et al. [50] observed a lignin degradation temperature of approximately 200 ºC for hardwood (*Castannea sativa*, *Eucaliptus globulus*, and *Quercus robur*), and for softwood (*Pinus pinaster* and *Pinus sylvestris*) in the range of 290–300 ºC, this fact was explained by the different behavior of softwood and hardwood.

In this sense, the onset temperature of around 225 ºC was also reported by Martín-Sampedro et al. [30] for hardwood lignin, namely organosolv lignin from *Robinia pseudoacacia L*. In the current study, which involves a mixture of hardwood and softwood, the T_{onset} obtained are between these values. However, the results do not represent the real behavior of lignin, as the isolation process makes significant chemical changes in its structure and removes interactions between fractions [51]. Nevertheless, T_{onset} is an important thermal property of the material, which may limit its application in plastics, for example [52].

Regarding the T_{max}, these ranged from 359.23 to 380.55 ºC. Wen et al. [53]verified maximal degradation of an organosolv lignin from birch, a hardwood, at 355.7 ºC, suggesting that more stable lignin structures, such as condensed structures, were formed after the organosolv process, as also reported by the authors. Nonetheless, Martín-Sampedro et al. [30] reported the maximal lignin degradation at 475 ºC for hardwood. As mentioned, the T_{max} from OL of the current work, which involves a mixture of hardwood and softwood, are among these values.

The mass remaining at 800 ºC (char residue) varied from 28.71% to 38.38%. This is in agreement with the work of Ross et al. [54], which reported at 800 ºC, the mass remaining between 34.7% and 36.9% for lignin extracted from wheat straw, triticale straw, and flax shives with ionic liquid. This amount of residue is also comparable to that reported by Elshafie et al. [55] for lignin from black liquor.

In general, as reported by Prime et al. [56], if the polymeric portion of a material is relatively stable, up to 300 ºC, only a low molecular mass will be lost; between 300 ºC and 600 ºC, most polymers will degrade and volatilize; and above 600 ºC, some polymers based on aromatic structures will not be converted into gaseous products and will form a char residue, as was verified in OL samples of the current work.

3.2.2.2 Glass transition temperature

The glass transition temperature (T_g) is an important property of using lignin as a polymer for industrial applications, and it limits its usability in the glassy state (for example, polystyrene and epoxy) or in the rubbery state (for example, polysoprene or polybutadiene). At T_g (on heating), the glassy state changes to the rubbery or melt state. Nonetheless, the glass transition does not occur at a precise temperature but in a temperature range, thus being a kinetic transition [57].

In this study, to determine the T_g for the different lignin fractions from the organosolv process, was applied first heating to remove the thermal history in all lignin fractions. **Table 3** presents the values of T_g obtained from DSC. The studied lignins had a glass transition temperature between 73–85 ºC, and it was not possible to detect T_g in treatment with the highest ethanol concentration (80%). The results showed that, in general, increasing treatment hardness led to a decrease in T_g values.

The low values of T_g are related to the decline in the rigid aromatic backbone of macromolecules due to the replacement of hydroxyl groups (namely phenolic hydroxyl groups) by ester, which leads to a reduction in the number of hydrogen bonds in the lignin

molecule, implying an increase of the free volume and thus the mobility of the chains [52,58].

In general, T_g of lignin has been reported to be between 90 and 180 ºC [59,60], but lower T_g has been reported for organosolv lignin [22,61–63]. For example, Hussin et al. [61] reported T_g values of 54.65 ºC and 64.65 ºC for organosolv and kraft lignin, respectively, while for soda lignin, it was 81.79 ºC, suggesting that the T_g values are affected by the relation between the molecular weight of lignin samples and the free volume [61]. Li and McDonald [52] studied the fractionation of commercial lignin with methanol, and reported a lower T_g for the methanol soluble lignin fractions, 117 ºC for Indulin AT lignin (softwood kraft), and 132 ºC for Protobind 1000 lignin (agricultural fiber soda pulp), than the original lignin, which was related to the fewer condensed structures of the methanol soluble lignin fractions [52]. On the other hand, Martín-Sampedro [30] showed a T_g at 138 ºC for lignin obtained from organosolv treatment of *Robinia pseudoacacia L.*. Low T_g temperatures, as those of the current work, may be related to more thermoplastic lignins. Thus, these OL are more susceptible to being mixed with synthetic and bio-based polymers [30].

3.2.3 Radical scavenging activities

DPPH method was used to determine the antioxidant activity of the lignin fractions, which were compared in terms of IC_{50}.

