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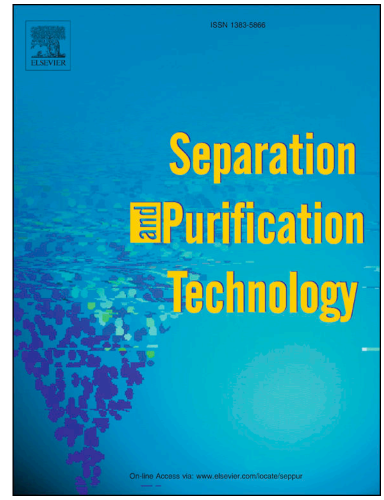

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Evaluating the integration of olive tree pruning fractionation with ternary eutectic solvent after aqueous pre-extraction or dilute acid pre-treatment

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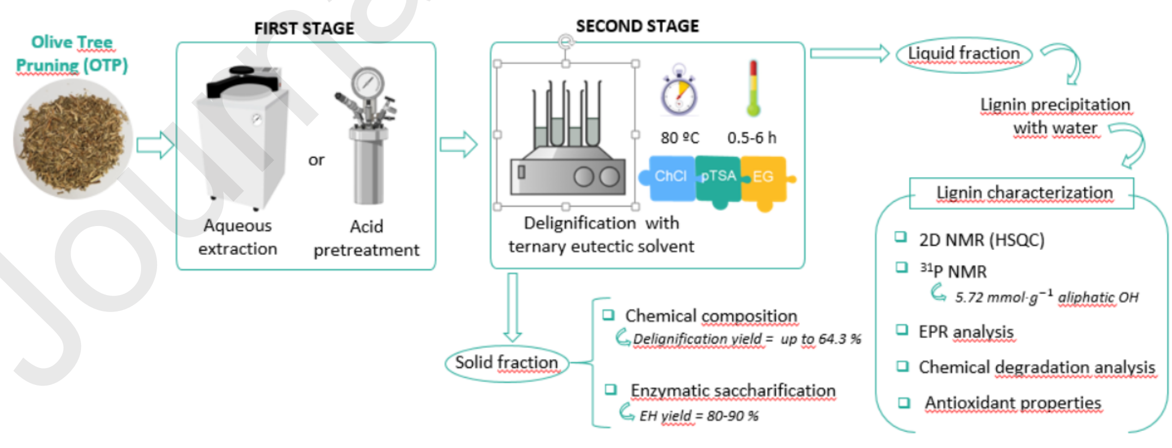

ABSTRACT

Developing lignocellulosic biomass fractionation approaches that valorize lignin besides the conventional polysaccharide conversion routes have been a growing interest in achieving full biomass cascade processing. Additionally, integrating and designing technologies that use environmentally friendly and recyclable solvents following green chemistry is required. Within this context, the present work aims at investigating the integration of pre-treatments, such as aqueous pre-extraction or dilute acid pre-treatment, with the fractionation of the olive tree pruning biomass (OTP) assisted by ternary eutectic solvent (ES) composed of cholinium chloride, *p*-toluenesulfonic acid and ethylene glycol (ChCl:pTSA:EG; 1:1:9). The impact of both technological integrations on lignin isolation yield and on lignin physicochemical, thermal and antioxidant properties were the major parameters under evaluation. Overall, the most efficient integration was accomplished with aqueous pre-extraction plus ternary ES treatment. Around 64.3 % and 36.6 % delignification were achieved with ES, when using solid fractions obtained from aqueous pre-extraction and dilute acid pre-treatment, respectively. The lignin isolated after aqueous pre-extraction and ES treatment showed a remarkable aliphatic OH content (5.72 $mmol \cdot g^{-1}$). Finally, the integration of these processes enabled cellulose enrichment of remaining solid fractions that were accessible to enzymes (80.1-90.3 % hydrolysis yield).

KEYWORDS: ternary eutectic solvents; process integration; lignin valorization; antioxidant properties; biorefinery;

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1. INTRODUCTION

The olive sector plays a vital role in the Mediterranean region from a socioeconomic and cultural perspective (Donner et al., 2022). Tree pruning is a biennial operation performed after olive harvesting to increase productivity, growth and health of olive trees for a new season. However, this activity produces a large amount of lignocellulosic material known as olive tree pruning (OTP). Currently, the area under olive cultivation in Mediterranean countries is approximately 5 million ha (FAOSTAST, 2024) with an estimated OTP annual production of 3 t/ha (Najafi et al., 2021). Although OTP has been explored for energetic purposes during the last decades (Mamaní et al., 2021), its high abundance and valuable composition make this residue a promising bio-based raw material to be processed in the frame of the biorefinery and circular bioeconomy concepts (Donner et al., 2022). Ensuring an efficient processing of OTP is expected to contribute with significant economic, environmental and social benefits to the Mediterranean region, by increasing energetic valorization, environmental protection, resource sustainable management and simultaneously decreasing undesired green-house gas emissions (Yin et al., 2021). Moreover, the development of biorefinery processes envisaging technological integration and the use of green solvents emerges as hot research topics towards a more sustainable fractionation and conversion of OTP into a wide range of value-added bio-based products, including fuels, chemicals and materials (González et al., 2023; New et al., 2022).

Cost-effectiveness, recyclability of solvents/catalysts/reagents, and ease of operation are criteria that must be met to deliver an efficient OTP valorization (George et al., 2015). Additionally, the integration of different processes capable of delivering high fractionation efficiency without degrading biomass fractions is also of utmost importance to maximize the valorization pathway of such raw material. One of the critical factors upon OTP fractionation relies on the high content of non-structural components, namely extractives, including phenolic compounds, tannins, terpenes, fatty acids, and proteins, among others (Fernandes-Klajn et al., 2018). If these components are not removed, the fractionation efficiency of structural components, including cellulose, hemicelluloses and lignin can be negatively affected. To address this, pre-extraction processes using water or organic solvents (e.g. ethanol) are usually applied to enable the removal of extractives and to expose the lignocellulosic matrix, facilitating further fractionation processes.

Regarding to the fractionation of structural components, conventional pre-treatments, such as alkaline extraction based on aqueous solutions of NaOH, KOH, NH_4OH and Na_2CO_3 (Seok Kim et al., 2016), acid hydrolysis based on aqueous H_2SO_4 and HCl (Swiatek et al., 2020), hydrothermal treatments (e.g. autohydrolysis) (Scapini et al., 2021), organosolv (Gómez-Cruz et al., 2021), steam explosion (Romero-García et al., 2022), and pyrolysis (Zadeh et al., 2020), can be applied. Particularly, aqueous diluted acid pre-treatments have been used for the selective hydrolysis of hemicelluloses to produce oligosaccharides and/or pentoses as main products, leaving a solid composed mostly of cellulose and lignin (Díaz-Blanco et al., 2018) that requires further processing. As following step, lignin is dissolved (either by alkali or organic solvents), leaving a cellulose-rich solid with better enzyme accessibility towards saccharification. However, isolated lignin is prone to chemical modifications (e.g. oxidation and condensation reactions) during alkaline processes (Narron et al., 2016), resulting in a low-value lignin fraction that will negatively affect its applicability. Although low-value lignin can be employed in the manufacture of pellets, binders, dispersants, soil stabilizer and cement (Gaspar and Fardim, 2023); high-quality lignin, exhibiting for instance high phenolic content, radical scavenging ability and broad adsorption in the UV-Vis range, can be advantageous as an antioxidant and UV protectant material in the formulation of high value products (Collins et al., 2019). In this sense, low-thermal and environmentally friendly biomass processing, using alternative reaction media, such as eutectic solvents (ES), could stand as suitable alternative to alkaline processes towards the recovery of high-quality lignin fraction from pre-extracted biomass (eg from OTP).

