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Lignin extraction from acacia wood: Crafting deep eutectic solvents with a systematic D-optimal mixture-process experimental design

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ABSTRACT

Lignin is a complex biopolymer whose efficient extraction from biomass is crucial for various applications. Deep eutectic solvents (DES), particularly natural-origin DES (NADES), have emerged as promising systems for lignin fractionation and separation from other biomass components. While ternary DES offer enhanced fractionation performance, the role of each component in these mixtures remains unclear. In this study, the effects of adding tartaric acid (Tart) or citric acid (Cit) to a common binary DES mixture composed of lactic acid (Lact) and choline chloride (ChCl) were investigated for lignin extraction from acacia wood. Ternary Cit-based DES showed superior performance compared to Tart-based DES. Using a combined mixture-process D-Optimal experimental design, the Lact:Cit:ChCl DES composition and extraction temperature were optimized targeting maximum lignin yield and purity. The optimal conditions (i.e., Lact:Cit:ChCl, 0.6:0.3:0.1 molar ratio, 140 °C) resulted in a lignin extraction yield of 99.63 ± 1.24 % and a lignin purity of 91.45 ± 1.03 %. Furthermore, this DES exhibited feasible recyclability and reusability without sacrificing efficiency.

1. Introduction

The increasing demand for resources and shortage of feedstock are driving the research efforts toward alternative renewable and sustainable sources that can be used in the production of chemicals and materials. In this sense, lignocellulosic biomass has emerged as an attractive platform to replace non-renewable fossil fuels. Lignocellulosic biomass is composed of three main structural polymers: cellulose, hemicellulose, and lignin. While carbohydrate-based products find wide industrial applications in chemicals, pharmaceuticals, and papermaking, lignin remains underutilized, being usually incinerated for electricity and heat generation [1,2]. However, lignin holds potential for producing polymers, carbon fibers, adhesives, etc., that can be introduced in different sectors [2]. As a highly abundant and natural polyphenol, it can be regarded as a sustainable alternative to petroleum-derived phenol for the production of novel materials [3]. To obtain valuable bio-based materials, an efficient extraction process must be applied to fractionate biomass and separate it into its constituent polymers [2]. The complex molecular structure of lignin and the establishment of lignin-carbohydrate complexes render lignin to be considered a recalcitrant material and makes its isolation from the other components a challenging task [4]. Conventional extraction procedures, such as those adopted in the papermaking industry (e.g., Kraft or sulfite pulping), are highly effective at separating lignin. However, they are

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optimized for the by-product extraction rather than for the recovery of high-quality lignin. Consequently, lignin obtained from the pulp and paper industry is often of poor quality and contains contaminants [5]. To improve the quality of the extracted lignin, research on novel fractionation methods has been conducted. In this respect, deep eutectic solvents (DES), namely those of natural origin, have emerged as promising candidates for biomass fractionation, aiming superior lignin extraction and recovery, as documented in recent literature reviews [6–9].

DES have been studied not only for lignin extraction but also for extracting multiple compounds from biomass, such as hyaluronic acid [10], carotenoids [11], sugars [12], and other phenolic compounds [12–14], due to their high versatility and tunability. The definition of DES in the literature is not always clear, leading to widespread misconceptions. DES are commonly described as mixtures comprising, at least, one hydrogen bond donor (HBD) and one hydrogen bond acceptor (HBA), forming a homogeneous mixture with a melting point lower than that of the individual compounds. Nevertheless, this definition is insufficient to define a mixture as a DES, as the presence of an eutectic point in the solid–liquid phase diagram is a characteristic of all mixtures of compounds that are fully or partially immiscible in the solid phase [15]. Martins, et al. [15] proposed a new definition, stating that a DES is a mixture of two or more pure compounds that, simultaneously, fulfills two criteria: i) exhibits an eutectic point temperature below that of an ideal liquid mixture; and ii) the mixture is liquid at operating temperature for a certain composition range. Therefore, any composition within this liquid range at room temperature can be regarded as a DES. Including a range in the definition allows for a broader classification of mixtures as DES, enhancing their tunability, since the composition of the mixture is not limited to a fixed stoichiometric ratio as it happens in the thermodynamic definition of eutectic mixture.

Due to the extensive list of compounds that can form DES, their solvent properties, such as polarity, functional groups, or viscosity, can be easily tuned to improve their efficiency for extracting targeted compounds. DES can be considered green solvents because there is a wide range of natural compounds, such as sugars or amino acids, that can be used to prepare natural DES, thereby improving their eco-friendly features, such as biocompatibility or biodegradability [16,17]. Despite their advantageous properties, the use of DES also presents some drawbacks. The composition of DES and the intermolecular interactions between its components, particularly hydrogen bonding, greatly affect their physicochemical properties and increase their viscosity [2]. The high viscosity of DES is the main challenge in using them as solvents. To address this issue, various strategies have been explored, including the addition of a third component. For example, the addition of small amounts of water can substantially reduce the DES viscosity and improve the mass transfer during extraction processes [18]. While this approach has proven beneficial for the extraction of certain compounds, such as rosmarinic acid and salviaflaside [19] and alkaloids [20], it may hinder the efficient extraction of compounds insoluble in water. This phenomenon has been previously reported for lignin dissolution [21–24] and extraction [25,26], and depends on the DES used. Kumar, et al. [22] reported an increase in lignin extraction efficiency from rice straw when 5 % (v/v) water was added to the lactic acid–choline chloride (ChCl) DES, but the extraction yield decreased when the water content was further increased above 5 %. Conversely, the addition of water to the citric acid–ChCl (1:1) mixture was found to negatively affect the extraction of lignin from pine wood [25]. This trend was similarly observed by Soares, et al. [26] during the delignification of eucalyptus wood. In their study, using neat lactic acid–ChCl DES significantly improved wood delignification when compared to its aqueous counterpart, highlighting the detrimental effect of water on this process [26]. Despite its viscosity-reduction properties, water addition does not improve lignin extraction because lignin is not water-soluble and changes in polarity can hinder its dissolution [25]. Moreover, the addition of large amounts of water can disrupt the DES hydrogen bonding network, destabilizing its structure [27]. Recently, ternary DES have emerged as highly tunable solvents with enhanced properties and extraction performance compared to binary mixtures. This strategy has proved to be beneficial when combining the properties of two binary mixtures, resulting in enhanced extraction efficiency and selectivity. In our recent work on lignin extraction from pine wood, lactic acid–ChCl exhibited high extraction efficiency (32 wt% of extracted residue) but the extracted lignin showed a purity of only 71 % [25]. On the other hand, the tartaric acid–ChCl DES yielded a lower residue (15 wt%) but a higher purity was achieved (87 %). When combining these two acids in a ternary DES (i.e., lactic acid–tartaric acid–ChCl), an extraction yield of 27 wt% and a purity of 93 % were obtained. A similar effect was recently reported by Wang, et al. [28] for chitin extraction from crustacean waste using binary and ternary DES. When employing ChCl–formic acid (FA), a yield of 66.2 % and a purity of 93.4 % are obtained. On the other hand, the DES ChCl–*N*-acetyl-D-glucosamine (AG) results in a higher yield of 90.6 % but with a lower purity of 60.9 %. While FA contributed to the selective extraction of chitin, the strong acidic environment of ChCl–FA resulted in some chitin degradation. By using the ternary DES ChCl–AG–FA, an extraction yield of 85.6 % and purity of 90.2 % were attained. Other works have also reported the successful biomass pretreatment using ternary DES [29–31]. Ternary DES represent a new type of solvent with enhanced tunability, allowing for the addition of extra components in the solvent mixture. However, the precise impact of adding a third component and the role of the molar ratio between all compounds in the mixture have not been well elucidated yet. In this work, we investigated binary lactic acid–ChCl mixtures for lignin extraction from acacia wood and evaluated the effect of adding tartaric or citric acid to form ternary DES. The influence of the components' molar ratio in a lactic acid–citric acid–ChCl mixture and the extraction temperature were further assessed using a mixture–process D-Optimal design of experiments (DOE). DOE is a mathematical and statistical approach widely used to model, design, and evaluate the relationship between several independent variables and responses [32]. It aims the optimization of processes for a wide range of applications, including lignin extraction [33–36]. After the optimization, the extracted lignin was further characterized regarding its structure, content and type of hydroxyl groups, thermal stability, and molecular weight.

2. Materials and methods

2.1. Materials

Acacia wood (*Acacia dealbata* Link) was obtained in Tábua, Coimbra, Portugal. The DES were prepared using lactic acid (Lact) (88–92 %) from Panreac, tartaric acid (Tart) (> 99.7 %) and citric acid (Cit) (99.9 %) from José Manuel Gomes dos Santos, Lda., and choline chloride (ChCl) (> 98.0 %) from TCI. Acetone was purchased from José Manuel Gomes dos Santos, Lda., sulfuric acid (72 %) from Chem-lab, L-cysteine (> 98.0 %) from TCI. All chemicals were used as received except for ChCl, which, due to its hygroscopic nature, was oven-dried before use to ensure dryness. Dimethyl sulfoxide (DMSO, HPLC grade, > 99.7 %) and lithium bromide (> 99 %) were purchased from Sigma Aldrich and used for GPC analysis. For the ^{31}P NMR analysis, anhydrous pyridine, deuterated chloroform, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) and chromium (III) acetylacetonate were all purchased from Sigma-Aldrich (Germany).

2.2. Biomass source

In this work, lignin was extracted from acacia wood branches (*Acacia dealbata* Link) which was harvested in Coimbra, Portugal. The wood was oven-dried until reaching constant weight and then ground to powder using a Retsch cross beater mill. The resulting sawdust was sieved to a mesh size between 0.25 and 0.84 mm, oven-dried and stored at constant temperature until further use. The lignin content in the acacia wood was determined as described in Section 2.6.1 and found to be 21.49 ± 0.61

%.

2.3. DES preparation

Several DES were prepared combining ChCl as HBA and Lact, Cit, or Tart as HBD. All DES components were weighted according to their molar ratios and magnetically stirred at 80 °C until a clear and homogeneous mixture was obtained. For samples that did not form a liquid mixture under these conditions, the stirring was stopped after 1 h. An initial screening of the ratios was performed to determine the liquid region, which corresponds to compositions that form liquid mixtures at room temperature. The molar ratios of the mixtures used in the different parts of the work are specified in the respective section.