Radical scavenging activity of DPPH of all OL fractions did not strongly differ between samples (IC_{50} from 0.14 to 0.21 mg/mL). Although their antioxidant potential was lower than that of Trolox (IC_{50} of 0.06 mg/mL), they were comparable to BHT (IC_{50} of 0.16

mg/mL), a commercial antioxidant often used as standard (**Table S3**). **Figure 5** compares the RSA of OL10 (the optimal condition for OL extraction and the most severe condition studied), OL2 (one of the mildest conditions studied), and the standards trolox and BHT, confirming the antioxidant potential of OL is comparable to BHT.

It has been reported that the antioxidant activity is related to the lignin extraction technique, as well as the lignocellulose source [22, 54]. For example, Aguié-Béghin et al. [64] reported IC_{50} of around 0.31 mg/mL for organosolv lignin and 0.37 mg/mL for alkali lignin (DPPH method) [64]. Michelin et al. also reported low IC_{50} for organosolv lignin (0.17 – 0.26 mg/mL, DPPH method) [22]. Lu et al. [28] compared the antioxidant capacity of the organosolv lignin extracted with different organic solvents, and the lignin of the acetic acid-water mixture was the most effective with IC_{50} of 0.66 mg/mL, which showed lower radical scavenging capacity when compared with our results.

3.3 Enzymatic hydrolysis of delignified biomass mixture

The glucose yield (GY) after 72 h of EH is shown in **Figure 6**, using the solid fraction resulting from the optimal organosolv condition (200 ºC, 60 min, 80% EtOH) at 5% and 10% solids load. This condition was selected considering the highest delignification since lower lignin content could be related to higher enzymatic susceptibility of cellulose, as cellulases can irreversibly bind to lignin [44].

The current work obtained the maximum GY of 50% at 10% solids, being the maximum glucose concentration of 37.51 g/L, as shown in Figure 6. Other authors have reported GY in this range for two-stage pretreatments. For example, Mesa et al. [65] obtained a maximum GY of 29% using 5% solids at the most severe condition studied (195 ºC, 60

minutes, and 60% of ethanol concentration) of the sequential pretreatment of acid pretreatment and organosolv of sugarcane bagasse. This yield was similar to the current work for a solid load of 5% solids (33% GY). Obama et al. [44] used a Miscanthus biomass in two combined treatments of autohydrolysis + organosolv (AO), and EH + organosolv (EO) and obtained a maximum of CGC (cellulose-to-glucose conversion) of approximately 40%, in conditions similar to those of this work. This conversion increased to approximately 60% with the enzymatic prehydrolysis step instead of autohydrolysis [44], suggesting a possible repolymerization of small lignin fragments promoted by autohydrolysis. Santos et al. [66] discussed in a recent work the EH yield as a function of the insoluble lignin content of the pretreated solids, showing that the higher the lignin content, the lower the EH yield. Tong et al. [21,67] also verified lignin inhibition on enzymatic hydrolysis of cellulose caused by lignin repolymerization after acid pretreatment and the strategies used to surpass this barrier, but in this work, 2% solid loading was used. On the other hand, crystallinity has also been reported as one of the most important factors affecting enzymatic hydrolysis efficiency since higher cellulose crystallinity makes biomass inaccessible to enzymatic attack [68]. However, they verified that the changes in cellulose crystallinity in imidazole treatment are related exclusively to the great lignin removal once delignification contributes to increase the cellulose content in the treated material.

Despite that, the sequential process of autohydrolysis and organosolv allowed biomass fractionation into hemicellulose, lignin, and cellulose, respectively, for further valorization as a biorefinery overview. This sequential process uses renewable feedstocks and green processes, recovering high-purity lignin that proved to be a promising resource to produce aromatic compounds to replace fossil fuels.

4. Conclusion

A mixture of biomass was pretreated by autohydrolysis and organosolv processes. The isolated lignins showed high purity and kept the main structure, according to FTIR and NMR results. Process hardness seemed to influence their thermal properties. Moreover, lignins showed antioxidant activity as high as the commercial antioxidant BHT and a 50% GY was obtained with the pretreated solids. The organosolv process proved to be an effective method to separate lignin from autohydrolyzed biomass, with high purity, structure relatively unaltered, and high antioxidant activity. A new pathway for a mixture of unexploited resources was identified, contributing to the biobased society and industry development.