ES are basically a mixture of two or more components in which the melting temperature of the mixture is lower than that of the individual components. The chief technical advantageous of an ES relies on its intrinsic and unique characteristics, i.e., it is a liquid at a given desired temperature where at least one of its components would, otherwise, be a solid unfit to be applied as a solvent (Abranches and Coutinho, 2023). Recently, numerous studies have demonstrated the efficacy of ES in extracting lignin from different lignocellulosic biomass materials (Amesho et al., 2023; Gundupalli et al., 2022; Tan et al., 2023). The enhanced ability of ES, particularly acid-based, to selectively break aryl ether bonds between lignin units (Gómez-Cruz et al., 2024), being a major reason behind the high delignification yields achieved with such solvents (Loow et al., 2017). Binary ES, including cholinium chloride (ChCl):lactic acid (LA) or ChCl:ethyleneglycol (EG), have demonstrated advantages in respect to conventional biomass treatments. These include higher selectivity for extracting lignin and hemicelluloses (Kumar et al., 2016) and improvements of glucan conversion to glucose in enzymatic hydrolysis (Can et al., 2023). However, ternary ES have shown to be more effective in the fractionation of lignocellulosic biomass under mild operating conditions and simultaneously have enabled to isolate lignin with unconventional structures (Gómez-Cruz et al., 2024; Poy et al., 2023; Zhai et al., 2020). A particular example is the combination of cholinium chloride (ChCl), *p*-toluenesulfonic acid (pTSA) and ethylene glycol (EG) at a molar ratio 1:1:9, that recently demonstrated the potential to extract a unique lignin fraction from olive tree pruning, characterized by EG grafting and oleic acid esterification (Gómez-Cruz et al., 2024), while simultaneously preserving the cellulose content in the resulting solid towards one of the highest butanol production titer achieved by fermentation (Poy et al., 2023).

Taking the advantages of ChCl:pTSA:EG (1:1:9) in biomass fractionation, the present work aimed at studying for the first time the integration of already optimized aqueous pre-extraction (120 °C, 60 min) or dilute acid pre-treatment (2.4 % w/v H_2SO_4, 130 °C, 84 min) with the fractionation of the olive tree pruning (OTP) assisted by the ternary ES. The impacts of those two upstream steps on the biomass fractionation efficiency as well as on the quality of isolated lignin, particularly its chemical, thermal and antioxidant properties, were evaluated. Envisaging the integral valorization of OTP within the biorefinery and circular economy framework, the chemical composition of delignified solids was determined, while their enzymatic digestion into fermentable sugars was also assessed.

2. MATERIALS AND METHODS

2.1. Biomass pre-treatments

Olive tree pruning (OTP) was obtained from an olive orchard in Jaén (Andalucía, Spain). OTP was subjected to a first aqueous pre-extraction (120 °C, 10% w/v solid, 60 min) in autoclave to remove extractives according to a study published elsewhere (Martínez-Patiño et al., 2017). The resulting solid was called AQ-OTP. Afterwards, dilute acid treatment was performed on AQ-OTP in a benchtop reactor fitted with magnetic stirring (Parr Instrument Company, Moline, IL, USA) under previously conditions optimized (130 °C, 84 min, 20 % w/v solid, 2.4% w/v H_2SO_4) (Martínez-Patiño et al., 2017). The acid pre-treated solid, called as PT-OTP, was washed with deionized water and dried at 40 °C. The AQ-OTP and PT-OTP materials were characterized following the standard method provided by the National Renewable Energy Laboratory (NREL, Golden, CO, USA) as described in Section 2.4.1. The obtained data is shown in Table S1.

Subsequently, AQ-OTP and PT-OTP were milled to a size of ~1 mm using a ZM 200 ultracentrifugal ball mill (Retsch, Haan, Germany) and sieved to a particle size between 0.250-1 mm. Moisture content was determined using the HE53 moisture analyzer (Mettler Toledo, Barcelona, Spain). Approximately 4.49% and 3.78% w/w of moisture content was determined for AQ-OTP and PT-OTP, respectively.

2.2. Eutectic solvent preparation

The preparation of ternary ES composed of ChCl, pTSA and EG in molar ratio 1:1:9 was performed as described elsewhere Gómez-Cruz et al. (2024). A known mass of the three components was added to a closed glass flask and gradually heated in an oil bath to a maximum temperature of 80 °C until a homogeneous liquid was obtained.

2.3. Delignification of OTP fractions with ternary ES

The delignification of AQ-OTP and PT-OTP was carried out following the experimental conditions by Gómez-Cruz et al. (2024), including solid to liquid ratio of 1:9 (w/w), 80 °C and residence time varying from 0.5 to 6 h (Fig. 1). Approximately 9 g of prepared ternary eutectic mixture was poured into a tube of a Radleys Tech Carousel reaction station (Saffron Walden, UK) along with approximately 1 g of dry biomass. Afterwards, the reaction tubes were hermetically sealed with polytetrafluoroethylene (PTFE) caps and incubated in the carousel station at a temperature of 80 °C controlled by a probe under constant magnetic stirring of 500 rpm.

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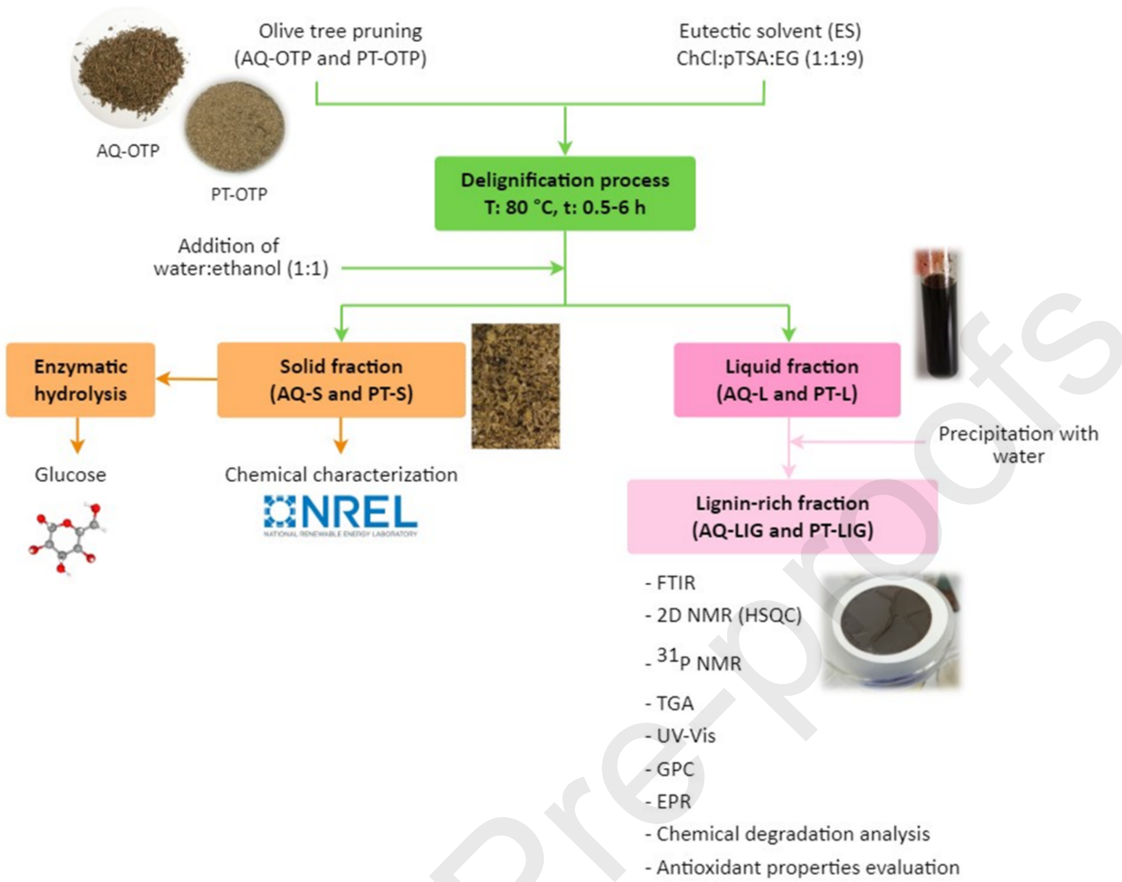

Fig. 1. Scheme of AQ-OTP and PT-OTP fractionation process developed in this study using ternary eutectic solvent.