2.4. Viscosity determination

The shear flow viscosities (for simplicity, abbreviated as viscosity) of a wide range of compositions were measured and used to define and limit the range of molar ratios to be tested. Note that highly viscous solvents have limited extraction performance due to restricted mass transfer and limited penetration into the lignocellulosic matrix for lignin dissolution [37]. A total of 25 different ratios of the Lact:Cit:ChCl mixtures were prepared, as listed in Table 1. For those mixtures that were liquid at room temperature, their viscosities were measured at 20 °C in a HAAKE RheoStress 1 rheometer (Thermo Fisher Scientific) using a plate-plate geometry (20 mm, 0.2 mm gap). A HAAKE Phoenix II bath (Thermo Fisher Scientific) was used for strict temperature control. The experiments were conducted at a fixed shear stress of 50 Pa for 60 s. Each DES ratio was prepared in duplicate, and two replicate measurements were performed for each sample.

2.5. Lignin extraction

Ternary and binary DES mixtures were screened for lignin extraction from acacia sawdust, at different conditions, to study the effect of the solvent composition and fractionation conditions on the lignin extraction efficiency. Throughout all experiments, the solute-to-solvent ratio was kept constant at 1:10 weight ratio. Briefly, ca. 1 g of acacia sawdust and 10 g of DES were added to a Teflon-lined stainless-steel reactor. The reactor was then placed in a pre-heated oven at the defined temperature (i.e., 120–180 °C) and time (i.e., 1–6 h). After the extraction, the remaining solid residue (cellulose-rich fraction) was separated from the liquid fraction, containing the isolated lignin, by low pressure filtration. Due to the high viscosity of some DES mixtures, 10 mL of 70 % acetone aqueous solution was added prior to the filtration to decrease the viscosity and facilitate the filtration process. The solid fraction was washed with 70 % acetone until no colour was observed in the washing solvent. This washing step is important to remove all lignin dissolved during the DES treatment, which reprecipitates at the surface of the solid residue after cooling [38,39]. Note that a careless separation procedure could reduce both the lignin extraction yield and purity. To recover the extracted lignin, acetone was evaporated and recovered, and an excess of water was added to induce lignin precipitation and allow its separation by low pressure filtration. The recovered lignin was then oven-dried and weighed. A schematic representation of the main steps of the lignin extraction process is presented in Fig. 1.

The method selectivity, i.e., lignin purity, was evaluated by determining the lignin content (described in Section 2.6) in the lignin-rich fraction, while the efficiency of the extraction was assessed from the lignin extraction yield, calculated as expressed in Eq. (1).

$$Lignin\ yield\ (\%) = \frac{extracted\ weight \times lignin\ content_{sample}\ (\%)}{initial\ biomass\ weight \times lignin\ content_{biomass}\ (\%)} \times 100 \quad (1)$$

The statistical analysis of the lignin yields was performed using one-way ANOVA (with a 95 % of confidence, $\alpha = 0.05$) together with a post-hoc Tukey test to assess the significance of the different mixtures and conditions.

Table 1
Molar ratios of the DES prepared for viscosity determination.

Type of mixture	Components	Ratio	State
Initial components	Lactic acid (Lact)	1	Liquid
	Citric acid (Cit)	1	Solid
	Choline chloride (ChCl)	1	Solid
Binary	Lact:ChCl	0.25:0.75	Solid
		0.5:0.5	Liquid
		0.75:0.25	Liquid
	Cit:ChCl	0.25:0.75	Liquid
		0.5:0.5	Liquid
		0.75:0.25	Solid
Ternary	Lact:Cit:ChCl	0.1:0.1:0.8	Solid
		0.1:0.25:0.65	Liquid
		0.1:0.4:0.5	Liquid
		0.1:0.5:0.4	Liquid
		0.1:0.65:0.25	Liquid
		0.1:0.8:0.1	Solid
		0.25:0.1:0.65	Liquid
		0.25:0.25:0.5	Liquid
		0.25:0.5:0.25	Liquid
		0.25:0.65:0.1	Solid
		0.33:0.33:0.33	Liquid
		0.4:0.1:0.5	Liquid
		0.4:0.5:0.1	Solid
		0.5:0.1:0.4	Liquid
		0.5:0.25:0.25	Liquid
		0.5:0.4:0.1	Liquid
		0.65:0.1:0.25	Liquid
		0.65:0.25:0.1	Liquid
		0.8:0.1:0.1	Liquid

2.6. Determination of lignin purity

To assess the selectivity of the extraction method, lignin purity was determined as the total lignin content in the lignin-rich extracts. This quantification was made using the Cysteine–Assisted Sulfuric Acid (CASA) method proposed by Lu, et al. [40]. The CASA procedure enables rapid quantification of the lignin content using a smaller amount of sample, which is advantageous for small-scale extraction experiments where the extracted amounts are often lower than the minimum required for standard procedures [41]. For lignin quantification by UV spectroscopy, the determination of its absorptivity value is crucial to obtain reliable results. However, the absorptivity value of lignin varies among sources and pretreatments, so it is recommended to determine the absorptivity for the samples being analyzed [42]. Consequently, the CASA method relies on the initial development of a standard calibration curve by plotting the absorbance obtained for the samples against the lignin concentration determined by a standard quantification procedure. For this, six independent lignin samples extracted from acacia wood were selected, and acid hydrolysis (procedure described in Section 2.6.1) was performed to determine their lignin content. Lignins from the same samples were also subjected to the CASA method (procedure described in Section 2.6.2) and their absorbances were plotted against their real lignin concentrations (calculated based on the previously obtained lignin content) to obtain a calibration curve. An accurate linear correlation was obtained, with a coefficient of correlation (R^2) of 0.98. The calibration curve was then used for quantifying the lignin content in the other samples studied.

2.6.1. Lignin content determination by acid hydrolysis

The total lignin content of the acacia wood and lignin samples used for the calibration curve mentioned in Section 2.6, was determined by the standard procedure of the National Renewable Energy Laboratory [41]. Approximately 100–300 mg of sample and 1–3 mL of 72 % sulfuric

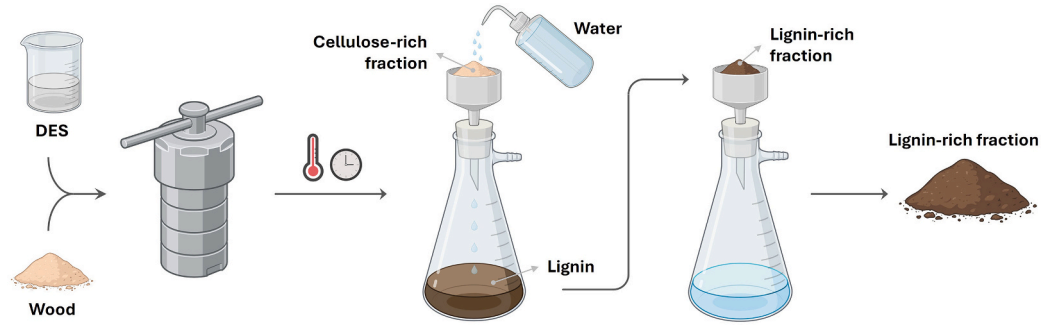

Fig. 1. Schematic representation of the lignin extraction process.

acid were added to a glass tube and heated at 30 °C for 1 h, with manual stirring every 5–10 min. Then, the samples were transferred to sealed 50 mL glass vials, diluted to 4 % sulfuric acid with deionized water, and autoclaved for 1 h at 121 °C. After the hydrolysis, the solids (i.e., acid-insoluble lignin, AIL) were filtered, and the solvent was collected to measure the acid-soluble lignin (ASL) before washing the AIL residue with water. AIL was determined by the ratio between the weight of the sample after and before the hydrolysis. The ASL was diluted, if needed, and measured by UV–Vis spectrophotometry (Jasco V–550 spectrophotometer) at 205 nm according to Eq. (2), using the absorptivity value of 110 $L \cdot g^{-1} \cdot cm^{-1}$.

$$ASL(\%) = \frac{Abs_{205\ nm} \times V \times DF}{\varepsilon \times b \times w_{sample}} \tag{2}$$

Where $Abs_{205\ nm}$ is the absorbance for the sample at 205 nm, V is the volume of the filtrate, DF is the dilution factor, ε is the absorptivity for the specific wavelength (110 $L \cdot g^{-1} \cdot cm^{-1}$), b is the optical path length, and w_{sample} is the dry weight of the sample.

2.6.2. Lignin content determination by CASA method

Briefly, ca. 5–10 mg of the extracted lignin-rich fraction was placed in a vial and 1 mL of a solution of L-cysteine in 72 % sulfuric acid (0.1 $g \cdot mL^{-1}$) was added. The mixture was magnetically stirred at room temperature for 1 h. The solution was then diluted to a final volume of 250 mL with deionized water and the absorbance of the final solution was measured at 283 nm using a Synergy HTX multi-mode microplate reader from BioTek. The lignin concentration was calculated based on the obtained absorbance using the calibration curve previously established and used to calculate the lignin purity.

2.7. Optimization of the extraction conditions

An initial screening of different DES mixtures, molar ratios, and extraction conditions (temperature and time) was performed to identify the most important variables and their limits, aiming to reduce the number of variables under analysis. For the preliminary screening, two different mixtures (Lact:Cit:ChCl and Lact:Tart:ChCl) were selected based on the good performance of ternary systems for biomass fractionation observed in our previous study [25]. These mixtures were prepared at three different molar ratios (i.e., 0.5:0:0.5, 0.33:0.33:0.33 and 0.5:0.25:0.25) and tested for lignin extraction at four temperatures (i.e., 120, 140, 160 and 180 °C) and four extraction times (i.e., 1, 2, 4 and 6 h). The lignin extraction yield was used as the response variable to assess the influence of these variables, employing a one-way ANOVA test with post-hoc Tukey test at 95 % of confidence.

Based on the results of the preliminary screening (as presented in Section 3.1), the Cit-based mixtures were selected due to their superior performance, and the extraction time was excluded and fixed at 2 h. The following ranges were established and used for the design of experiments (DOE) using a combined mixture-process D-Optimal design: the mixture factors were from the DES mixture (Lact:Cit:ChCl) and the process factor was the temperature, which ranged between 120 and 140 °C.

