Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61997R2
Full Title:	Ultrafast Lignin Extraction from Unusual Mediterranean Lignocellulosic Residues
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TITLE:

Ultrafast Lignin Extraction from Unusual Mediterranean Lignocellulosic Residues

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KEYWORDS:

Lignocellulosic, Choline chloride, Oxalic acid, Urea, Lactic acid, Lignin, Microwave

SUMMARY:

Deep eutectic solvent-based, microwave-assisted pretreatment is a green, fast, and efficient process for lignocellulosic fractionation and high-purity lignin recovery.

ABSTRACT:

Pretreatment is still the most expensive step in lignocellulosic biorefinery processes. It must be made cost-effective by minimizing chemical requirements as well as power and heat consumption and by using environment-friendly solvents. Deep eutectic solvents (DESs) are key, green, and low-cost solvents in sustainable biorefineries. They are transparent mixtures characterized by low freezing points resulting from at least one hydrogen bond donor and one hydrogen bond acceptor. Although DESs are promising solvents, it is necessary to combine them with an economic heating technology, such as microwave irradiation, for competitive profitability. Microwave irradiation is a promising strategy to shorten the heating time and boost fractionation because it can rapidly attain the appropriate temperature. The aim of this study was to develop a one-step, rapid method for biomass fractionation and lignin extraction using a low-cost and biodegradable solvent.

In this study, a microwave-assisted DES pretreatment was conducted for 60 s at 800 W, using three kinds of DESs. The DES mixtures were facilely prepared from choline chloride (ChCl) and three hydrogen-bond donors (HBDs): a monocarboxylic acid (lactic acid), a dicarboxylic acid (oxalic acid), and urea. This pretreatment was used for biomass fractionation and lignin recovery from marine residues (Posidonia leaves and aegagropile), agri-food byproducts (almond shells and olive pomace), forest residues (pinecones), and perennial lignocellulosic grasses (*Stipa tenacissima*). Further analyses were conducted to determine the yield, purity, and molecular weight distribution of the recovered lignin. In addition, the effect of DESs on the chemical functional groups in the extracted lignin was determined by Fourier-transform infrared (FTIR) spectroscopy. The results indicate that the ChCl–oxalic acid mixture affords the highest lignin purity and the lowest yield. The present study demonstrates that the DES–

microwave process is an ultrafast, efficient, and cost-competitive technology for lignocellulosic biomass fractionation.

INTRODUCTION:

Sustainable biorefinery processes integrate biomass processing, its fractionation into molecules of interest, and their conversion to value-added products[1]. In second-generation biorefining, pretreatment is considered essential for fractionating biomass into its main components[2]. Traditional pretreatment methods utilizing chemical, physical, or biological strategies have been widely applied[3]. However, such pretreatment is considered the most expensive step in biorefining and has other disadvantages such as long processing time, high heat and power consumption, and solvent impurities[4]. Recently, DESs, whose properties are similar to those of ionic liquids[3], have emerged as green solvents owing to advantages such as biodegradability, environmental-friendliness, ease of synthesis, and recovery after treatment[5].

DESs are mixtures of at least one HBD, such as lactic acid, malic acid, or oxalic acid, and a hydrogen-bond acceptor (HBA) such as betaine or choline chloride (ChCl)[6]. HBA–HBD interactions enable a catalytic mechanism that permits cleavage of chemical bonds, causing biomass fractionation and lignin separation. Many researchers have reported the DES-based pretreatment of lignocellulosic feedstocks such as ChCl–glycerol on corn's cob and stover[7,8], ChCl–urea, and ChCl–oxalic acid on wheat straw[9], ChCl–lactic acid on *Eucalyptus* sawdust[10], and ChCl–acetic acid[11] and ChCl–ethylene glycol on wood[11]. To improve DES efficiency, the pretreatment should be combined with microwave treatment to accelerate biomass fractionation[5]. Many researchers have reported such a combined pretreatment (DES and microwave) of wood[8] and of corn stover, switchgrass, and Miscanthus[5], which provides new insight into the capacity of DESs for lignocellulosic fractionation and lignin extraction in one easy step over a short period.

Lignin is a phenolic macromolecule valorized as a raw material for the production of biopolymers and presents an alternative for the production of chemicals such as aromatic monomers and oligomers[12]. In addition, lignin has antioxidant and ultraviolet absorption activities[13]. Several studies have reported lignin applications in cosmetic products[14,15]. Its integration in commercial sunscreen products has improved the sun protection factor (SPF) of the product from SPF 15 to SPF 30 with the addition of only 2 wt % lignin and up to SPF 50 with the addition of 10 wt % lignin[16]. This paper describes an ultrafast approach for lignin-carbohydrate cleavage, assisted by combined DES–microwave pretreatment of Mediterranean biomasses. These biomasses consist of agri-food byproducts, particularly olive pomace and almond shells. Other biomasses that were investigated were those of a marine origin (Posidonia leaves and aegagropile) and those originating from a forest (pinecones and wild grasses). The focus of this study was to test low-cost green solvents to evaluate the effects of this combined pretreatment on feedstock fractionation, to investigate its influence on lignin purity and yield, and to study its effects on the molecular weights and chemical functional groups in the extracted lignin.