After the delignification process, 20 g of a water:ethanol solution (50 % v/v) was added to the reaction tube to reduce the viscosity of the resulting liquid and to simultaneously maintain the fractions extracted from biomass dissolved. A vacuum filtration system fitted with 0.45 µm porosity Cytiva Whatman nylon membrane filters (Fisher Scientific, Pittsburgh, PA, USA) was used to separate the solid fraction (AQ-S or PT-S) from the liquid fraction (AQ-L or PT-L). The solids were then washed with deionized water to remove traces of ES and oven dried (40 ºC for 24 h) to determine the solid recovery yield according to Eq. (1):

$$Solid\ recovery\ (\%) = \frac{Delignified\ solid\ fraction\ weight\ (g)}{initial\ biomass\ weight\ (g)} \cdot 100 \qquad (1)$$

The lignin precipitation process consisted of pouring ice into a 100 mL beaker to half of its capacity. Afterwards, approximately 20 mL of the liquid fraction (AQ-L and PT-L) was added, followed by 40 mL of deionized water, and the mixture obtained was left to stand for 24 h at 5 ºC. Finally, the precipitate was filtered under vacuum, washed with 2 volumes of 50 mL of deionized water and oven dried at 40 ºC for 24 h. The isolated lignin was named AQ-LIG and PT-LIG, considering the pre-treatments performed before delignification with ES, namely aqueous pre-extraction or acid pre-treatment, respectively.

Calculations of delignification and isolated lignin yields were performed according to the following Eq. (2) and Eq. (3):

$$Delignification\ yield\ (\%) = 1 - \frac{Lignin\ content\ in\ resulting\ solid\ fraction\ (g)}{Lignin\ content\ in\ raw\ biomass\ (g)} \cdot 100 \quad (2)$$

$$Isolated\ lignin\ (mg\ lignin \cdot g^{-1}\ biomass) = \frac{Precipitated\ lignin\ (mg)}{Raw\ biomass\ (g)} \quad (3)$$

2.4. Chemical analysis of solid fractions

The chemical characterization of delignified solids (AQ-S and PT-S) under the experimental conditions studied was performed according to NREL protocols. Carbohydrate (glucan and hemicelluloses) and lignin content were determined according to NREL/TP-510-42618 (Sluiter et al., 2012) after two-step acid hydrolysis. The liquid fraction was analyzed by high performance liquid chromatography (HPLC) (Section 1.3 supplementary material) to quantify monomeric sugars, while acid-soluble lignin (ASL) was determined spectrophotometrically at 240 nm. Acid insoluble lignin (AIL) and ash contents were determined gravimetrically according to NREL/TP-510-42622 (Sluiter et al., 2008).

2.5. 2D HSQC NMR analysis

^{1}H-^{13}C two-dimensional nuclear magnetic resonance (2D NMR) analyses of AQ-LIG and PT-LIG were performed on a Bruker AVANCE 500 MHz instrument (Bruker, Billerica, MA, USA) equipped with a z-gradient double resonance probe. Both lignin samples were dissolved in DMSO-d_6 and heteronuclear single quantum correlation (HSQC) spectra were recorded under the conditions reported by Gómez-Cruz et al. (2024) and Sosa et al. (2020). Residual DMSO (at δH/δC 2.50/39.6 ppm) was used as internal calibration reference. MestReNova 12.0.0 software (Chemistry-Biology Library, Cincinnati, OH, USA) was used to analyze the spectra and identify the different ^{1}H-^{13}C correlation signals of samples.

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2.6. ^{31}P NMR analysis

Lignin samples were dried in a vacuum oven (Thermo Scientific VT 6025, Waltham, MA, USA) for 6 h at 40 °C to remove moisture. Subsequently, 30 mg of dried samples were weighed and dissolved in 500 μL of anhydrous pyridine and $CDCl_3$ (1.6:1, v/v) (solvent solution). Afterwards, 100 μL of a solution of cyclohexanol (20 mg·mL^{-1}) and chromium (III) acetylacetonate (derelaying agent) (3.6 mg·mL^{-1}) dissolved in the same solvent solution were added as internal standard and, finally 100 μL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) phosphatidation reagent was added. The obtained solution was shaken until complete homogenization was achieved and then transferred to an NMR tube (Wilmad-LabGlass, Vineland, NJ, USA) for immediate analysis in Bruker AVANCE 400 III MHz NMR spectrometer instrument (Billerica, MA, USA).

The quantification of the different hydroxyl group content was performed according to Eq. (4):

$$Functional\ group\ \left(\mathrm{mmol}\cdot g^{-1}\ lignin\right) = \frac{normalized\ peak\ area}{sample\ weight}\cdot IS\ concentration\cdot 0.1 \quad (4)$$

2.7. Electron Paramagnetic Resonance analysis

EPR analyses were run on an X-band (9 GHz) Bruker Elexys E-500 spectrometer endowed with a superhigh sensitivity probe head. A flame-sealed glass capillary was filled with a weighted amount of sample and coaxially inserted in a 4 mm quartz sample tube. Spectra were collected at 25 °C with a sweep width of 100 G, a 1024-point resolution and a scan time of 20.97 s. The amplitude of the field modulation (1.0 G) and the microwave power were preventively checked to avoid detectable signal overmodulation and microwave saturation, respectively. For power saturation experiments, the microwave power was gradually increased from 0.002 to 164 mW. Mn^{2+}-doped MgO was used as an internal standard for the evaluation of the g value and of the radical concentration (Muñoz-García et al., 2015).

2.8. Chemical degradation analysis

2.8.1. Alkaline hydrogen peroxide degradation

10 mg of each lignin sample was suspended in 1 M aqueous NaOH solution (1 mL) and treated with 1.5 % aqueous H_2O_2 solution (final concentration) at room temperature and under vigorous stirring (Greco et al., 2009; Panzella et al., 2012). After 24 h, the mixtures were treated with 5 % w/v sodium metabisulfite aqueous solution (200 μL), the pH was adjusted to 3 with 85 % aqueous H_3PO_4, and the mixtures were filtered through nylon membranes (13 mm, 0.45 μm), and analyzed by LC-UV-MS on an Agilent (Cernusco sul

Naviglio, Milan, Italy) 1260/6230DA ESI-TOF instrument coupled with a UV-Vis detector set at 254 nm under the following conditions: negative ion mode; 35 psig nebulizer pressure; drying gas (nitrogen) flushed at 10 $L·min^{-1}$ at a temperature of 325 °C; 3500 V capillary voltage; 175 V fragmenter voltage. An Eclipse Plus (Cernusco sul Naviglio, Milan, Italy) C18 column (150 × 4.6 mm, 5 µm) was used and a gradient elution was performed using 0.1 % formic acid (solvent A)/methanol (solvent B): 5 % B, 0–10 min; from 5 to 80 % B, 10–47.5 min at a flow rate of 0.4 $mL·min^{-1}$.

2.8.2. Alkali Fusion

Solid and finely grinded KOH (100 mg), NaOH (100 mg), and sodium dithionite (2 mg) were mixed in a pyrex tube and kept at 240 °C until fusion of the reagents. Around 20 mg of each lignin sample were then added and the mixtures were kept at 240 °C for additional 10 min (Panzella et al., 2018). After cooling to room temperature, 10 mL of 1% w/v sodium dithionite aqueous solution was added. After acidification with 6 M aqueous HCl, the mixtures were extracted with ethyl acetate (3 × 15 mL) and the combined organic layers were dried over sodium sulfate anhydrous and evaporated to dryness. The solid residue was reconstituted in 2 mL of methanol and analyzed by LC-UV-MS as above.

2.9.Antioxidant activity evaluation

2.9.1. DPPH assay

5-20 µL of a 10 $mg·mL^{-1}$ lignin sample solution in DMSO were added to 2 mL of a freshly prepared 0.2 mM solution of DPPH in methanol, and the mixtures were kept under vigorous stirring at room temperature (Goupy et al., 2003). After 10 min, the absorbance at 515 nm was measured. Trolox was used as reference antioxidant. Trolox, butylhydroxytoluene (BHT), and ascorbic acid (AA) were used as reference antioxidants. Experiments were run in triplicate.