The lignin extraction yield and lignin purity were selected as independent response variables to be optimized by the extraction process. Each one of these estimated responses, generically referred as Y, can be fitted by a third-degree polynomial expression as described in Eq. (3). This expression results from the combination of a quadratic Scheffé model with a process model (the use of a Scheffé model is necessary due to the natural constraint imposed to mixture variables, that must sum up 1, which would lead to a collinearity situation if a standard model was used). For more detailed information on the model selection process, the reader is referred to Section A of the Supplementary material.

$$\begin{aligned} Y = &\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{1\cdot2} X_1 X_2 + \beta_{1\cdot3} X_1 X_3 + \beta_{1\cdot4} X_1 X_4 + \beta_{2\cdot3} X_2 X_3 \\ &+ \beta_{2\cdot4} X_2 X_4 + \beta_{3\cdot4} X_3 X_4 + \beta_{1\cdot2\cdot4} X_1 X_2 X_4 + \beta_{1\cdot3\cdot4} X_1 X_3 X_4 \\ &+ \beta_{2\cdot3\cdot4} X_2 X_3 X_4 + \varepsilon \end{aligned} \tag{3}$$

In Eq. (3), Y is the estimated response (lignin extraction yield or lignin purity), β_i and $\beta_{i\cdot j}$ are the linear and crossed regression coefficients, respectively, and X_i are the input factors (X_1: Lact; X_2: Cit; X_3: ChCl; and X_4: temperature); ε is the residual term of the model, which is assumed to be an independent random variable with zero mean, constant variance, and following a Gaussian distribution.

The results obtained were analyzed using the JMP® Pro 17 software, employing ANOVA and Standard Least Squares regression with a significance level of 5 %.

2.8. DES recovery and reuse

The recyclability of the DES used in this work was assessed to infer on its potential for recovery and reuse across multiple extraction cycles before disposal. Assessing solvent recyclability is crucial for both environmental and economic reasons, especially if the recovery process is simple, cost-effective, and does not significantly compromise the solvent's efficiency and/or selectivity.

For the assessment of DES recyclability, lignin extraction was performed at 140 °C for 2 h using the DES Lact:Cit:ChCl (0.6:0.3:0.1), following the procedure outlined in Section 2.5, differing only on the amount of woody biomass used. Specifically, 15 g of acacia sawdust and 150 g of DES were used in each experiment. Upon completion of the lignin extraction process, the DES was recovered as follows: the lignin dissolved in the DES was precipitated by adding an excess of water, followed by its removal at low pressure filtration. Subsequently, the water present in the DES was evaporated in a rotary evaporator. No additional purification steps were conducted on the recovered DES before its reuse in subsequent extraction cycles. It's noteworthy that the recovered DES retained some hemicellulose and extractives that were also extracted from acacia wood. The extractions were performed in duplicate, and the DES was used for three successive lignin extraction cycles.

2.9. Lignin characterization

2.9.1. Lignin structural features by FTIR

The structural characterization of the extracted lignin was evaluated by Fourier-transform infrared spectroscopy (FTIR). FTIR-ATR spectra of lignin extracted with freshly prepared DES and recovered DES were recorded on a Jasco FT/IR-4200 spectrometer from 450 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and 64 scans.

2.9.2. Thermogravimetric analysis (TGA)

Thermal stability of the extracted lignin was determined by thermogravimetric analysis (TGA) using a TGA-Q500 equipment (TA Instruments). Approximately 5 mg of lignin were used in the measurements, temperature ranging from 25 °C to 600 °C, under nitrogen flow and with a heating rate of 10 °C•min^{-1}.

2.9.3. ^{31}P NMR

The quantification of the phenolic, aliphatic, and carboxylic hydroxyl groups of lignin was performed by Phosphorous-31 Nuclear Magnetic Resonance (^{31}P NMR) based on the procedure described elsewhere [43]. The samples were analyzed in a Bruker Avance II+ 600 MHz spectrometer, using the following parameters: 64 scans with an acquisition time of 1.0 s and a relaxation delay of 5.0 s. All the other settings were defined as described elsewhere [43]. Cholesterol was used as internal standard as it allows the samples to remain stable in long-term experiments or extended sample storage [43].

2.9.4. Lignin molecular weight determination

The molecular weight (Mw) and polydispersity index (PDI) of the extracted lignin (extraction conditions: Lact:Cit:ChCl (0.6:0.3:0.1), 140 °C, 2 h) were estimated by gel permeation chromatography (PL-HPC 50 Plus Integrated GPC system, Polymer Laboratories, Varian Inc.). The system is equipped with two 300 × 7.5 mm PolarGel-M columns, one 50 × 7.5 mm PolarGel-M guard column and one UV detector operating at 280 nm. DMSO with 10 mM LiBr was used as the mobile phase and the flow rate was 0.5 mL•min^{-1} at 50 °C. The UV detector was calibrated with 10 Pullulan standards ranging from 0.180 to 708 kDa (Varian PL2090–0100, Varian Inc). Samples were prepared by dissolving ca. 10 mg in 1 mL of mobile phase under stirring overnight, then diluted to 0.25 mg•mL^{-1} and filtered with 0.2 μm syringe filters. The data was analyzed with the Cirrus GPC Software 3.2.

3. Results and discussion

3.1. Preliminary screening of the variables

A preliminary study to understand the effect of different DES compositions and extraction conditions was conducted to identify the most relevant variables and define their limits for subsequent optimization. Three types of DES were employed: one binary DES (Lact:ChCl) and two ternary DES (Lact:Tart:ChCl and Lact:Cit:ChCl). Lignin extraction was carried out at temperatures ranging from 120 to 180 °C, at a fixed extraction time of 4 h (Fig. 2). Then, the effect of the extraction time (from 1 to 6 h) was evaluated for lignin extraction at 140 °C, using the ternary DES (Fig. 3). The results indicated a wide range of lignin extraction yields, varying from 0 % (0.5:0:0.5, 120 °C, 4 h, Fig. 2) to 82.1 % (Lact:Cit:ChCl 0.33:0.33:0.33, 140 °C, 2 h, Fig. 3). Notably, the choice of solvent ratio and composition, as well as the extraction conditions, greatly influence the extraction performance. Specifically, temperatures of 160 and 180 °C were found to be unsuitable for lignin extraction due to the formation of a black foam-like mixture, likely resulting from DES components degradation and crosslinking with the biomass. Such harsh conditions may facilitate crosslinking between polycarboxylic acids and wood components [44]. Interestingly, Cit and Tart have been recognized as modification agents to improve wood properties [45]. For instance, Cit exhibits optimal crosslinking with cellulose at 180 °C, a temperature within the range tested in this work [46]. Cit has also been reported as a crosslinking agent to produce lignocellulosic foams [47]. Nonetheless, an increase in the extraction yield is observed when rising the extraction temperature from 120 to 140 °C for all mixtures and ratios, especially for the binary DES (0.5:0:0.5) (Fig. 2). Temperature increase has been reported to favor lignin extraction [25,48,49]. Cheng, et al. [50] studied the structural changes in lignin extracted from poplar wood with γ-valerolactone and found a positive correlation between temperature and lignin yield.

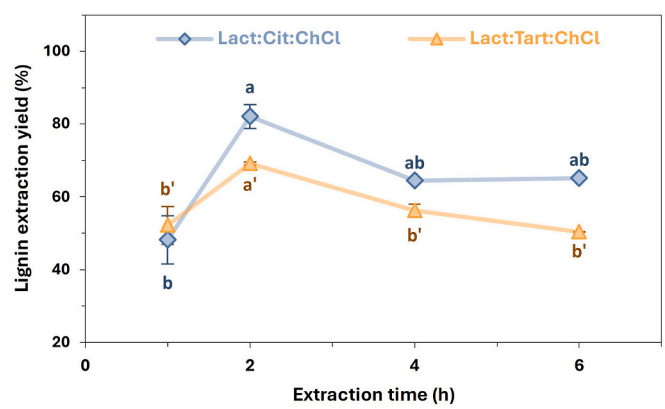

Fig. 3. Effect of the extraction time on the lignin extraction yield using Lact:Tart:ChCl (orange symbols) and Lact:Cit:ChCl (blue symbols). Extraction conditions: temperature of 140 °C and DES molar ratio of 0.33:0.33:0.33. The same letter above the bars indicates no significant differences (Tukey test, $p \leq 0.05$).

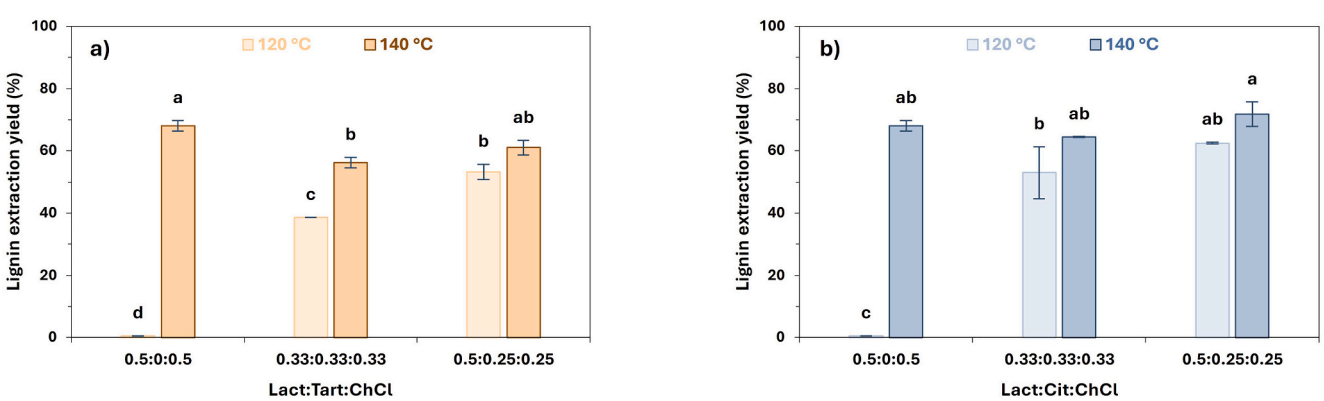

Fig. 2. Effect of the molar ratio and temperature on the lignin extraction yield using a) Lact:Tart:ChCl and b) Lact:Cit:ChCl. Extraction time: 4 h. The same letter above the bars indicates no significant differences (Two-way ANOVA with Tukey test, $p \leq 0.05$).