CRediT authorship contribution statement

Pontes Rita: Conceptualization, Investigation, Methodology, Data curation, Writing-Original draft preparation. **Michele Michelin**: Conceptualization, Validation, Supervision, Writing-Reviewing. **Aloia Romaní**: Conceptualization, Validation, Supervision, Writing-Reviewing. **Alice Dias**: Methodology (NMR analysis), Writing-Reviewing. **José Teixeira**: Writing-Reviewing, Supervision and Funding acquisition. **João Nunes**: Writing- Reviewing, Supervision, and Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UIDB/04469/2020 unit and Centre Bio R&D Unit (UIDB/05083/2020 and UIDP/05083/2020), and by LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechanical Systems, LA/P/0029/2020. Centre CQUM (UIDB/00686/2020), and National NMR Network (RNRMN) (PINFRA/22161/2016) and Project Excelencia ED431F 2022/09 k251 (program funded by FEDER and Xunta de Galicia). Rita Pontes (SFRH/BDE/10916/2015) thank FCT for their doctoral felowship. Aloia Romaní acknowledges the Ministry of Science and Innovation (MICIN) through the grant RYC2020-030690-I.

Appendix A. Supplementary material

Supplementary data to this article can be found online at ...

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Table 1. Assignments of FTIR bands of lignin [47].

OL_1	OL_2	OL_3	OL_4	OL_5	OL_6	OL_7	OL_8	OL_9	OL_{10}	Assignments
Bands (cm^{-1})										
3414	3420	3447	3420	3416	3420	3420	3420	3420	3420	O-H stretch
2938	2938	2938	2930	2928	2930	2930	2934	2932	2932	C-H stretch in methyl and methylene groups
1595	1593	1595	1595	1595	1595	1593	1595	1595	1595	aromatic skeletal vibrations plus C = O stretch; S > G; G condensed > G etherified
1514	1508	1508	1514	1514	1514	1508	1508	1514	1508	aromatic skeletal vibrations; G > S
1460	1460	1460	1462	1462	1460	1460	1462	1460	1462	C-H deformations; asym.in $-CH_3$ and $-CH_2-$
1423	1423	1423	1423	1423	1423	1423	1423	1423	1423	aromatic skeletal vibrations combined with C-H in-plane deform.
1325	1325	1325	1325	1325	1325	1325	1325	1325	1325	S ring plus G ring condensed; (i.e. G ring substituted in pos. 5)
1221	1221	1221	1221	1221	1221	1221	1221	1221	1221	C-C plus C-O plus C = O stretch; G condensed > G etherified
1086	1086	1086	1086	1086	1086	1086	1086	1086	1086	C-O deformation in secondary alcohols and aliphatic ethers
1030	1030	1030	1030	1030	1030	1030	1030	1030	1030	aromatic C-H in-plane deformation, G > S; plus C-O deform, in primary alcohols; plus C = O stretch (unconj.)
829	833	833	833	829	829	831	831	829	827	C-H out-of-plane in positions 2,5, and 6 of G units

Table 2. Structural characteristics (lignin interunit linkages, aromatic units, and S/G ratio) from the integration of $^{13}C–^{1}H$ correlation peaks in the HSQC spectra

Lignin Structures	OL2	OL10
lignin interunit linkages (%)		
β-O-4' aryl ethers (A)	78.6	79.1
α-oxidized β-O-4' aryl ethers (Aox)	2.1	1.6
phenylcoumarans (B)	11.3	9.2
resinols (C)	8.0	10.1
dibenzodioxocins (D)	n.d.	n.d.
spirodienones (SD)	n.d.	n.d.
total	100	100
lignin aromatic units		
H (%)	3.1	5.6
G (%)	24.9	25.9
S (%)	72.0	68.5
total	100	100
G'/(G'+G) (%)	4.1	5.2
S'/(S+S') (%)	2.1	1.5
S'/S ratio	0.02	0.15
G'/G ratio	0.04	0.05
S/G ratio	2.9	2.6
H/G ratio	0.12	0.21

n.d.: not detected

Table 3. Thermal analysis of lignins

Samples	T_{onset} (ºC)	$T_{max.}$ (ºC)	Residue at 800 ºC (%)	T_g (ºC)
OL_1	234.30 ± 0.37	365.41 ± 1.68	36.84 ± 0.27	80.67 ± 2.04
OL_2	246.23 ± 0.50	359.23 ± 2.04	38.38 ± 0.12	84.78 ± 2.26
OL_3	255.68 ± 2.87	371.86 ± 8.66	33.89 ± 1.30	83.93
OL_4	258.60 ± 0.00	367.75 ± 3.45	34.61 ± 1.16	80.01
OL_5	247.29 ± 2.00	380.55 ± 1.97	29.61 ± 0.18	76.77 ± 1.73
OL_6	245.17 ± 2.25	367.18 ± 7.16	34.88 ± 0.66	72.87 ± 0.05
OL_7	247.29 ± 0.75	364.00 ± 0.98	30.97 ± 0.05	n.d.
OL_8	252.59 ± 0.00	372.74 ± 0.00	28.71 ± 0.29	n.d.
OL_9	262.45 ± 2.80	359.58 ± 0.44	29.64 ± 0.47	n.d.
OL_{10}	248.26 ± 2.12	366.91 ± 3.89	30.78 ± 0.14	n.d.