PROTOCOL:

1. Preparation of biomasses

1.1. Biomass drying

1.1.1. Place the Posidonia leaves and aegagropile balls (*Posidonia oceanica*), harvested from Mediterranean beaches, in an oven at 40 °C for 72 h.

1.1.2. Place the almond shells (*Prunus dulcis*), generated from food industries, and olive pomace (*Olea europaea L.)*, obtained from olive oil mills, in an oven at 40 °C for 72 h.

1.1.3. Place the pinecones (*Pinus halepensis*), collected from a forest, and alfa leaves (*Stipa tenacissima*), collected from the southern Mediterranean basin, in an oven at 40 °C for 72 h.

NOTE: If the biomass contains sand, it must be rinsed with distilled water before it is placed in the oven. The biomasses are shown in **Figure 1A–F**.

1.2. Biomass grinding

1.2.1. Place 20 g of each biomass in a hammer cutter equipped with a 1 mm sieve. Collect the resultant powder in a 0.25 L beaker and feed it to a hammer cutter equipped with a 0.5 mm sieve. Collect the powder in a 0.25 L beaker.

2. Microwave-assisted, ultrafast lignin extraction

2.1. Deep eutectic solvent (DES) preparation

2.1.1. Prepare DES1 (ChCl–oxalic acid) in a molar ratio of 1:1 by mixing 174 g of ChCl with 126 g of oxalic acid dihydrate in a 500 mL round-bottom flask and melting them in a bath at 70 °C for 4 h until a homogenous and transparent liquid is formed.

2.1.2. Prepare DES2 (ChCl–lactic acid) in a molar ratio of 1:1 by mixing 174 g of ChCl with 90 g of lactic acid in a 500 mL round-bottom flask and melting them in a bath at 70 °C for 4 h until a homogenous and transparent liquid is formed.

2.1.3. Prepare DES3 (ChCl–urea) in a molar ratio of 1:12 by mixing 174 g of ChCl with 120 g of urea in a 500 mL round-bottom flask and melting them in a bath at 70 °C for 4 h until a homogenous and transparent liquid is formed.

NOTE: Stir these mixtures continuously with a stir bar at 500 rpm.

2.2. Combined microwave–DES treatment

2.2.1. Place 5 g of the feedstock in a microwave in a closed polytetrafluoroethylene reactor. Add 50 mL of DES, and place a stirring bar in the sample. Close the microwave container with an appropriate cap, and attach the temperature cap.

2.2.2. Place the microwave container on the edge of the turntable, ensuring that it is constantly agitated. Set the microwave power at 800 W for 1 min. Using suitable gloves, take the container out of the microwave, and let the mixture cool. Repeat this treatment using the three DESs for each biomass sample.

NOTE: Check and ensure that the temperature probe is correctly placed, and the microwave container has a homogenous temperature.

2.3. Lignin isolation

2.3.1. Prepare a homogeneous antisolvent solution by mixing ethanol:water in a 50:50 (v:v) ratio. Add 50 mL of the antisolvent solution to the treated feedstock, place the mixture in a centrifugation container (250 mL), and centrifuge for 5 min at 3,000 × *g*.

2.3.2. After centrifugation, filter the supernatant (lignin-rich fraction) using a glass filter crucible (porosity 4, 10–16 µm, diameter 10 mm). Wash the remaining cellulose residue collected after centrifugation with 25 mL of the antisolvent solution.

2.3.3. Centrifuge at 3,000 × *g* for 5 min after each wash. Repeat washes 4x, and collect and filter the washes through the glass filter crucible (porosity N 4, 10–16 µm, diameter 10 mm).

2.3.4. Add the filtered lignin-rich fraction from step 2.3.2 to the filtered washes from step 2.3.3 in a 500 mL round bottom flask. Evaporate ethanol using a rotary evaporator at 50 °C and 110 mbar.

2.3.5. Add 150 mL of deionized water to the concentrated liquor (lignin-rich fraction), and precipitate the lignin by centrifugation. Collect lignin as a pellet, and wash it with 25 mL of distilled water; repeat the washes 4x. Lyophilize lignin, or dry it in an oven at 40 °C.

NOTE: If necessary, wash lignin >4x to remove the salts from the solvents.

2.3.6. Use the following formula to determine the yield:

$$Yield\ (\%) = \frac{\text{g of lignin precipatate}}{\text{g of initial lignin}} \times\ 100$$

NOTE: Lignin extraction was also performed with two other DESs: choline chloride + resorcinol and choline chloride + butyric acid at 1 min. However, the amounts of lignin recovered using these DESs were extremely small (and unrecoverable) compared with the amounts obtained using the other three DESs.