2.9.2. Ferric Reducing/Antioxidant Power (FRAP) assay

5-30 µL of a 10 $mg·mL^{-1}$ lignin sample solution in DMSO were added to 3.6 mL of a freshly prepared solution containing $FeCl_3$ (1.7 mM) and 2,4,6-tris(2-pirydyl)-s-triazine (0.83 mM) in 0.3 M acetate buffer (pH 3.6) (Benzie and Strain, 1996). The mixtures were kept under vigorous stirring at room temperature and the absorbance of the solution at 593 nm was measured after 10 min. Results were expressed as Trolox equivalents (eqs). BHT and AA were used as reference antioxidants. Experiments were run in triplicate.

2.9.3. ABTS assay

Firstly, ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS solution (7 mM in water) with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h (Re et al., 1999). The $ABTS^{\bullet+}$ solution was diluted with 0.1 M sodium phosphate buffer at pH 7.4 to an absorbance of ca. 0.7 at 734 nm before use. 5-20 µL of a 10 mg·mL^{-1} lignin sample solution in DMSO were then added to 2 mL of the $ABTS^{\bullet+}$ working solution. After stirring the resulting solution at room temperature for 10 min, the absorbance at 734 nm was measured. Trolox BHT, and AA were used as reference antioxidants. Experiments were run in triplicate.

2.10. Enzymatic hydrolysis of solid fractions

The initial biomass samples (AQ-OTP and PT-OTP) and obtained solid fractions (obtained AQ-S and PT-S) after treatment with ternary ES at 80 ºC for 4 h were used as substrates for enzymatic hydrolysis (EH). The commercial enzyme solution Cellic® CTec2 (Novozymes A/S, Bagsværd, Denmark) at 15 filter paper units (FPU)/g substrate and β-glucosidase (Novozymes A/S, Bagsværd, Denmark) at 15 international units (IU)/g substrate were used to hydrolyze the aforementioned solids. The experiments were carried out at 5%, 10%, and 20% (w/v) solids loading, respectively, in citric acid-citrate buffer 0.05 M at pH 4.8 and Avicel® PH-101 (Sigma-Aldrich, St. Louis, MO, USA) was used as enzymatic control. The samples were processed on an orbital shaker (IKA KS 4000 i control, Staufen, Germany) for 72 h at 50 °C and 150 rpm. Samples were taken at 8, 24, 32, 48 and 72 h, and the released glucose and xylose concentrations were determined by HPLC-RID analysis as detailed in supporting information. Enzymatic digestibility (Y_{WIS}) and enzymatic hydrolysis yield (EH yield) were calculated as follows:

$$Y_{WIS}\ (\%) = \frac{g\ glucan\ by\ EH}{100\ g\ glucan\ in\ S{-}ES} \quad (5)$$

$$EH\ yield\ (\%) = \frac{g\ glucan\ \text{by}\ EH}{100\ g\ glucan\ in\ OTP} \quad (6)$$

3. RESULTS AND DISCUSSION

3.1. Evaluation of the delignification capacity of ES on OTP-based raw materials

This work addressed an evaluation of the delignification performance of ChCl:pTSA:EG (1:1:9) on OTP materials that were pre-extracted and pre-treated beforehand to forecast an efficient integration and fractionation process. The aqueous pre-extraction conditions adopted in this work (120 °C, 10 % w/v solid, 60 min) lied on another work data related to olive tree pruning valorization with hydrothermal treatments (Martínez-Patiño et al., 2017). The aqueous pre-extraction was performed in an autoclave and aimed at removing highly soluble and non-structural polar components, (e.g. free sugars, polar phenolic compounds, proteins, among others), reducing the overall content of the fraction called “extractives” by 49.7 % with respect to the original OTP material (Table S1, AQ-OTP sample) (Martínez-Patiño et al., 2017). These conditions are not aggressive to the lignocellulosic matrix, preventing chemical reactions of such non-structural components with cellulose,

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hemicelluloses and lignin during following fractionation processes or inhibitory effects on cellulases. On the other hand, acid pre-treatment enables hydrolysis and dissolution of OTP hemicellulosic fraction, leaving a solid (indicated as PT-OTP) mostly composed of cellulose and lignin (Table S1). The conditions (130 °C, 84 min, 20 % w/v solid, 2.4 % w/v H_2SO_4) were previously optimized through central composite experimental design and response surface methodology (Martínez-Patiño et al., 2017).

The impact of reduced contents of “extractives” and hemicelluloses on the delignification efficiency mediated by ES was thus assessed in the following sections. The obtained data were also compared with delignification of native OTP (without pre-extraction or pre-treatment) using the same ES under the same conditions as reported in previous study (Gómez-Cruz et al. (2024)).

3.1.1. Chemical compositions of solid and liquid fractions after delignification step

After delignification of pre-extracted and pre-treated OTP materials with ChCl:pTSA:EG (1:1:9) at 80 °C and different residence times, the recovery yields and chemical compositions of obtained solids, AQ-S and PT-S, respectively, were determined as depicted in Fig. 2.

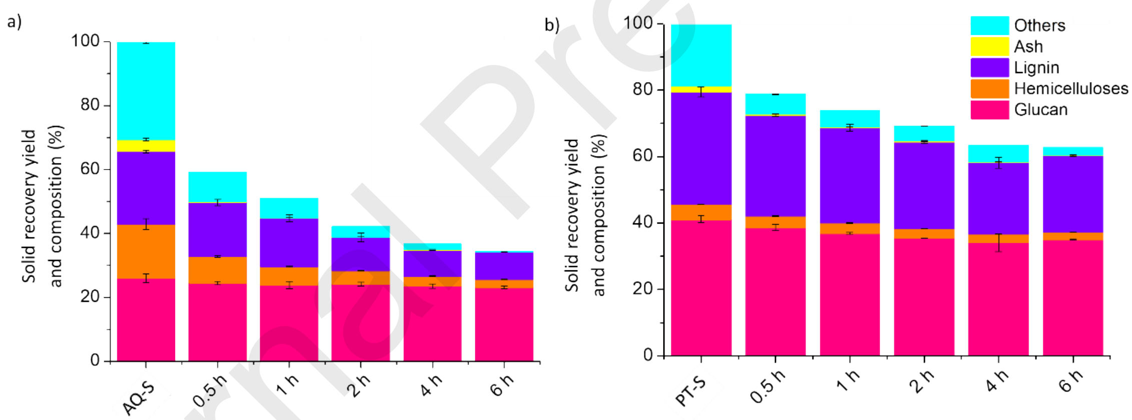

Fig. 2. Solid recovery yield (total bar) and composition of main fractions (stacked bar) after biomass delignification by ternary eutectic solvent. a) using aqueous pre-extracted solid (AQ-S) and b) using acid pre-treated solid (PT-S). Composition is based on dried biomass.

The overtime delignification of AQ-OTP and PT-OTP resulted in continuous solubilization of biomass components as explicitly demonstrated by the reducing solid recovery yield during the time of both treatments. The obtained yields shifted between 60.8 % and 36.1 % for AQ-OTP delignification, while PT-OTP delignification showed a solid recovery yield reduction from 78.8 % to 62.7 %, for the same period (from 0.5 h to 6 h). This is consistent with decreasing contents of hemicelluloses, lignin and components accounted as “others”,

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yet at the different extensions. The maximum extraction yields of lignin and hemicelluloses were reached at 4 h, namely 64.3 % and 82.2 % in AQ-OTP treatment, respectively, while only 36.6 % and 44.4 % yields were obtained from PT-OTP processing, correspondingly. It can be concluded that ES is more effective in extracting lignin when aqueous pre-extraction is applied rather than dilute acid pre-treatment. This is a consequence of the Notably, cellulose dissolution was negligible for both treatments, leading to considerable increase in the relative content of cellulose in the obtained solids, ranging from 41.1-65.5% and 49.1-55.8 % for AQ-S and PT-S, respectively. (Figs. S1a and S1b).