Specifically, as the temperature increased from 80 to 120 °C, a decrease in the β–O–4 content was observed which facilitates lignin dissolution in the solvent [50]. While the binary DES Lact:ChCl (0.5:0.5) is not suitable for lignin extraction at 120 °C, the inclusion of Tart or Cit significantly increases the extraction yields at lower temperatures. At 140 °C, no significant differences were observed in the extraction yields between the binary and ternary DES, except for Lact:Tart:ChCl (0.33:0.33:0.33). These findings suggest that ternary DES holds promise for achieving more efficient extraction processes at milder conditions.

Regarding the extraction time, for both Cit and Tart-based mixtures, the best results were obtained at 2 h (Fig. 3). An initial increase in the lignin extraction yield occurs when increasing the extraction time from 1 to 2 h, but beyond this a decline in performance is evident. These results are in agreement with literature where an optimal extraction time is often reported. For instance, Saadat Ghareh Bagh, et al. [51] performed the optimization of lignin extraction from black liquor using Lact:ChCl (11:1), noting that while increasing the extraction time from 0.5 to 3.5 h boosted extraction yield, subsequent increase to 6.5 h resulted in a slight decrease. Similarly, Wang, et al. [52] observed a decline in lignin regeneration with prolonged treatment with Lact:ChCl (2:1), attributing it to lignin fragmentation under harsher conditions, leading to smaller and less recoverable soluble fractions. On the other hand, some authors state that extensively long extraction times may cause a decrease in the lignin extraction yield due to condensation of lignin into insoluble oligomers [50]. This was actually supported by the observed increase in the percentage of condensed structures and decrease in the molecular weight of the extracted lignin [50].

When comparing the different DES, it is observed that those containing Cit appear to be more efficient for lignin extraction than Tart-based ones. The presence of extra carboxylic groups in the HBD increases its ability to form extra hydrogen bonds, facilitating the establishment of more favorable interactions with lignin [25]. In our previous work, when using a DES composed of ChCl and one HBD, Tart exhibited higher extraction capacity than Cit [25]. Although Cit offers more groups that can favorably interact with lignin, its higher viscosity may hinder the extraction process. Interestingly, when these acids are used in combination with Lact in ternary DES, the opposite behavior is observed. In this case, the viscosities of Lact:Cit:ChCl and Lact:Tart:ChCl are similar (6.82 and 8.39 Pa•s, respectively, for 0.33:0.33:0.33 ratio, and 1.99 and 2.00 Pa•s for the 0.5:0.25:0.25 molar ratio) and the presence of extra carboxylic groups in Cit favors the extraction using this type of ternary mixture.

Based on these preliminary results, the following variables and limits were defined: extraction temperature from 120 to 140 °C, extraction time fixed at 2 h and only the ternary DES containing Cit was further evaluated.

3.2. DES viscosities

In the optimization of an extraction process, viscosity emerges as one of the most important parameters to consider. A solvent with low viscosity not only facilitates mass transfer but also favors processing and handling [53]. DES are generally highly viscous, which stands as a drawback to their generalized application as solvents. The hydrogen bonded network that is formed between HBA and HBD components in DES is believed to be responsible for the typically observed high viscosity [54]. Nevertheless, DES viscosity can be adjusted by careful selection of the compounds used and/or their molar ratio [53].

The boundaries for the molar ratios that can be applied for the extraction will depend on the properties of the mixture, particularly its melting point and viscosity. Therefore, we have constrained this variable to encompass mixtures that remain liquid at room temperature and present low viscosity. The viscosities of the prepared DES are represented in Fig. 4a.

As can be observed in Fig. 4a, the viscosity of Lact:Cit:ChCl DES is greatly dependent on the molar ratio. Among the components, Lact stands out as the only one being liquid at room temperature. Moreover, an increase in its molar fraction within the mixture clearly reduces the viscosity. The Lact:ChCl binary DES finds widespread use as an extraction solvent, including lignin extraction processes, where higher Lact concentrations often correlate with enhanced extraction performance [55–58]. This is commonly attributed to the acidity of the solvent, which can promote the cleavage of lignin-carbohydrates bonds within lignocellulose complexes and also disrupt internal lignin bonds, thereby favoring its dissolution [57,59]. It should be noted that when changing the molar ratio to higher Lact concentrations, the decrease in viscosity may also contribute to promote the solute-solvent interactions and mass transfer phenomenon.

Based on these results, the following limits were defined for the mixtures to be tested: $0 \leq x_{Lact} \leq 0.9$; $0 \leq x_{Cit} \leq 0.75$ and $0.1 \leq x_{ChCl} \leq 0.8$. Note that these limits are graphically represented in Fig. 4b by the gray regions. On the other hand, the blue regions in Fig. 4b represent the linear constraints defined by Eqs. (4) and (5), that exclude the compositions which are solid at room temperature:

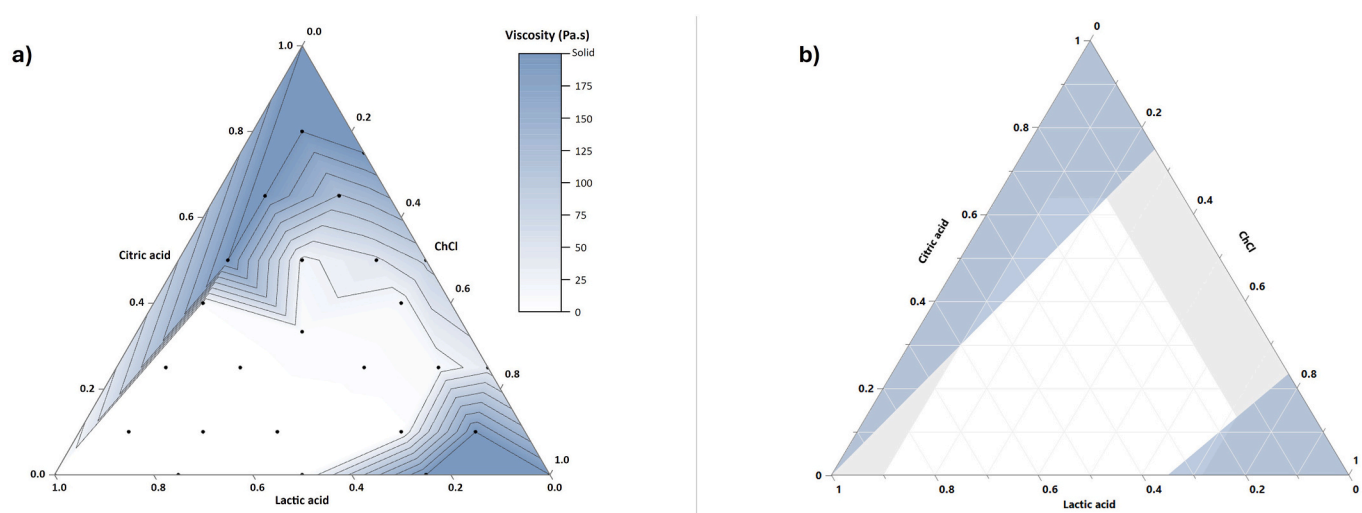

Fig. 4. a) Viscosities of the ternary mixtures composed of Lact, Cit, and ChCl. Black dots on the plot represent the compositions of the tested mixtures; b) variable limits (gray area) and linear constraints (blue area) defined for the concentration of the mixture components presented as molar fractions. The blue area represents the linear constraints defined by Eqs. (4) and (5).

$$1\ \text{Lact} + 0\ \text{Cit} + 4\ \text{ChCl} \geq 1 \quad (4)$$

$$1\ \text{Lact} + 1.5\ \text{Cit} + 0\ \text{ChCl} \geq 0.35 \quad (5)$$

3.3. Combined mixture-process D-optimal design of experiments

After defining the extraction variables and their ranges, a combined mixture-process D-Optimal design was applied to establish the experimental conditions that allow fitting a surface model which ultimately could point to optimal conditions for the lignin extraction, focusing on both the extracted quantity (lignin extraction yield) and the purity of the extracted fraction. A total of 15 different runs were performed in triplicate. Table 2 provides an overview of the experimental conditions tested and a summary of the results obtained for the lignin extraction yield and for the lignin purity. Additionally, the predictions obtained from the fitted surface models for both responses, as described by the Eqs. (6) and (7), are also listed.

Regarding the lignin purity, it is notable that for all the conditions tested, it exceeds 80 %, varying from 81.61 ± 2.64 to 101.20 ± 3.88 %. Interestingly, the DES 0.35:0:0.65 yielded the highest purity when the extraction was performed at 120 °C. However, when the temperature increased to 140 °C, it resulted in extracts with the lowest purity. Nonetheless, all extracted lignins display high purity, which indicates that the selected DES and the temperature range enable selective extraction of lignin from acacia wood. On the other hand, the lignin extraction yield is highly influenced by the experimental factors, ranging from 2.64 ± 0.48 to 99.63 ± 1.24 %. The temperature has a notable effect in almost all conditions, except for the 0.15:0.64:0.21 ratio. This effect is somehow anticipated and aligns with the results presented in Section 3.1 and with existing literature [25,48–50]. While the extraction efficiency can be improved by increasing the temperature, the solvent molar ratio also plays a crucial role. At 140 °C, the lignin extraction yield ranges from 23.13 ± 14.7 to 83.12 ± 2.23 % for Lact:ChCl mixtures and from 45.23 ± 3.58 to 99.63 ± 1.24 % for the mixtures also containing Cit. This suggests that the presence of Cit can enhance lignin extraction but the percentage and overall ratio of all components in the mixture are pivotal. For instance, the binary DES 0.9:0:0.1 demonstrated superior performance for lignin extraction at both temperatures compared to the ternary DES 0.15:0.13:0.72. It is important to consider that when working with mixtures, a change in the content of one component also affects the content of the other components [60]. Therefore, to better rationalize the effect of each component on each response (i.e., lignin extraction yield and purity), an optimization study was performed for each response based on the results of the D-optimal design outlined in Table 2.

3.3.1. Lignin extraction yield optimization

Upon observing the impact of the selected variables on both lignin extraction yield and purity, an in-depth statistical analysis was undertaken to elucidate their influence and optimize them to maximize the response variables. Concerning lignin extraction yield, the values obtained from the 15 experiments conducted (Table 2) were cross-referenced with the predicted values derived from the model, yielding an average error of 5.98 %. Furthermore, the model demonstrated a determination coefficient of $R^2 = 0.98$, meaning a good fit to the experimental data. To conclude this assessment, a Durbin-Watson test was conducted, resulting in a *p*-value of 0.2746 (exceeding the significance level of 0.05), thus confirming the absence of serial structure in the residuals, which further consubstantiates that the important trends were captured by the fitting model.