The values expressed are the average of three replicates
n.d.: not detected

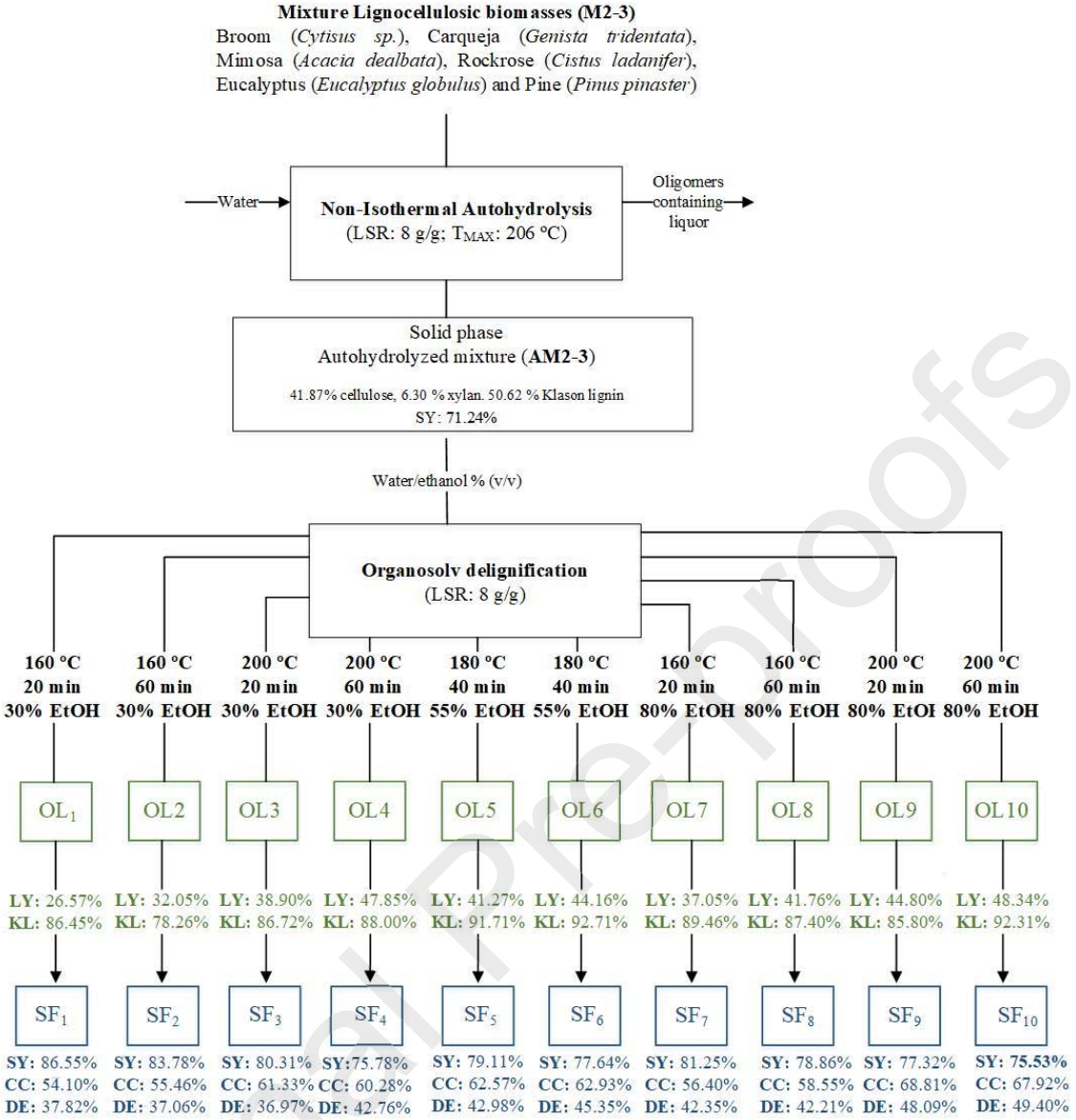