In a previous study, the delignification of OTP (without pre-extraction or pre-treatment) with ChCl:pTSA:EG (1:1:9) at 80 °C and 4 h enabled a total of 62.7 % and 87.3% of lignin and hemicelluloses, respectively. The maximum cellulose content achieved in the obtained solid was 62.5 % (Gómez-Cruz et al., 2024). This indicates that an aqueous pre-extraction of OTP slightly enhances the capacity of ternary ES to extract lignin and increase cellulose relative content in the solid fraction, albeit at the expense of a slight reduced capacity to dissolve hemicelluloses (82.2 wt% in this work *vs* 87.3% in Gómez-Cruz et al., 2024). Compared to other studies, the application of ChCl:pTSA:EG allowed excellent solubilization of lignin and hemicelluloses from switchgrass (71.2 % and 74.7 %, respectively) (Chen et al., 2019) and bamboo wood (93.4 % and 90.3 %, respectively) (Zhai et al., 2020). The higher extraction yields obtained in these studies are linked to more severe conditions applied (>110 ºC in both studies), which in turn provided less preservation of cellulose content in the obtained solid. For instance, at the best conditions, Zhai et al. presented cellulose preservation of 86.0 % in the solid fraction (Zhai et al., 2020), while the present study shows 90.8 % preservation at the best conditions found for AQ-OTP treatment (80 °C, 4 h). Furthermore, these harsher conditions may negatively influence the final properties of isolated lignin as well, a criterion that must be also considered in current development of biorefinery processing schemes.

As argued in our previous study (Gómez-Cruz et al., 2024), the presence of pTSA makes the ternary system more reactive to OTP biomass favoring the cleavage of critical chemical bonds (e.g. aryl ether bonds in lignin, ester bonds between lignin and hemicelluloses and glycosidic bonds in hemicelluloses), facilitating the separation between lignin and structural polysaccharides (Yang et al., 2022). Furthermore, as reported elsewhere (Da Costa Lopes et al., 2020), the presence of ChCl, particularly the chloride anion, catalyzes the cleavage of lignin aryl ether bonds (e.g. β-O-4). Although pTSA has demonstrated hydrotropic properties to enhance lignin dissolution (Li et al., 2020), the high EG content provides the dissolving ability to ternary ES.

HPLC analysis of the liquid fractions (AQ-L and PT-L) showed low hydrolysis of glucan, especially for PT-L (0.68-0.99 g glucan·100 g^{-1} biomass). This is important to stress out, since without aqueous pre-extraction or dilute acid pre-treatment, ternary ES enables a slightly higher glucan hydrolysis (2.74-3.78 g glucan·100 g^{-1} biomass) as reported elsewhere (Gómez-Cruz et al., 2024). Particularly, the dilute acid treatment, which provides the hydrolysis of the hemicelluloses, enables also a simultaneous hydrolysis of the amorphous parts of cellulose. In this scenario, treating PT-OTP biomass with ternary ES led to slightly lower glucan hydrolysis yield, since amorphous cellulose content was reduced in the acid pre-treatment. Regarding the hemicellulosic fraction, solubilization and hydrolysis was observed after ES treatment. Around 8.70-13.90 g hemicelluloses 100 g^{-1} biomass was dissolved and hydrolyzed in AQ-L, while an interval of only 1.79-2.93 g hemicelluloses·100 g^{-1} biomass was observed for PT-L as expected (Table S2). Another advantage of using ternary ES as OTP fractionation solvent is the absence of sugar degradation products, namely furfural and hydroxymethylfurfural. This suggests that this ternary ES, combined with the experimental conditions tested, does not degrade the hydrolyzed sugars produced during AQ-OTP and PT-OTP treatments. This feature is a clear advantage for the subsequent valorization of both liquid and solid fractions, since those sugar degradation products can negatively affect for instance the direct fermentation of the liquid stream (pentose-rich) or the two-step enzymatic hydrolysis/fermentation of the solid stream (cellulose-rich) (López-Linares et al., 2020).

3.1.2. Isolation and characterization of lignin

Both delignification and lignin isolation yields achieved with AQ-OTP and PT-OTP processing with ternary ES, at 80 ºC during time, are presented in Table 1. The obtained results demonstrated a correlation between increasing delignification yield and increasing lignin isolation yield up to 4 h of treatment. At this time of treatment, maximum values were reached for both evaluation parameters. Note that the recovery of lignin-rich samples totalized 76.0 and 142.1 mg lignin·g^{-1} biomass from AQ-OTP and PT-OTP, respectively. However, the delignification yield of PT-S (36.6 %) was lower than that of AQ-S (64.3 %), since higher lignin content is found in the initial PT-OTP (33.9 %) material in contrast with AQ-OTP (22.7 %). On the other hand, the delignification and lignin isolation yields decreased for both cases (AQ-S and PT-S) after 6 h of treatment. For high treatment time, lignin condensation reactions that originate insoluble products overcome the dissolution ability of ES, which in turn reduces both delignification and lignin isolation yields.

The differences in the ES capacity to delignify PT-OTP and AQ-OTP could be associated to the distinct lignin structure existing in each of those biomass materials. While aqueous pre-extraction (120 ºC, 6 h) is expected to maintain lignin structure intact in the resulting solid (AQ-S) close to native form in raw OTP biomass, the dilute acid treatment may substantially change the lignin structure (in PT-S) as consequence of the strongly acidic and oxidizing action of sulfuric acid. For instance, dehydration (removal of hydroxyl groups) and subsequent formation of Hibbert ketones and new C-C bonds in lignin structure are promoted during acid treatment, which may reduce in the subsequent step the solubility efficacy in ternary ES (Wu et al., 2023).

Table 1. Delignification yield (%) and isolated lignin yield (mg lignin·g^{-1} biomass) achieved with ChCl.pTSA:EG (1:1:9).

	AQ-S	PT-S

Time (h)	Delignification yield (%)	Isolated lignin (mg lignin·g^{-1} biomass)	Delignification yield (%)	Isolated lignin (mg lignin·g^{-1} biomass)
0.5	25.4 ± 3.2	24.3 ± 1.2	10.1 ± 0.8	60.8 ± 1.9
1	33.3 ± 2.6	34.5 ± 5.4	15.3 ± 1.4	87.8 ± 1.7
2	54.3 ± 2.5	71.9 ± 2.3	23.1 ± 3.0	95.0 ± 2.8
4	64.3 ± 1.2	76.0 ± 1.2	36.6 ± 3.0	142.1 ± 0.5
6	62.5 ± 0.0	75.2 ± 1.5	31.9 ± 0.9	126.0 ± 0.8

An in-depth understanding of the structural characteristics of lignin is essential for ascertaining its potential applications. FTIR, 2D NMR (HSQC), ^{31}P NMR, TGA, GPC, EPR, and chemical degradation analysis were used to obtain information on the structure and physicochemical characteristics of the isolated lignins, namely AQ-LIG and PT-LIG, isolated under the best experimental conditions (80 °C and 4 h). Both lignin samples were also compared with Kraft lignin and a lignin sample obtained from pristine OTP (P-LIG) by applying the same conditions, i.e., fractionation process using ChCl:pTSA:EG (1:1:9) at 80 °C and 4 h, as published elsewhere (Gómez-Cruz et al., 2024).

Overall, the chemical analysis with FTIR and 2D NMR (HSQC) (Figs. S2-S4) showed very similar characteristics between AQ-LIG, PT-LIG and P-LIG. The detailed chemical characterization is reported in previous study (Gómez-Cruz et al., 2024). Yet some differences can be highlighted in this work. For instance, the band at 1090 cm^{-1} (Table S3) associated with an increase in the content of aliphatic hydroxyl groups (Joffres et al., 2014) appears more pronounced in the AQ-LIG and PT-LIG spectra than that of P-LIG. On the other hand, the HSQC spectra (Fig. S4) demonstrated a high level of EG grafting onto lignin structure by etherification, since the HSQC signals for the EG grafting (71.01/3.36, 80.97/4.61, 85.13/4.26 ppm) (Table S4) present in AQ-LIG and PT-LIG spectra are the same as those reported in previous study (P-LIG) (Gómez-Cruz et al., 2024). The EG grafting is generally caused by the presence of a strong acid, such as oxalic (Wang et al., 2022), trifluoromethanesulfonic (Liu et al., 2021), or pTSA (Gómez-Cruz et al., 2024), in the eutectic formulation that favors etherification eactions. In addition, neither aqueous pre-extraction or dilute acid pre-treatment were able to remove oleic acid, since esterification between this fatty acid and lignin (e.g. C-H resonance of double bond at 129.90/5.34 ppm) (Table S4) is observed for AQ-LIG and PT-LIG samples, analogously to P-LIG reported in previous work (Gómez-Cruz et al., 2024). The ethylene glycol grafting and oleic acid esterification makes the isolated lignin distinct from other lignin isolated fractions in literature. The obtained data supports the conclusion that the fractionation process with ChCl:pTSA:EG at 80 °C and 4 h provides homogeneous chemical properties to the isolated lignin samples, independently of the upstream processing, *i.e.*, no treatment, applying aqueous pre-extraction or dilute acid pre-treatment.