Subsequently, an ANOVA analysis was also performed, as delineated in Table 3, where it was confirmed that certain variables exhibited a considerable influence over the lignin extraction yield. Specifically, the variables [Lact], [Cit], [Lact] · [Cit], [Lact] · temperature, [Cit] · [ChCl], [Cit] · temperature, [Lact] · [Cit] · temperature, and [Cit] · [ChCl] · temperature, all show highly significant *p*-values, lower than 0.0001. Furthermore, the interaction of [Lact] · [ChCl] · temperature also exerts a noteworthy influence, as indicated by a *p*-value of 0.0104. Notably,

Table 2
Experiments obtained from DOE, and the experimental data obtained for lignin extraction yield and lignin purity. Results are presented as the means of three replicates. Values connected by the same superscript letter show no significant difference in lignin extraction yield (lower case) and lignin purity (capital letter) (Tukey test, $p \leq 0.05$).

Mixture Ratio (Lact:Cit:ChCl)	Temperature (°C)	Lignin extraction yield (%)	Predicted Lignin extraction yield (%)	Error (%)	Lignin purity (%)	Predicted Lignin purity (%)	Error (%)
0.15:0.13:0.72	120	5.98 ± 0.71^{i}	5.29	11.50	$97.05 \pm 1.18^{A,B}$	98.54	1.54
	140	45.23 ± 3.58^{f}	40.07	11.42	$85.52 \pm 1.86^{F-H}$	84.75	0.90
0.15:0.39:0.46	120	29.73 ± 4.35^{g}	31.85	7.13	$88.92 \pm 1.31^{C-G}$	88.90	0.02
	140	$78.58 \pm 0.56^{b,c}$	82.76	5.33	$95.94 \pm 3.01^{A-C}$	96.97	1.07
0.15:0.64:0.21	120	$66.77 \pm 3.42^{d,e}$	66.12	0.98	$90.10 \pm 1.05^{B-G}$	90.11	0.00
	140	$71.83 \pm 2.71^{c,d}$	70.55	1.78	$93.22 \pm 2.23^{B-F}$	92.91	0.34
0.35:0:0.65	120	2.64 ± 0.48^{i}	2.55	3.74	101.20 ± 3.88^{A}	99.72	1.47
	140	$23.13 \pm 1.47^{g,h}$	26.73	15.60	81.61 ± 2.64^{H}	82.00	0.48
0.6:0.3:0.1	120	62.48 ± 3.12^{e}	64.19	2.74	$88.06 \pm 2.57^{E-H}$	88.70	0.72
	140	99.63 ± 1.24^{a}	102.20	2.58	$91.45 \pm 1.03^{B-F}$	92.41	1.05
0.61:0:0.39	120	17.31 ± 2.51^{h}	19.29	11.46	$88.44 \pm 0.84^{D-H}$	90.38	2.19
	140	60.94 ± 0.43^{e}	59.31	2.67	$90.56 \pm 0.42^{B-G}$	91.14	0.64
0.9:0:0.1	120	46.25 ± 3.53^{f}	45.37	1.90	$82.89 \pm 0.93^{G-H}$	82.21	0.82
	140	83.12 ± 2.23^{b}	83.15	0.04	$95.54 \pm 2.66^{A-D}$	95.32	0.23
0.42:0.23:0.35	130	$68.86 \pm 3.39^{d,e}$	61.41	10.83	$94.90 \pm 3.99^{A-E}$	92.12	2.93

Table 3
Results from combined mixture-process design analysis for lignin yield obtained from acacia wood extraction. Values with * represent the variables that significantly affect the lignin extraction yield at a significance level of 0.05.

Term	Term	Sum of Squares	F Ratio	*p*-value
X_1	(Lact-0.15)/0.75	25,686.03	1,329.53	<0.0001*
X_2	Cit/0.75	7,187.64	372.04	<0.0001*
X_3	(ChCl-0.1)/0.75	41.17	2.13	0.1551
$X_1 \bullet X_2$	Lact · Cit	1,086.69	56.25	<0.0001*
$X_1 \bullet X_3$	Lact · ChCl	6.72	0.35	0.5601
$X_1 \bullet X_4$	Lact · Temperature	2,182.12	112.95	<0.0001*
$X_2 \bullet X_3$	Cit · ChCl	895.47	46.35	<0.0001*
$X_2 \bullet X_4$	Cit · Temperature	505.51	26.17	<0.0001*
$X_3 \bullet X_4$	ChCl · Temperature	0.29	0.01	0.9036
$X_1 \bullet X_2 \bullet X_4$	Lact · Cit · Temperature	590.36	30.56	<0.0001*
$X_1 \bullet X_3 \bullet X_4$	Lact · ChCl · Temperature	145.19	7.51	0.0104*
$X_2 \bullet X_3 \bullet X_4$	Cit · ChCl · Temperature	1,286.74	66.60	<0.0001*

most of the selected variables and their interactions demonstrate statistically significant effects on the lignin extraction yield derived from acacia wood. These findings were visually represented in a Pareto Chart (Fig. 5), where the positive effects of all influential variables, except for the [Cit] · temperature interaction term, are shown. This supports the hypothesis that when most of the variables are set at the uppermost limit of their designated ranges, the resulting lignin extraction yields are correspondingly maximized. The negative interaction of Cit and temperature indicates that the effect of each of these variables is lower when the value of the other is higher. This means that increasing the Cit content of a DES has a greater influence on the extractions performed at lower temperatures.

In addition, the coefficients for the model equation used to estimate the lignin yield according to the different variables were determined, and resulted in Eq. (6):

$$\begin{aligned} Y_{pred} = {} & 64.26\,X_1 + 65.64\,X_2 - 5.18\,X_3 + 76.59\,X_1X_2 - 6.65\,X_1X_3 \\ & + 18.89\,X_1X_4 + 104.43\,X_2X_3 - 18.25\,X_2X_4 \\ & + 0.46\,X_3X_4 + 62.38\,X_1X_2X_4 + 34.34\,X_1X_3X_4 \\ & + 138.56\,X_2X_3X_4 \end{aligned} \tag{6}$$

In this equation, Y_{pred} corresponds to the predicted lignin extraction yield; X_1: Lact; X_2: Cit; X_3: ChCl; and X_4: temperature. The contour profile plots for the lignin extraction yield at 120 and 140 °C are represented in Fig. S5 of the supplementary information (SI).

Finally, the optimal conditions for achieving the highest lignin extraction yield were determined: 1 g of acacia wood with 10 g of solvent (a solution of 0.62:0.28:0.1 (Lact:Cit:ChCl)) at 140 °C for 2 h. As observed, the optimal temperature value falls within the highest extreme of the studied range. However, since elevated temperatures have proved to induce substantial changes in the matrices, complicating the extraction processes, higher values were not evaluated. The predicted optimal DES is mainly composed of Lact and Cit. These are the most significant compounds, with a positive effect on the lignin extraction yield. This emphasizes the preference for DES with higher acid content, aligning with prior literature which suggests that higher acid:ChCl ratios enhance lignin solubilization and recovery. A recent study from Zhou, et al. [61] evaluated the influence of the DES composition, particularly the molar ratio of acidic HBD on the separation efficiency of poplar lignin. For this, different contents of 4-chlorobenzene sulfonic acid (4-Cl-BSA) were added to a DES composed of ChCl and ethylene glycol (EG) (1:2). The authors found that a higher amount of acidic HBD improves lignin dissolution: for the same extraction conditions, the delignification was 64.67 %, 84.53 %, and 97.01 % when using DES with the molar ratio of 1:2:0.1, 1:2:0.3, and 1:2:0.7 (ChCl:EG:4-Cl-BSA), respectively. The higher capability of the acidic HBD for donating protons contributed to an increase in the extraction efficiency [61]. While Lact is the major contributor to an efficient fractionation, Cit also plays an important role in the process. In addition, the significant positive effect of the [Lact] · [Cit] term suggests a synergistic effect between these two acids, demonstrating that ternary DES are promising solvents for biomass fractionation. This is in agreement with our previous study, where the benefits of adding Tart to Lact:ChCl were highlighted [25].

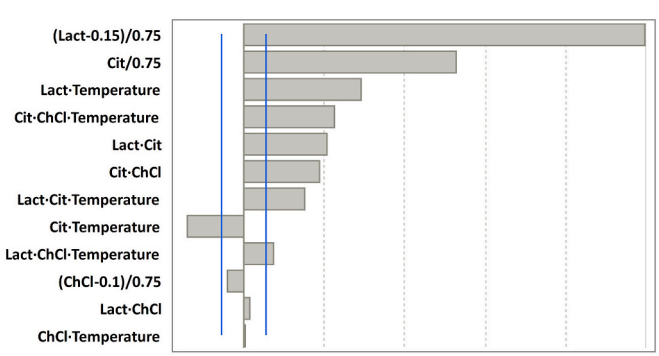

Fig. 5. Sorted parameter estimates obtained from the combined mixture-process design analysis of the lignin extraction yield obtained from acacia wood. The vertical blue line indicates the 95 % of confidence.

3.3.2. *Lignin purity optimization*

After evaluating the lignin extraction yield, the subsequent variable analyzed was lignin purity. Its assessment was performed for the 15 conducted experiments (Table 2) and correlated with predicted values, resulting on an average error of 0.96 %, being lower than 5 % in all cases. The developed methodology exhibits a determination coefficient of $R^2 = 0.85$. Moreover, the Durbin-Watson test yielded a *p*-value of 0.8904, exceeding the significance threshold of 0.05, thereby confirming the absence of significant serial structure in the residuals.

Subsequently, an ANOVA test was conducted, and the list of most influential variables is displayed in Table 4. These variables include [Lact], [Cit] and [ChCl], as well as the interactions [Lact] · temperature, [ChCl] · temperature, and [Cit] · [ChCl] · temperature, all displaying *p*-values lower than 0.0001, thus indicating their substantial impact. These results are depicted in a Pareto chart (Fig. 6). As can be observed, all three mixture components have a positive influential effect on the purity of lignin, in contrast to what was observed for lignin extraction yield, where ChCl displayed a negative non-influential contribution. Additionally, no significant synergistic effects were observed in the second order interactions between any DES components.