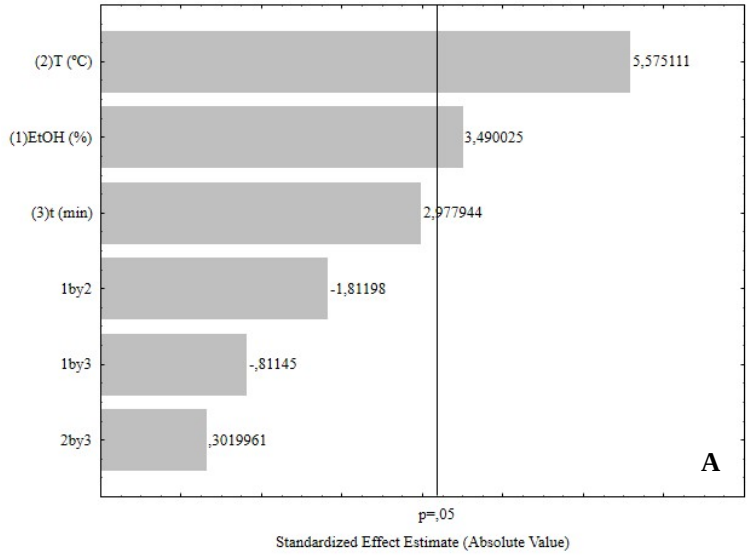
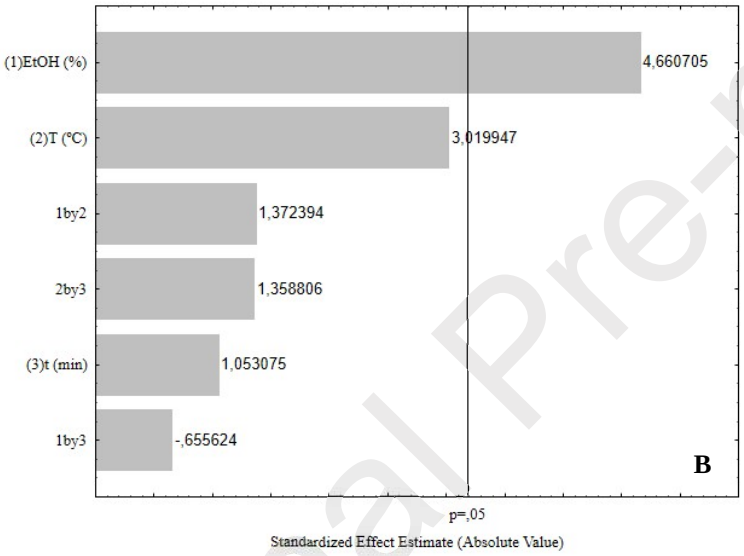