The EG grafting, particularly onto lignin unit α carbon (80.97/4.61) (Table S4), enabled a substantial increase of aliphatic OH content as evidenced by the ^{31}P NMR analysis. The aliphatic OH contents in AQ-LIG and PT-LIG were found much higher than that observed for Kraft lignin as depicted in Fig. 3 (spectra are shown in Fig. S5). A value of 5.2 mmol·g^{-1} lignin was previously determined for P-LIG as one of the highest values reported in literature (Gómez-Cruz et al., 2024). This work showed that prior aqueous-extraction of OTP is beneficial to EG grafting, enabling a remarkable number of 5.7 mmol·g^{-1} aliphatic OH content. The grafting of EG onto the lignin structure is positive, since it effectively prevents condensation reactions, from lignin dehydration to benzylic carbocation formation and finally to Hibbert ketones, as previously noted by (Ee et al., 2023). Among examined lignin samples, Kraft lignin presented the highest content of condensed structures (C5-substituted), while among isolated lignins, PT-LIG presented the highest value, which might be associated to the prior acid treatment.

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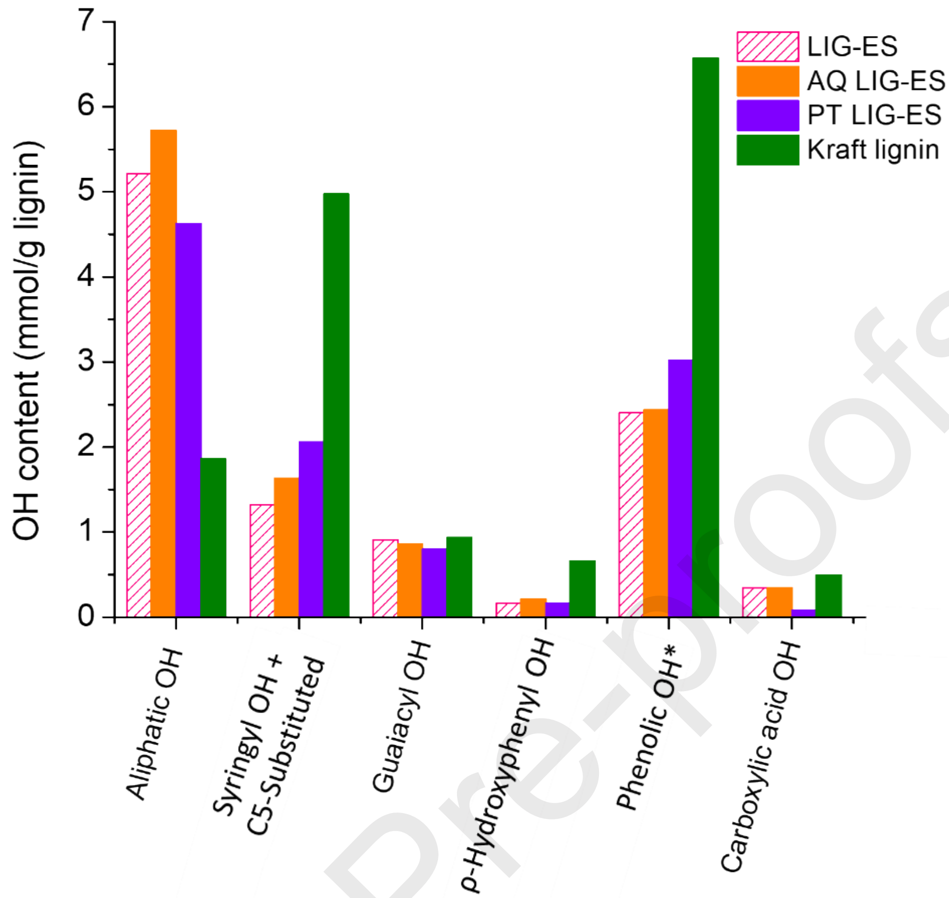

Fig. 3. Hydroxyl group (OH) contents of AQ-LIG and PT-LIG isolated with ChCl:pTSA:EG (80 ºC, 4h) determined by ^{31}P NMR. OH contents of Kraft lignin and P-LIG (Gómez-Cruz et al., 2024) are also depicted for comparison purpose. *Sum of "syringyl OH+C-5 substituted", "guaiacyl OH" and "p-hydroxyphenyl OH".

To gather further information about the structural characteristics of the lignin samples, additional experiments were conducted in which the samples underwent chemical degradation treatments commonly applied for analyzing phenolic polymers, namely alkaline hydrogen peroxide degradation and alkali fusion. These techniques are indeed commonly employed to analyse insoluble and structurally complex phenolic polymers such as melanin pigments and lignins, and are based on the identification of low-molecular weight markers, deriving from oxidative breakdown of the polymer (Greco et al., 2009; Moccia et al., 2020; Panzella et al., 2012). The LC-UV-MS profiles of the alkaline hydrogen peroxide degradation and alkali fusion mixtures of the samples (see Fig. S6 for a representative example) showed in particular the presence of three compounds eluted at 29.5, 31.5, and 32.5 min, identified as *p*-hydroxybenzoic, vanillic and syringic acid, in that order, based on the MS spectra and on the comparison of the chromatographic properties with those of reference standards. In agreement with NMR analysis, these compounds were indicative of the presence of H, G, and S units in the lignin samples, with no significant quantitative differences between the P-LIG, AQ-LIG, and PT-LIG samples.

The thermal stability of isolated lignin samples was also analysed by thermogravimetric analysis (TGA) (Fig. 4). TGA data revealed that the degradation of P-LIG, AQ-LIG, and PT-LIG occurred across a broad temperature range. This behaviour can be attributed to the intricate nature of the lignin structure (Schorr et al., 2014) and the presence of various oxygen-containing functional groups with differing thermal stabilities (de Hoyos-Martínez et al., 2018). Additionally, the weight loss observed in all lignin samples isolated from OTP was notably higher in comparison to Kraft lignin. This difference might be associated with the condensation degree of lignin samples. As explained above, the incorporation of EG into lignin structure is expected to reduce the occurrence of condensation reactions among lignin macromolecules during the extraction process (Choi et al., 2021; Gómez-Cruz et al., 2024), thus higher weight losses are observed during TGA. On the other hand, Kraft lignin, which exhibits more condensed structures as demonstrated in ^{31}P NMR, presented lower weigh loss. In the same context, P-LIG, which only suffered ES treatment, showed the highest weight loss after TGA, suggesting less content of intricate structures than any other examined lignin sample.