The coefficients for the model equation used to estimate the lignin

Table 4
Results from combined mixture-process design analysis for lignin purity of extracts from acacia wood. Values with * represent the variables that significantly affect the lignin purity at a significance level of 0.05.

Term	Term	Sum of Squares	F Ratio	*p*-value
X_1	(Lact-0.15)/0.75	49,010.71	7,034.48	<0.0001*
X_2	Cit/0.75	13,508.96	1,938.93	<0.0001*
X_3	(ChCl-0.1)/0.75	12,402.65	1,780.15	<0.0001*
$X_1 \bullet X_2$	Lact · Cit	5.42	0.78	0.3851
$X_1 \bullet X_3$	Lact · ChCl	6.60	0.95	0.3385
$X_1 \bullet X_4$	Lact · Temperature	262.58	37.69	<0.0001*
$X_2 \bullet X_3$	Cit · ChCl	11.89	1.71	0.2018
$X_2 \bullet X_4$	Cit · Temperature	26.10	3.75	0.0628
$X_3 \bullet X_4$	ChCl · Temperature	442.19	63.47	<0.0001*
$X_1 \bullet X_2 \bullet X_4$	Lact · Cit · Temperature	0.47	0.07	0.7980
$X_1 \bullet X_3 \bullet X_4$	Lact · ChCl · Temperature	23.66	3.40	0.0756
$X_2 \bullet X_3 \bullet X_4$	Cit · ChCl · Temperature	241.33	34.64	<0.0001*

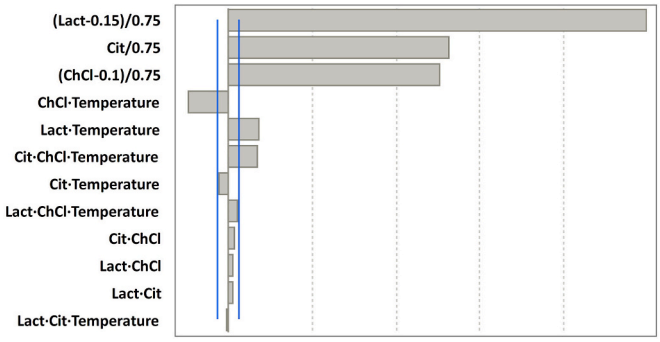

Fig. 6. Sorted parameter estimates obtained from combined mixture-process design analysis for lignin purity of extracts obtained from acacia wood.

purity obtained according to the different variables evaluated were determined, and resulted in the Eq. (7):

$$Y_{pred} = 88.77\,X_1 + 89.99\,X_2 + 89.86\,X_3 – 5.41\,X_1X_2 – 6.59\,X_1X_3 + 6.55\,X_1X_4 + 12.03\,X_2X_3 – 4.15\,X_2X_4 – 18.16\,X_3X_4 – 1.75\,X_1X_2X_4 + 13.86\,X_1X_3X_4 + 60.00\,X_2X_3X_4 \quad (7)$$

where, Y_{pred} corresponds to the predicted lignin purity of the extracts; X_1: Lact; X_2: Cit; X_3: ChCl; and X_4: temperature. The contour profile plots for the lignin purity at 120 and 140 °C are represented in Fig. S6 (SI).

The optimal conditions for achieving the highest lignin purity were determined, corresponding to 1 g of acacia wood extracted with 10 g of DES (Lact:Cit:ChCl) with a composition of 0.35:0:0.65 for 2 h at 120 °C. In contrast to the lignin extraction yield, these optimal conditions lie within the lowest value of the studied temperature range. Although higher temperatures favor lignin dissolution, it may also promote the dissolution of other biomass components, leading to the presence of impurities in the extracts. Previous research has shown that lignin purity increases with temperature until reaching a maximum value and then decreases at higher temperatures [25,62,63]. Regarding DES composition, the predicted optimal DES does not contain Cit. While its presence facilitates the extraction of higher amounts of lignin, it is possible that its high fractionation ability may also compromise the selectivity of the DES toward lignin when using high concentrations of Cit. Additionally, it is worth noting that the influence of each DES component also depends on temperature. The contour profiles in Fig. S6 (SI) show that, at 120 °C, lignin purity is higher for the DES containing a low fraction of Cit and high ChCl content. On the other hand, at 140 °C, the maximum purity of the extracts is obtained at a molar ratio of 0.15:0.45:0.40. Although lower temperatures often favor the selectivity of the fractionation process (with a maximum predicted purity of 99.7 % at 120 °C), at 140 °C, a maximum purity of 97.5 % was predicted.

3.3.3. Multi-objective optimization

The optimization of an extraction procedure aims at getting the conditions where both the extraction yield and the purity of the extracts are maximized. In this case, we observe that for the lignin purity, i.e., from a selectivity standpoint, the optimal conditions rely on an extraction temperature of 120 °C using a DES composed of 35 % of Lact, 0 % of Cit and 65 % of ChCl. On the other hand, when the optimization is focused on the overall efficiency of the method, maximizing lignin extraction yield, the optimal conditions are an extraction temperature of 140 °C using a DES composed of 62 % of Lact, 28 % of Cit and 10 % of ChCl. To find a compromise between optimizing both responses and to achieve an efficient and selective extraction method, a multi-objective optimization involving both variables was considered through a Desirability Function Approach (MO-DFA). Because lignin extraction yield is highly affected by the experimental conditions, while lignin purity remains consistently high for almost all conditions, the importance of the lignin extraction yield response was weighted higher than that of the

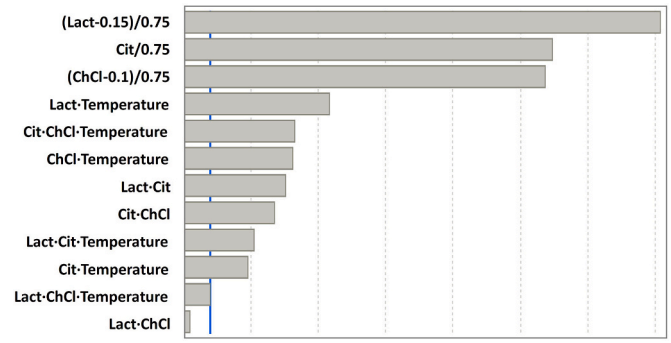

Fig. 7. Pareto chart of the effects for the multi-objective model using a combined mixture-process design for acacia wood extraction optimization. The vertical blue line indicates the 95 % of confidence.

lignin purity (5 to 1).

As can be observed in Fig. 7, all variables, except the interaction of [Lact] · [ChCl], are significantly influential for both lignin extraction yield and purity. These interactions were graphically represented in a response surface plot (Fig. 8). In this multi-objective optimization, all three mixture variables have high statistical impact on the overall efficiency of the extraction process. This contrasts with what was observed for the individual optimization of the lignin extraction yield, where ChCl showed no significant effect. In the single response optimization, second order interactions between Cit and the other mixture components ([Lact] · [Cit] and [Cit] · [ChCl]), only showed significance regarding the lignin extraction yield response, whereas for lignin purity, they were not significant. In the multi-objective optimization, the synergistic effects of Cit in enhancing the overall extraction are statistically significant, proving the benefit of adding Cit as a third component to the DES in order to improve its extraction ability.

The optimal conditions of the multi-objective models were found to be 1 g of acacia wood with 10 g solvent (0.6:0.3:0.1; Lact:Cit:ChCl) at 140 °C for 2 h. For these conditions, the model estimates a lignin extraction yield ranging from 96.36 to 108.19 %, with a predicted value of 102.3 % and a purity of 92.8 % (ranging from 89.25 to 96.35 %). These predictions are in good agreement with the results experimentally obtained: a lignin extraction yield of 99.63 ± 1.24 % and a lignin purity

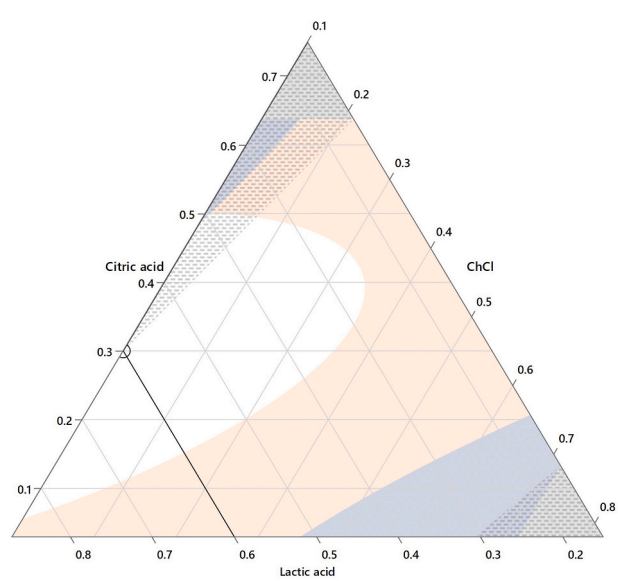

Fig. 8. Mixture profile at 140 °C showing the optimal mixture point. Highlighted regions represent the molar ratios that correspond to a lignin extraction yield (orange) and a lignin purity (blue) lower than 90 %. Dashed region represents the components limits and linear constraints.

of 91.45 ± 1.03 %. Since the experimental results for both responses are in the predicted ranges, this shows the high reliability and robustness of the model.

Based on this analysis, the experimental settings with DES Lact:Cit:ChCl at a molar ratio of 0.6:0.3:0.1 and an extraction temperature of 140 °C were selected as the best conditions and used in the following experiments.

3.4. Lignin characterization

3.4.1. FTIR analysis

The structural features of the extracted lignin were analyzed by FTIR. The FTIR spectra of acacia lignin is depicted in Fig. 9, and reveals characteristic bands indicative of a guaiacyl-syringyl (GS) type lignin, commonly found in hardwoods. The most prominent peaks are attributed to syringyl (S) and guaiacyl (G) units at 1111, 1209 and 1029 cm^{-1}, consistent with literature findings [58,64–67]. The assignment of all peaks is summarized in Table 5. The broad band in the region of 3400 cm^{-1} is attributed to the stretching vibration of phenolic and aliphatic O−H groups, while peaks at 2937 and 2839 cm^{-1} are assigned to C−H asymmetric and symmetric vibrations of methyl and methylene groups, respectively, which suggests the presence of side-chain units [64]. The bands at 1714 and 1597 cm^{-1} are assigned to ester groups and unconjugated and conjugated ketones. Aromatic skeleton vibrations and C−H deformation in aromatic groups occur in the region 1505–1422 cm^{-1}. As expected, acacia lignin clearly exhibits peaks associated with S and G units, characteristics of lignins extracted from hardwoods. Vibrations attributed to S units are present at 1322, 1209, and 1111 cm^{-1}, while peaks related to G units appear at 1264, 1029, and 822 cm^{-1}. The most intense peak at 1111 cm^{-1} is attributed to C−H in-plane deformation in S units.