Figure 1. Schematic representation of the organosolv delignification process. Each condition resulted in an organosolv lignin (OL) and a solid fraction (SL). The parameters evaluated were lignin yield (LY), Klason lignin (KL), solid yield (SY), cellulose content (CC), and delignification extend (DE).





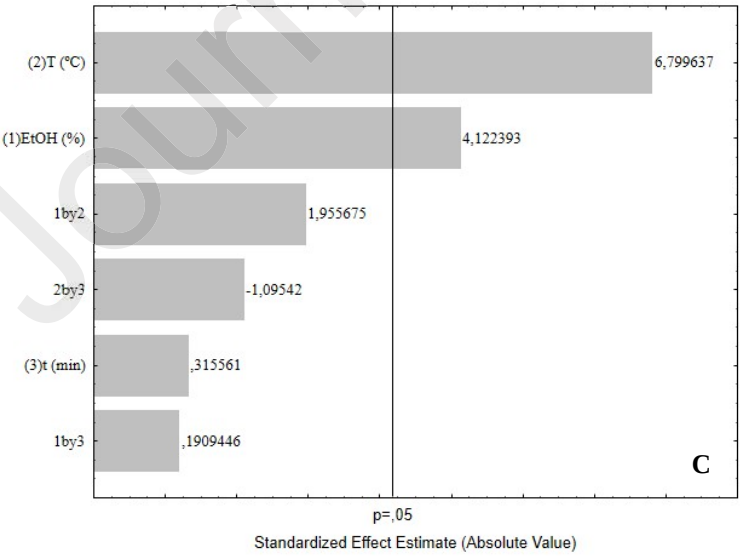
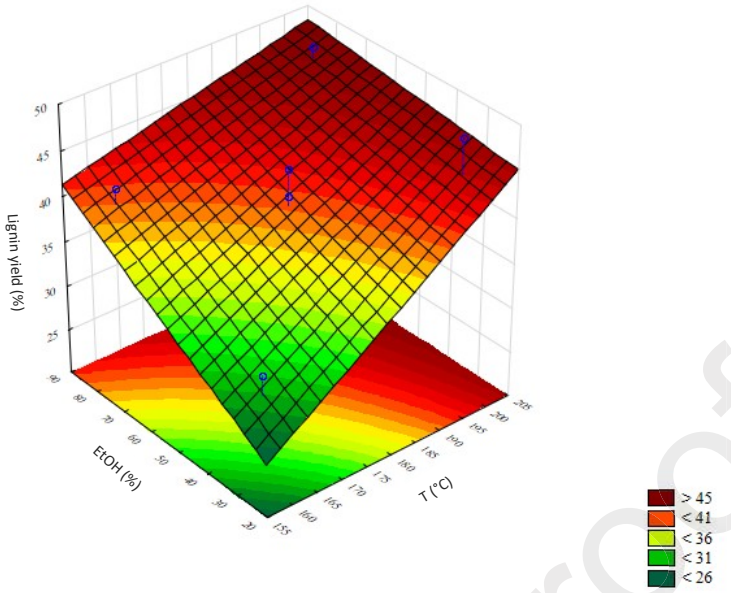
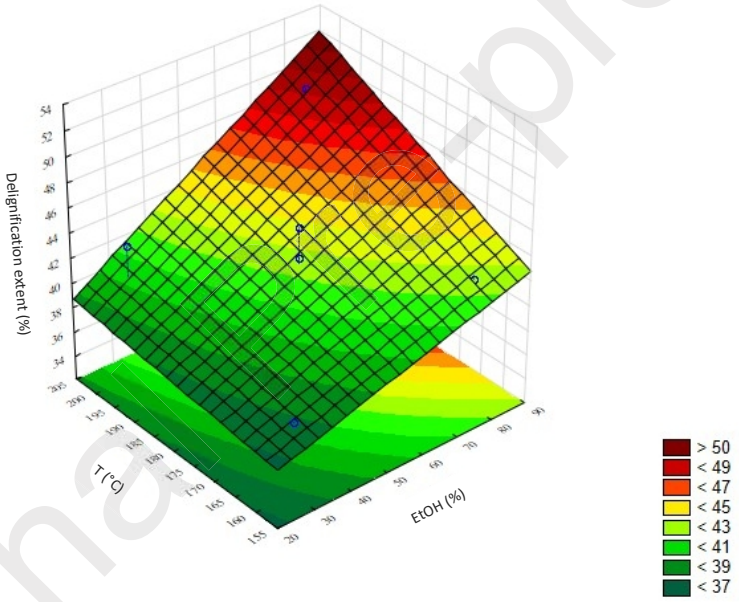


Figure 2. Pareto diagrams of the responses from the cubic experimental design. (1) EtOH (%) denotes the ethanol concentration, (2) T (ºC) the temperature, and (3) t (min) the time, 1by2 the interaction between EtOH and T, 1by3 interaction between EtOH and t, 2by3 interaction between T and t for lignin yield (A), delignification (B) and cellulose content (C).

A



B



C

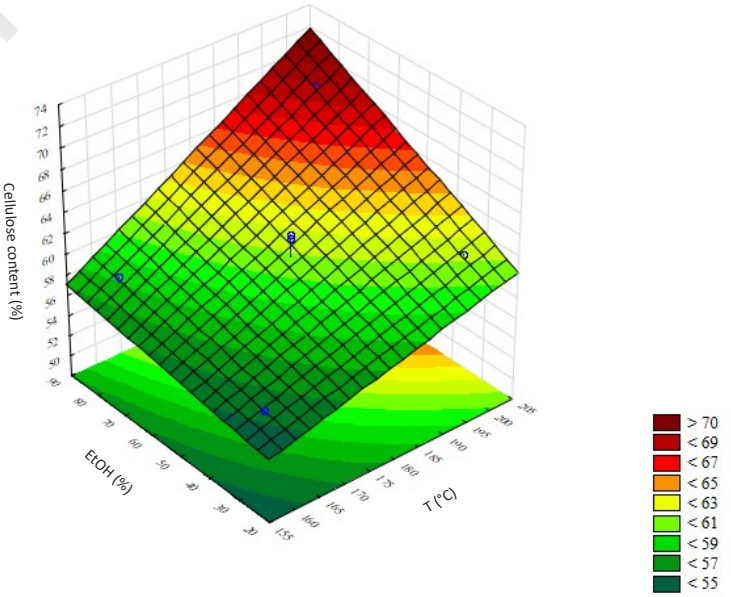


Figure 3. Response surface for (A) lignin yield (%), (B) delignification (%), and (c) cellulose content (%) with the variable temperature (˚C) and ethanol concentration (EtOH %),