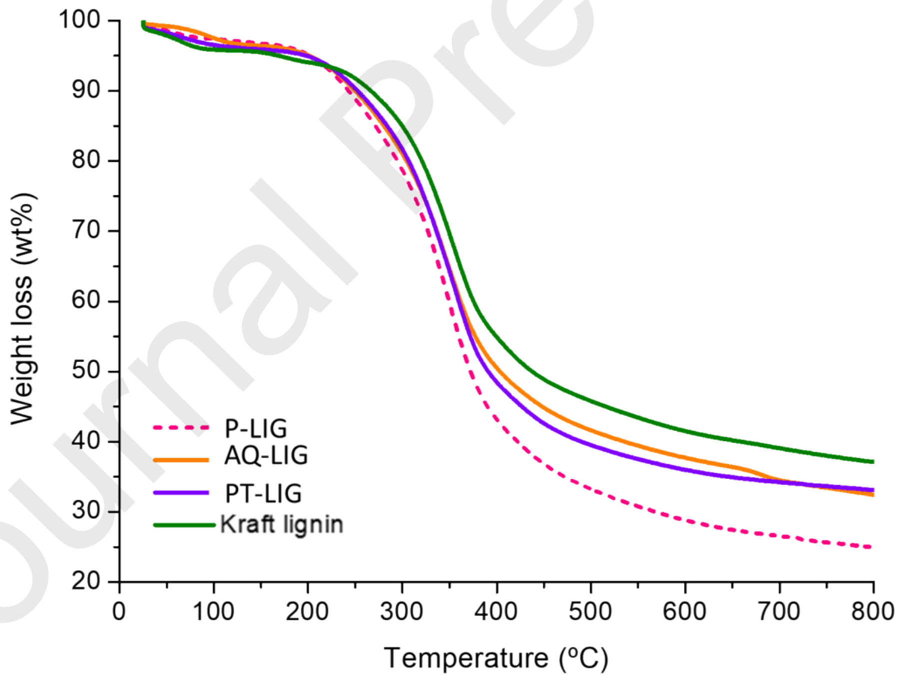

Fig. 4. Thermogravimetric curves of AQ-LIG and PT-LIG isolated with ChCl:pTSA:EG (80 ºC, 4h). Kraft lignin and P-LIG (Gómez-Cruz et al., 2024) are also depicted for comparison purpose.

The GPC data showed that AQ-LIG and PT-LIG had very similar average-weight molecular weight (Mw) (10824 and 11225 g·mol^{-1}, respectively) and average-weight molecular number (Mn) (1833 and 1812 g·mol^{-1}, respectively) (Table S5). These results show that isolated samples are heterogeneous (PDI of 5.91 and 6.20 for AQ-LIG and PT-LIG, respectively), similarly to P-LIG as previously reported (Gómez-Cruz et al. 2024).

The similarity between the three samples was evident also from EPR analysis, a technique exploited for the structural characterization of phenolic polymers exhibiting intrinsic free radical centers. Indeed, all spectra (Fig. S8) were characterized by a g value typical of carbon-centered radicals, indicating a comparable phenoxy radical concentration for all the samples (Table 2). The EPR spectra also exhibited a comparable signal broadening (ΔB), although the value determined for PT-LIG was slightly lower (Table 2). These values, together with the relatively high fraction of gaussian lineshape (Table 2), can provide information on the molecular structure and supramolecular organization of the radical species present in the samples. The obtained data suggests the presence of a rather heterogenous population of radicals, characterized by a quite different relaxation behavior, as evident also from the power saturation profiles (Fig. S8).

Table 2. EPR data for P-LIG, AQ-LIG, and PT-LIG.[a]

Sample	Radical concentration (g^{-1})	ΔB (G)	Fraction of gaussian lineshape	*g*-factor
P-LIG	7.9×10^{16}	6.1	0.46	2.0039
AQ-LIG	3.9×10^{16}	6.2	0.63	2.0037
PT-LIG	3.2×10^{16}	5.6	0.44	2.0032

[a]Experimental uncertainties are ± 0.0003 on g-factor, ± 0.2 G on ΔB, ± 10% on radical concentration.

The antioxidant properties of lignin are related to chemical structure. In particular, free phenolic OH groups and *ortho*-methoxy substitution in the aromatic rings are essential for its antioxidant potential (Cassoni et al., 2022; Zhou et al., 2024). In a last series of experiments, P-LIG, AQ-LIG and PT-LIG were characterized for their antioxidant properties by commonly used spectrophotometric assays. The results are reported in Fig. 5. In the DPPH and ABTS assays, PT-LIG was the most active material, exhibiting an EC_{50} value slightly lower than that of both P-LIG and AQ-LIG (Fig. 5a and 5b).. PT-LIG exhibited the highest reducing properties also in the FRAP assay (Fig. 5c). The higher antioxidant properties of PT-LIG would be in line with the higher content of phenolic OH groups as also shown by ^{31}P NMR analyses. In addition, the β-O-4 bonds cleavage taking place during DES delignification, releasing phenolic OH groups, enhances the antioxidant properties of the isolated lignin (Zhou et al., 2024). The boosting effect of acidic treatment on the antioxidant properties of phenolic polymers including lignin has been already reported (Alfieri et al., 2020; Moccia et al., 2020; Panzella et al., 2016; Panzella et al., 2019). Notably, although less active than ascorbic acid (AA), all lignin samples exhibited antioxidant properties comparable to or even exceeding those of the reference antioxidants butylated hydroxytoluene (BHT) and Trolox. Therefore, lignin may offer a sustainable and eco-friendly alternative to commercial antioxidants BHT and AA. The market price of lignin varies based on purity and application, but prices near $1 per Kg are already been achieved in large pulp and paper industries. While BHT and AA remain cost-effective for large-scale industrial applications, but they are generally synthesized – with BHT derived entirely from non-renewable petrochemical sources.

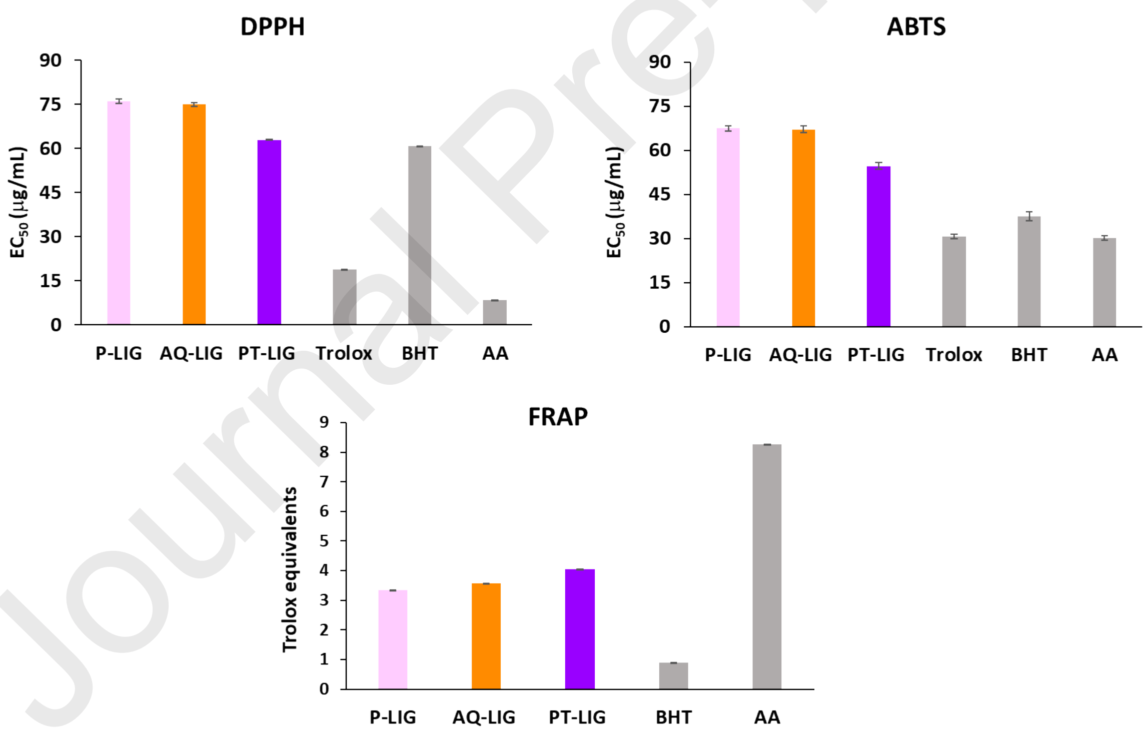

Fig. 5. Antioxidant properties of AQ-LIG, PT-LIG, and P-LIG. Reported are the mean ± SD values from at least three experiments. BHT: butylated hydroxytoluene; AA: ascorbic acid

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3.2. Enzymatic saccharification of solid fraction

In order to perform an integral valorization of OTP, the enzymatic digestibility of the samples AQ-OTP, PT-OTP, AQ-S and PT-S (80 °C and 4 h) into fermentable sugars was evaluated. Fig. 6 shows the saccharification efficiency (Y_{WIS}) expressed as g released glucan·100 g^{-1} glucan in AQ-S and PT-S and the enzymatic hydrolysis yield (EH yield) referred to g released glucan 100 g^{-1} glucan in original OTP.