Based on the relative intensity of these peaks, acacia lignin can be classified as a GS4 type lignin, according to the classification reported by Faix [68]. GS4 lignin has the lowest G content among all types of lignin. This classification is further supported by the analysis of C5-substituted and G units obtained by ^{31}P NMR (Section 3.4.3).

3.4.2. Thermal stability of lignin

The thermal degradation of the extracted lignin was evaluated by thermogravimetric analysis (TGA). The weight loss and the derivative weight of the acacia lignin were assessed across a temperature range of 30–600 °C. The obtained thermograms and derivative thermogravimetry (DTG) curves are depicted in Fig. 10. Overall, the thermal profile of lignin extracted from acacia wood with ternary DES aligns closely to other lignins described in the literature [25,69,70]. At 600 °C, nearly 60 wt% of the lignin initial weight is lost, yielding a final residue of 43.64 wt%. At temperatures below 100 °C, a minor loss (< 5 wt%) in lignin

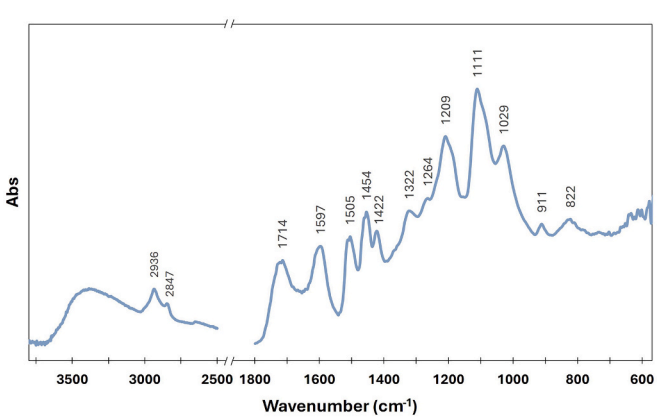

Fig. 9. FTIR spectrum of lignin extracted with Lact:Cit:ChCl (0.6:0.3:0.1), 140 °C, 2 h.

Table 5
FTIR absorption peak assignment for acacia wood lignin. Peaks were assigned according to the literature [58,64–67].

Wavenumber (cm^{-1})	Vibration mode [assignment]
3385	O–H stretching [phenolic and aliphatic OH]
2936	C–H asymmetric and symmetric [aliphatic methyl groups]
2847	C–H asymmetric and symmetric [aliphatic methylene groups]
1714	C=O stretching [unconjugated aryl ketones and lignin esters]
1597	C=O stretching [conjugated aryl ketones]
1505	C–C aromatic skeleton vibration [aromatic]
1454	C–H deformations [aromatic and methyl groups]
1422	C–C aromatic skeleton vibration [aromatic]
	C−H in plane deformations
1322	C–H bending [S and condensed G units]
1264	C–O stretching [G units]
	C=O stretching [aliphatic carbonyl groups]
1209	C–O stretching [S units]
	C=O stretching [carbonyl groups]
1111	C–H in-plane deformation [S units]
1029	Aromatic C−H in-plane deformation [G units]
	C−O deformation [primary alcohols or aliphatic ethers]
	C−H stretching [unconjugated]
911	C–H out of plane [aromatic]
822	C–H out of plane in positions 2, 5 and 6 [G unit]

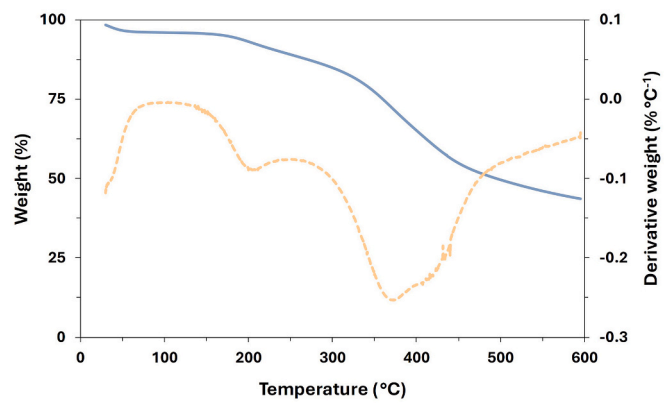

Fig. 10. TGA (blue line) and DTG (dashed orange line) curves of lignin extracted with Lact:Cit:ChCl (0.6:0.3:0.1) at 140 °C for 2 h.

weight is observed due to moisture evaporation. Two distinct weight loss stages related to lignin degradation are observed, with the maximum loss values at 202 and 372 °C. The first stage occurs between 150 and 250 °C and has a maximum loss of 0.09 wt%·$°C^{-1}$ at 202 °C. In this temperature range, partial degradation of the lignin side chains and of lower molecular weight lignin is expected to occur [69]. For example, acetic acid is reported to be formed at temperatures around 240 °C from the cleavage of bonds in alkyl side chains containing $–CH_2OH$ or –COOH [70]. The major weight loss occurs at a temperature range of 300–500 °C (maximum loss of 0.25 wt%·$°C^{-1}$ at 372 °C), corresponding to a more severe breakage of the lignin internal structure (C−C and ether bonds), potentially resulting in the formation of phenols or heterocyclic compounds [70].

3.4.3. ^{31}P NMR

The hydroxyl functional groups of the extracted lignin were determined by ^{31}P NMR. The ^{31}P NMR spectrum of the lignin sample can be found in the Supplementary material (Fig. S7). Detailed information of the content of carboxylic, aliphatic, and phenolic hydroxyl groups and the attribution of the NMR region for the determination of each functional group are presented in Table 6. Lignin extracted from acacia presents guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) OH groups, characteristic of a hardwood lignin. Due to signal overlap between

Table 6
Content of aliphatic, phenolic, and carboxylic hydroxyl groups of lignin extracted from acacia wood with ternary DES Lact:Cit:ChCl (0.6:0.3:0.1), determined by ^{31}P NMR.

Hydroxyl groups	Region (ppm)	Content (mmol•g^{-1} lignin)
Carboxylic acid OH	133.6–136	0.66 ± 0.03
Phenolic OH	137.6–144	3.01 ± 0.04
p-Hydroxyphenyl OH	137–138.6	0.14 ± 0.02
Guaiacyl OH	139–140.2	0.55 ± 0
C5-substituted OH	140–144.5	2.43 ± 0.02
Aliphatic OH	145.4–150	0.77 ± 0.02

syringyl and other types of condensed 5-substituted units (e.g., 5–5 and β–5), it is recommended to represent these units together as C5-substituted phenolic OH to prevent over/underestimation of syringyl and condensed units [43]. Because of this, an accurate quantification of the S units is challenging, but it is anticipated that these are the main contributors for the C5-substituted signal. Consistent with observations from the FTIR spectrum, the predominant phenolic group is derived from C5-substituted OH, mainly derived from S units, with minimal presence of H units. Lignin extracted from acacia using Lact:Cit:ChCl (0.6:0.3:0.1) presents a similar spectrum as reported for lignin extracted from acacia using ChCl:levulinic acid (Lev) (1:2) and (1:3), with the most intense band corresponding to C5-substituted OH groups [48]. This contrasts with the structure of commercial Kraft lignin, where the aliphatic OH groups predominate [48]. The high content of phenolic groups in acacia lignin is attributed to the acidic DES ability to cleave β–O–4 bonds [71,72], releasing phenolic OH groups during extraction [64,73]. Our finding contrasts with previous research by Magalhães, et al. [48], where acacia lignin exhibited a higher content of carboxylic than aliphatic OH groups: for each COOH group, only 0.35 and 0.5 aliphatic OH groups were present for lignin extracted with ChCl:Lev (1:2) and (1:3), respectively. Notably, lignin extracted with Lact:Cit:ChCl (0.6:0.3:0.1) has similar contents of COOH and aliphatic OH of 0.66 and 0.77 mmol•g^{-1}, respectively. This difference is probably caused by the harsher extraction conditions used by Magalhães, et al. [48] (4 h, 160 °C vs. 2 h, 140 °C in this work). The increasing harshness of the extraction conditions, such as temperature [74] and time [75,76], has been reported to decrease the content of aliphatic OH.

3.4.4. Lignin molecular weight

The determination of lignin molecular weight can offer insights into the fragmentation and/or recondensation processes that may occur during the extraction. The values of weight-average (M_w) and number-average (M_n) molecular weights, as well as the polydispersity index (PDI, M_w/M_n), are listed in Table 7. Lignin extracted with Lact:Cit:ChCl presents two peaks (Fig. 11), suggesting that the lignin has a heterogeneous structure with a wide range of molecular weights. The first peak, which accounts for >80 % of the extracted lignin, has a M_w of nearly 22 kDa, a M_n of 5.9 kDa and a PDI of 3.73. A smaller second peak, corresponding to a M_w of 747 Da, M_n of 609 Da and PDI of 1.23, suggests the presence of smaller lignin chains, potentially resulting from extra lignin fractionation and bond cleavage. The characteristics of lignin, including molecular weight, are highly dependent on the biomass source and on the extraction process parameters, such as the solvents, extraction conditions, and applied method. Thus, direct comparisons of molecular weight can be challenging. Nonetheless, the results obtained in this work align with those obtained for cellulolytic enzyme lignin extracted from poplar wood (M_w = 16.733 kDa, PDI = 4.2) [76]. However, lignin

Table 7
Molecular weight of lignin extracted with Lact:Cit:ChCl.