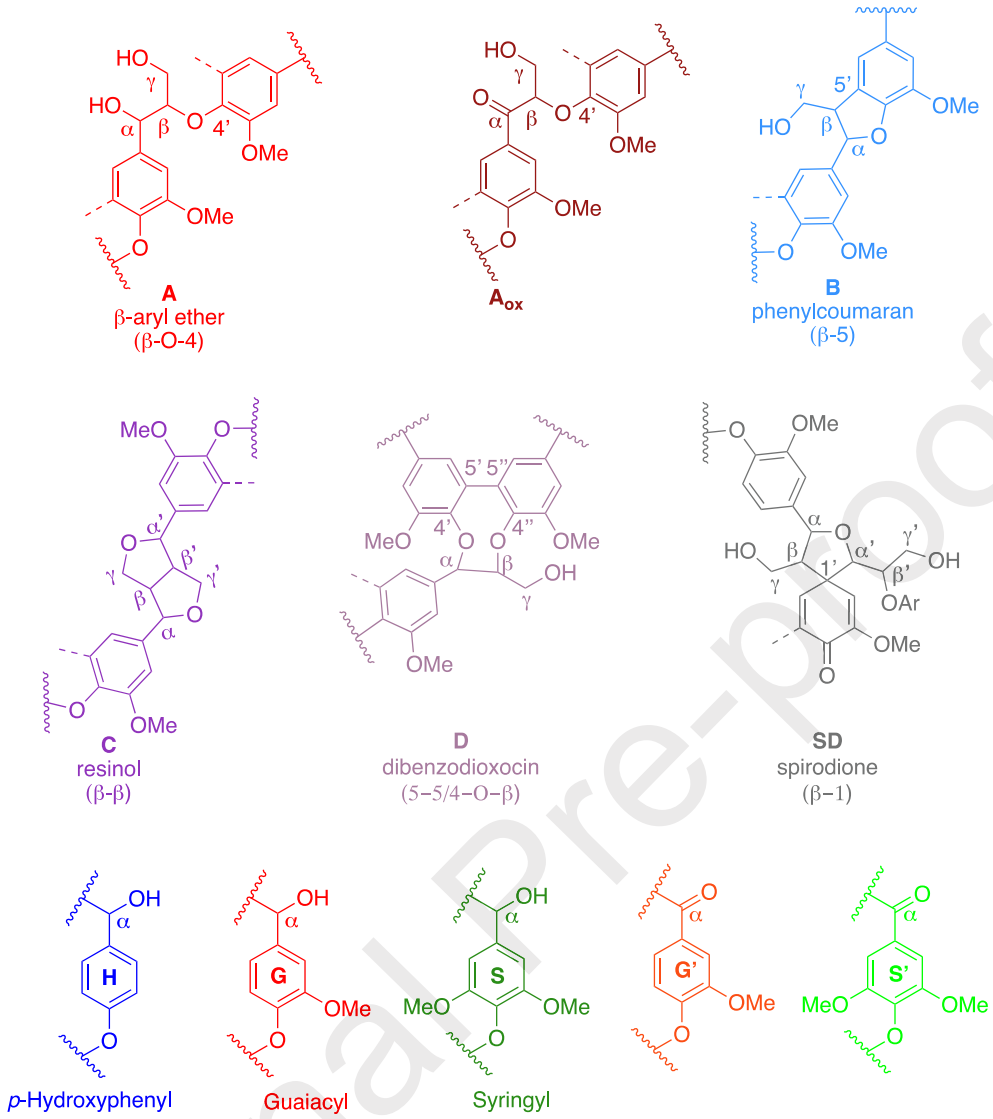


Figure 4. Main structures identified in lignins OL_2 and OL_{10} by heteronuclear single quantum correlation nuclear magnetic resonance (HSQC)