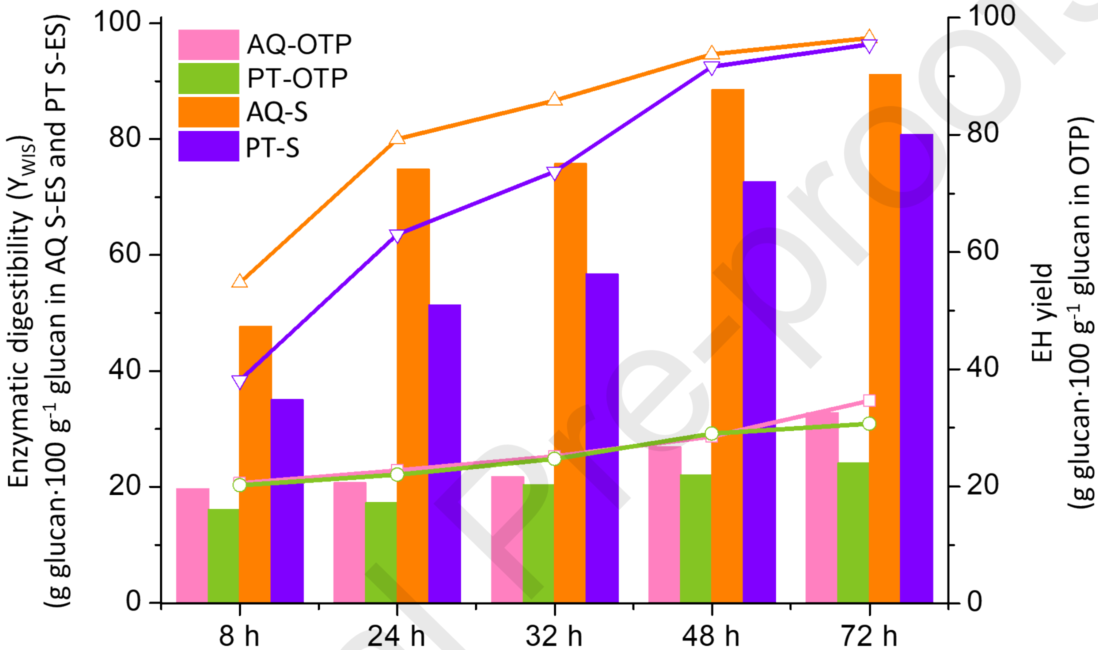

Fig. 6. Enzymatic digestibility (YWIS) and enzymatic hydrolysis yield (EH yield) of initial solids (AQ-OTP and PT-OTP) and delignified solids (AQ-S and PT-S) with ternary ES at 80 ºC during 4 h. Y_{WIS} and EH yields are represented by lines and bars, correspondingly.

Enzymatic hydrolysis (EH) of AQ-OTP and PT-OTP showed low values of both Y_{WIS} (34.87 % and 30.90 %, respectively) and EH yield (32.55 % and 23.97 %, respectively). In the first case, the intricate lignocellulosic matrix is still maintained after aqueous pre-extraction (only non-structural components were extracted), being a barrier to the accessibility of enzymes. On the other hand, although chemical disruption of lignocellulosic matrix was performed with dilute acid treatment in the second case (hydrolysis and dissolution of hemicelluloses), the high lignin content in PT-OTP (33.86 %) might have unfavored the cellulases performance (Miliotti et al., 2019).

However, when using AQ-S and PT-S as enzymatic substrates, near full hydrolysis of glucan was achieved (Y_{WIS} of 97.4 % and 96.4 %, respectively), exhibiting EH yields around 90.3 % and 80.1 %, respectively. The enzymatic performance when loading AQ-S as substrate was slightly better than that obtained with native OTP, as showed elsewhere (Y_{WIS} value of 97.7 % and EH yield of 81.8 %) (Gómez-Cruz et al., 2024). Although partial delignification was achieved with ChCl:pTSA:EG (1:1:9) for both biomass materials, it is expected that the reduction of lignin content favored enzymatic hydrolysis of both AQ-S and PT-S (Li et al., 2020; Varilla-Mazaba et al., 2022). The action of the acidic ternary ES in cleaving chemical bonds in lignin structure as well as between lignin and polysaccharides plays a role for enhanced enzymatic hydrolysis yields (Chen et al., 2019). Furthermore, as demonstrated in Fig. 2, the successful removal of other components (fraction "others"), such as lipids, proteins, phenolic compounds, among others, could be also beneficial for the enzymatic performance of cellulases.

4. CONCLUSIONS

This work revealed that applying aqueous pre-extraction or dilute acid pre-treatment to olive tree pruning biomass before delignification with ternary ES (ChCl:pTSA:EG; 1:1:9) substantially affects the OTP fractionation. The aqueous pre-extraction enables the removal of water-based "extractives" increasing the delignification efficiency mediated by ES. On the other hand, dilute acid pre-treatment, which allows hemicelluloses removal, did not offer a technical advantage for lignin extraction with ES. Although lignin content increases in the resulting solid fraction with dilute acid pre-treatment, the harsh conditions lead to the formation of lignin intricate structures that are difficult to solubilize in ES, reducing its delignification capacity. Therefore, the most efficient integration (aqueous pre-extraction plus treatment with ternary ES) enabled 64.3 % delignification yield at mild conditions (80 ºC, 4 h).

The proposed ES formulation allowed the isolation of lignin fractions exhibiting homogeneous chemical properties, independently of applying or not aqueous pre-extraction or dilute acid pre-treatment. The isolated lignin fractions exhibited very high aliphatic OH contents (5.7-4.6 $mmol \cdot g^{-1}$ lignin), resulting from the ethylene glycol grafting during treatment with ternary ES. Among the isolated lignins, the sample coming from dilute acid pre-treatment presented the highest antioxidant activity, an outcome related to the higher content of phenolic groups.

After delignification with ES, the resulting solid fractions showed high accessibility to cellulases cocktail. Once more aqueous pre-extraction was revealed to be a better choice before delignification, since the highest glucose yield was achieved (near 90 %) in this case.

Overall, this work provides new know-how on the valorization of OTP through a sustainable and integrative process with ES, targeting a cascade biomass valorization approach and contributing to the objectives of a circular bioeconomy and the principles of green chemistry. The obtained lignin presents distinct characteristics in contrast to other isolated lignin described in literature, while cellulose-rich solid can be further converted into high glucose yields. However, evaluating the economic and environmental impacts of using this ternary ES through life cycle assessment (LCA) and techno-economic analysis (TEA) is essential to assess the sustainability and feasibility of the proposed process integration for producing bio-based products from OTP.

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Irene Gómez-Cruz: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Filipe Sosa:** Data curation, Formal analysis, Writing – review & editing. **Maria Laura Alfieri:** Data curation, Formal analysis. **Rodolfo Esposito:** Data curation, Formal analysis. **Lucia Panzella:** Data curation, Formal analysis, Writing – review & editing, Validation. **Jalel Labidi:** Supervision, Writing – review & editing, Validation. **Brian Goodfellow:** Data curation, Formal analysis. **Eulogio Castro:** Supervision, Funding acquisition, Resources, Writing – review & editing, Validation. **Armando J.D. Silvestre:** Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – review & editing, Validation. **André M. da Costa Lopes:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing, Validation.

Highlights

- A maximum ~~olive tree pruning~~OTP delignification of 64.3% was achieved by integrating aqueous pre-extraction with ternary ~~eutectic solvent (~~ES~~)~~ treatment.
- The use of ternary ES enabled the isolation of lignin fractions with uniform properties, regardless of prior treatment.
- Dilute acid pretreatment altered the lignin structure, making it more intricate and less soluble in ES.
- Lignin fractions isolated with ES exhibited high levels of aliphatic OH groups (5.7-4.6 mmol·g^{-1} lignin).~~, a consequence of ES' ethylene glycol grafting into lignin structure.~~

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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