Peak No	Mn (Da)	Mw (Da)	PDI	Area (%)
1	5900	21,991	3.73	81.39
2	609	747	1.23	17.1

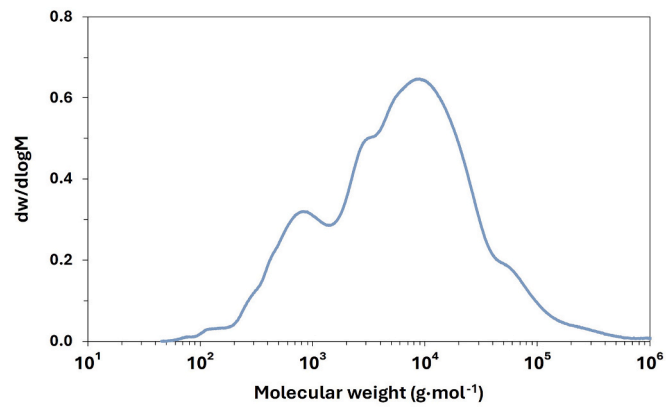

Fig. 11. GPC curve of extracted lignin.

extracted with acidic DES typically exhibits lower molecular weights. For instance, lignin extracted from willow using Lact:ChCl 10:1 shows a M_w ranging from 1040 to 1810 Da, decreasing with the increase of the extraction time [69]. Similarly, lignin extracted from softwood chips powder (SCP) and brewer's spent grains (BSG) with Lact:ChCl 10:1, also show two peaks with lower molecular weights compared to this work (M_w of 274–296 and 919–1124 Da, depending on the biomass source and conditions) [77].

3.5. DES recovery and reuse

One of the major advantages of using DES for biomass fractionation is their easy recovery at the end of the process, allowing for their reuse in subsequent extraction cycles. Throughout biomass fractionation, lignin, hemicellulose, and other extractives are solubilized, while the cellulose fraction remains insoluble. This fraction is separated by solid-liquid separation methods, such as filtration. Lignin can then be recovered by precipitation, by adding excess water, followed by filtration. The resulting solvent includes the DES, the added water and the dissolved hemicellulose and other extractives. The water can be removed by evaporation or freeze-drying, rendering the solvent ready to be reused in further extractions, even without any additional purification step, with evident economic advantages.

In this work, the potential for recovering and reusing the selected DES (Lact:Cit:ChCl, 0.6:0.3:0.1) was evaluated for three sequential biomass pretreatments. The results for the lignin extraction yield and purity of the extracts, after each extraction cycle, are depicted in Fig. 12.

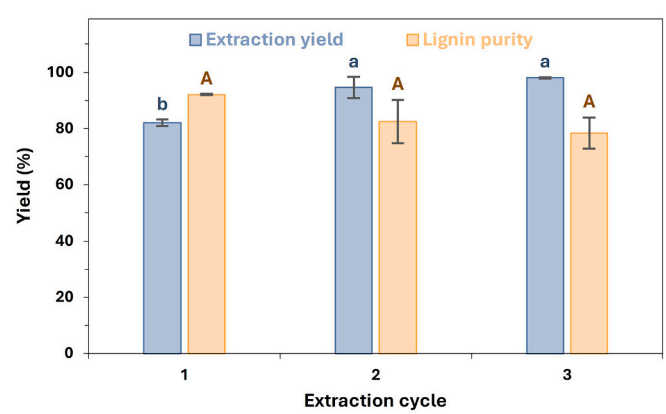

Fig. 12. Lignin extraction yield (blue columns) and purity (orange columns) from acacia wood fractionation using freshly prepared and recovered Lact:Cit:ChCl mixture (0.6:0.3:0.1). Extraction conditions: 2 h, 140 °C. The same letter above the bars indicates no significant differences in lignin extraction yield (lower-case) and lignin purity (capital) (Tukey test, $p \leq 0.05$).

It is worth noting that the lignin extraction yield using freshly prepared DES was slightly lower than previously observed during the optimization process for the same conditions (see Table 2, 0.6:0.3:0.1, 140 °C). This discrepancy may be attributed to anticipated differences in the acacia wood batches used, with the current batch exhibiting a slightly lower lignin content (19.34 wt% vs. 21.49 wt%). In addition, the experiments for DES recovery and reuse were performed in a reactor with a higher capacity, employing larger wood loadings (15 g vs. 1 g). Nevertheless, the solid-liquid ratio was kept the same (1:10). Due to the difference in the dimensions of the two reactors, further optimization of the extraction time may be needed to maximize the extraction yield. Although there was an observed decrease in the efficiency of the fresh DES, a still remarkable lignin extraction yield of 82 % was achieved, with the purity of the extracted lignin reaching 92.06 %, similar to the results previously observed (91.45 %). Interestingly, the lignin extraction yield improved in subsequent cycles, reaching 95 % and 98 %, in the second and third extraction assays, respectively. This improvement may be attributed to the presence of other compounds dissolved in the DES, which could act as catalysts, facilitating lignin extraction in the subsequent cycles. This is in agreement with related works where the presence of additives in the solvent (such as biopolymers) may boost phenomena, such as dissolution [78].

As for the purity of the extracts, a slight decrease when using recovered DES was observed, but without statistical significance. Lignin extracted in the third cycle had a purity of ca. 80 %, which can be acceptable depending on the desired application. Additionally, no significant differences were found in the structure of the lignins among the different cycles, as observed in the FTIR spectra of lignin extracted with fresh (Fig. 9, Section 3.4.1) and recovered DES (Fig. S8, SI). Both lignins exhibit a similar profile and no additional peaks were found in lignin extracted with recovered DES. The relative intensity of the peaks was also similar in both lignins. This suggests that DES reuse does not affect the structure of the extracted lignin and does not lead to the presence of contaminants.

Studies on DES recycling have been conducted in order to reduce costs and the environmental impact associated with biomass pretreatments using DES. Different performances have been reported in the literature for lignin extraction using recovered DES, caused by the great variability in biomass sources, DES composition and extraction methods. For example, the yield of regenerated lignin from eucalyptus decreased gradually from 44.8 % to 28.1 % after four cycle pretreatments using Lact:ChCl (10:1) [79]. Lignin extraction yield of *Pinus radiata* fractionation with benzyltrimethylammonium chloride:formic acid (1:2) ranged from 78.1 % to 66.6 % after five cycles [80], contrary to what was observed in the present study. The presence of contaminants in DES have been reasoned to reduce lignin selectivity and decrease the lignin yield [80]. The ability of a DES to be efficiently reused without compromising the subsequent extractions is not only dependent on its composition and thermal stability, but also on the recovery procedure. Yan, et al. [81] recently investigated the effect of different recovery methods on the pretreatment efficiency of recycled DES composed of ChCl and oxalic acid (1:1). The authors found that the content of oxalic acid decreases during the sequential extractions and, by adding oxalic acid to restore the composition of the original DES, they increase the number of recycling cycles from 5 to 8. Moreover, the same authors observed that, if a second precipitation of lignin with water followed by refrigeration is performed before the DES recovery, then the recovered DES would become less saturated, which allows for sustained efficiency even after 10 cycles.

DES reuse is a promising approach to enhance both the environmental sustainability and economic viability of these solvents. However, further studies should be performed to deeply analyze the composition of the recovered DES and to elucidate the effect of the other dissolved compounds on the efficiency and selectivity of the recycled solvent. Although this is out of scope of the present work, such detailed analysis will be explored in our subsequent studies.

4. Conclusions

In the current research, an in-depth study of the effect of the composition of a ternary DES on its ability to efficiently and selectively extract lignin from acacia wood was carried out. Tartaric (Tart) and citric (Cit) acids were introduced as third components alongside the commonly used lactic acid (Lact)–choline chloride (ChCl) mixture, forming a new ternary DES. A preliminary study revealed that Cit-based ternary DES are more efficient for lignin extraction due to the higher number of carboxylic acid groups that can favorably interact with lignin. In addition, it was found that 2 h is the optimal extraction time since extensively long exposure to high temperatures can cause partial degradation or recondensation of lignin.

The extraction temperature and the molar composition of Lact:Cit:ChCl DES were then optimized by a combined mixture-process D-optimal experimental design in order to maximize the lignin extraction yield and purity. This is indeed an innovative approach allowing a better understanding of the role that each component of the ternary DES mixture has in each of these responses. This process affords a more fulfilling design of solvent mixtures by finding the conditions that yield the maximum overall efficiency. Results highlighted Lact and Cit as pivotal variables positively impacting the extraction yield. Cit also showed a synergistic effect with Lact and ChCl, revealing that its addition positively affects DES ability to extract lignin. While all three compounds contributed positively to lignin purity, no synergistic effects were observed in the second-order interactions. Overall, the optimal conditions that simultaneously maximized the lignin extraction yield and purity were achieved when using Lact:Cit:ChCl at a molar ratio of 0.6:0.3:0.1 and at a temperature of 140 °C. These conditions led to an extraction of 99.63 ± 1.24 % of the lignin present in acacia wood with a purity of 91.45 ± 1.03 %, which represents a 19.9 % improvement when compared to the highest extraction yield obtained for the binary Lact:ChCl (83.12 % yield). Findings from this work are in agreement with those reported in the literature where ternary DES show an enhanced extraction performance when compared to their binary counterparts.

A detailed characterization of the extracted lignin revealed that it has a high distribution of molecular weights, with the main peak showing a M_w of nearly 22 kDa and a PDI of 3.73. It has a high content of phenolic hydroxyl groups, mainly composed of syringyl units, and its thermal stability is similar to other lignins described in the literature.

Additionally, the recycling properties of this DES were evaluated, and it was found that its efficiency is not compromised for three cycles, highlighting its sustainability and cost-effectiveness. Nonetheless, a slight decrease in the purity was observed when using recovered DES, yet with no significant impact. The high performance of the developed DES for lignin extraction, combined with its ability to be recovered, makes it highly appealing for further application in biomass pretreatment. In the future, it would be of great interest to deepen the characterization of the DES recovered along the cycles, not only to better understand its thermal stability but also to identify changes in its composition. In summary, our study adds valuable insights for solvent design and optimization of processes focused on lignocellulosic biomass fractionation, advancing our understanding and paving the way for enhanced sustainable practices in biomass valorization.

CRediT authorship contribution statement

Catarina Fernandes: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **María José Aliaño-González:** Writing – review & editing, Validation, Formal analysis. **Leandro Cid Gomes:** Writing – review & editing, Investigation. **Diana Bernin:** Writing – review & editing, Resources. **Rita Gaspar:** Writing – review & editing, Investigation. **Pedro Fardim:** Writing – review & editing, Resources. **Marco S. Reis:** Writing – review & editing, Formal analysis. **Luís Alves:** Writing – review & editing, Supervision. **Bruno Medronho:** Writing – review & editing,

Supervision, Conceptualization. **Maria Graça Rasteiro:** Writing – review & editing, Supervision. **Carla Varela:** Writing – review & editing, Project administration, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.135936.

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