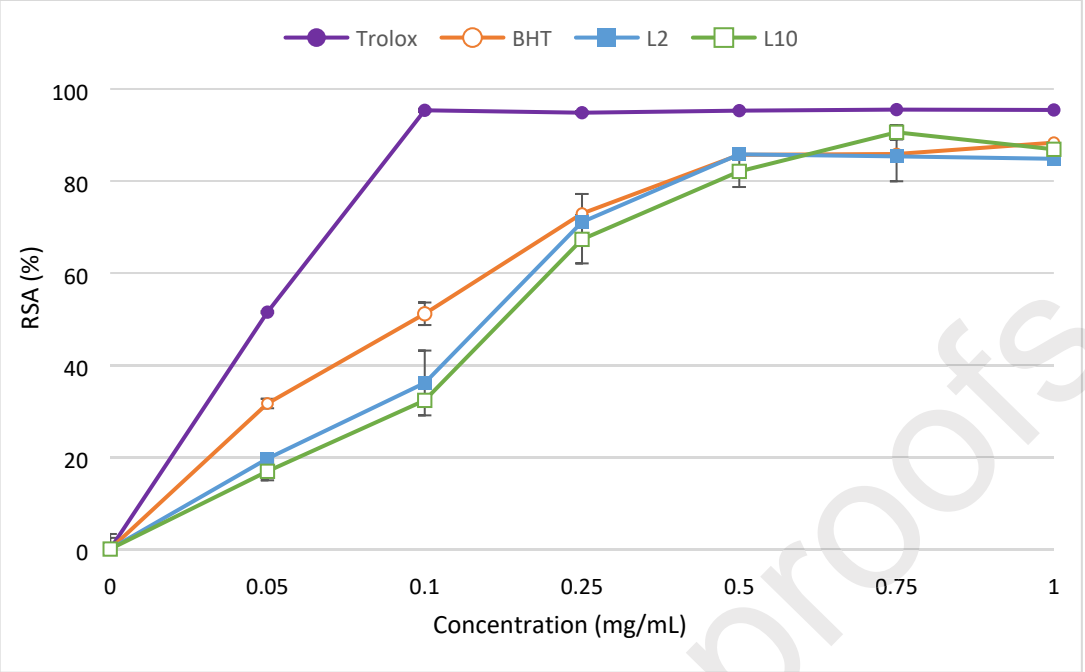


Figure 5. Radical scavenging activities of OL samples (OL_2 and OL_{10}) compared with commercial antioxidants (BHT and Trolox) for DPPH assay.

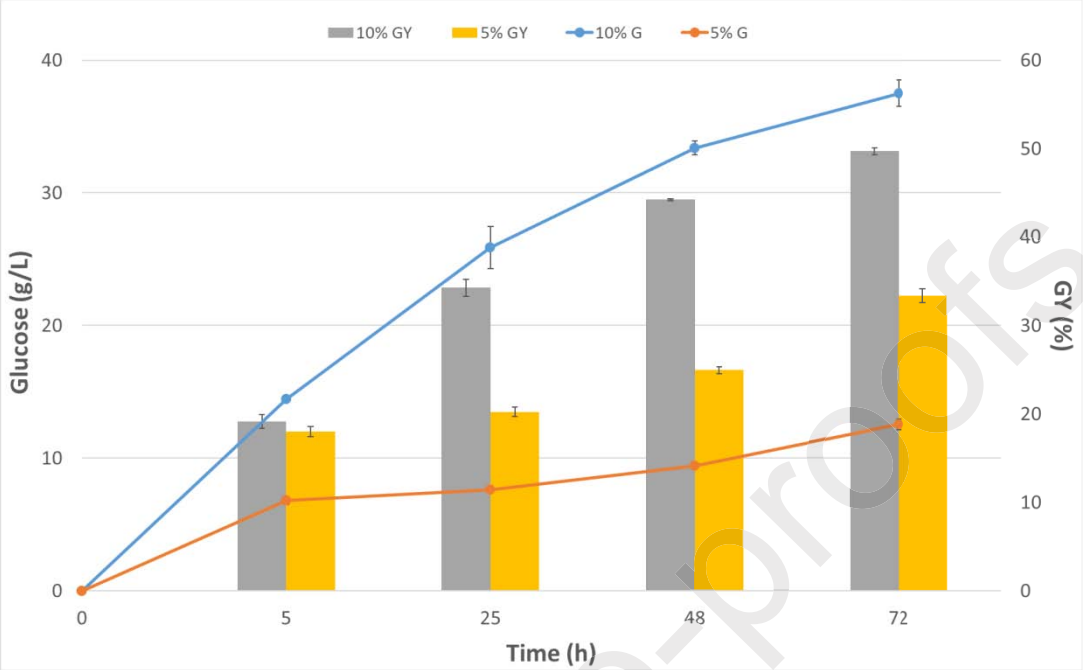


Figure 6. Glucose concentration (G) and glucose yield (GY) during enzymatic hydrolysis of the solid fraction from the optimal organosolv treatment (200 ºC, 60 min, and 80% EtOH) for 5% and 10% solids load (the standard deviation is presented on the graph). Gray and yellow bars refer to GY at 10% and 5% solids, and blue and orange lines refer to glucose released at 10% and 5% solids, respectively.

Highlights

A complex mixture of six lignocellulosic biomasses was pretreated for the first time.

High pure lignin was isolated from the sequential autohydrolysis and organosolv

The temperature had more effect on lignin yield than ethanol concentration and time

Lignin structure was almost unchanged by the organosolv process

Lignin showed antioxidant activity as high as the commercial antioxidant BHT