3. Purity determination of extracted lignin by Klason

3.1. Sample preparation for Klason hydrolysis

3.1.1. Place the filter crucible (porosity 4, diameter 4.5 mm) in a muffle furnace at 550 °C for 4 h (2 h ramp, from 25°C). Remove the crucible when the oven cools to 150 °C, place it in a desiccator to cool, and weigh.

3.1.2. Add approximately 30 mg of lignin into a borosilicate glass tube (see the **Table of Materials**), and note the weight of the sample. Add 1 mL of 72% sulfuric acid (H_2SO_4) to the sample, place the sample in a 30 °C bath for 60 min, and mix every 10 min by vortexing.

3.1.3. Remove the sample, transfer it to a 100 mL glass bottle, and add 28 mL of distilled water to dilute the acid to a concentration of 4%. Place the glass bottle in an autoclave at 121 °C for 60 min. Remove the glass bottle, and allow it to cool.

3.2. Analysis of acid-insoluble lignin

3.2.1. Filter the hydrolysate using a crucible under vacuum. Collect all the solids in a glass bottle containing deionized water. Rinse the crucible with 50 mL of deionized water.

3.2.2. Dry the crucible containing the solids by placing it in an oven at 105 °C for 16 h. Remove the crucible from the oven, place it in a desiccator, and allow it to cool. Weigh the sample.

3.2.3. Place the crucible in a muffle furnace at 550 °C for 4 h (2 h ramp). Remove it and place in a desiccator. Weigh the sample.

3.2.4. Use the following formula to calculate the percentage of the acid-insoluble residue (AIR):

$$\%\,AIR = \frac{(\mathrm{WCSA} - \mathrm{WC}) - (\mathrm{WCSMF} - \mathrm{WC})}{\mathrm{ODW}} \times 100$$

WCSA: weight of crucible + sample after removing them from the oven
WC: weight of crucible
WCSMF: weight of crucible after removing it from the muffle furnace
ODW: oven dry weight of the sample

3.3. Analysis of acid-soluble lignin

3.3.1. Measure the absorbance of the filtrate obtained in step 3.2.1 with a spectrophotometer at 205 nm using quartz cuvettes. Use distilled water as blank.

3.3.2. Use the following formula to calculate the percentage of the acid-soluble residue (ASL):

$$\%\,ASL = \frac{(\mathrm{UVabs} \times \mathrm{Volume\ of\ filtrate}) \times dilution}{\varepsilon \times \mathrm{ODW} \times \mathrm{Pathlength}} \times 100$$

NOTE: Absorbance should be between 0.2 and 0.7. Dilute the sample if necessary.
UVabs: absorbance at 205 nm
Pathlength: light path of the measuring cell (in cm)
ε: absorbency of biomass at a specific wavelength

4. Nitrogen content in extracted lignin

4.1. Preparation of alkali solution

4.1.1. In a 2.5 L volumetric flask, weigh 1 kg of sodium hydroxide (NaOH) and add deionized water up to the mark. Place a magnetic bar in the flask, and stir until the NaOH is completely dissolved.

4.2. Sulfuric acid solution preparation

4.2.1. Take 0.1 N H_2SO_4 (see the **Table of Materials**) in a 5 L volumetric flask, add deionized water up to the 5 L mark, place a magnetic bar, and stir until the contents dissolve.

4.3. Preparation of receiving solution

4.3.1. In a 5 L volumetric flask, dissolve 100 g of H_3BO_3 (boric acid) in deionized water, and bring up the volume to the mark.

4.3.2. Weigh 100 mg of bromocresol green in a 100 mL volumetric flask, and add technical methanol up to the mark.

4.3.3. Weigh 100 mg of methyl red in a 100 mL volumetric flask, and add technical methanol up to the mark.

4.3.4. Pour the 5 L of H_3BO_3 solution from step 4.3.1, 100 mL of bromocresol green solution from step 4.3.2, 70 mL of the methyl red solution from step 4.3.3, and 5 L of deionized water into a container. Shake the receiving solution well for 30 min.

NOTE: The final color of the solution must be green. If the color is not green, add 50 mL of 1 N NaOH solution.

4.4. Sample preparation

4.4.1. In a Kjeldahl tube, place 100 mg of lignin weighed on a nitrogen-free paper, add a tablet of Kjeldhal (1.5 g potassium sulfate (K_2SO_4) + 0.045 g copper sulfate pentahydrate ($CuSO_4.5H_2O$) + 0.045 g titanium dioxide (TiO_2)), and add 7.2 mL of concentrated H_2SO_4.

NOTE: Use four tubes with only nitrogen-free paper (without the samples) as blanks.

4.5. Sample digestion

4.5.1. Switch on the thermostat on the digester 1 h in advance at 360 °C.

4.5.2. Place the sample tubes on a rack, place the four blank tubes at the four corners of the rack, and fill in the holes (if any) of the rack with empty tubes.

4.5.3. Place the rack in the preheated digester, cover the suction system, and open the water pump.

NOTE: Take care to avoid fumes; increase the flow of water if fumes appear.

4.5.4. After 2 h, turn off the heating, remove the samples, and place them on a metal support. Allow the rack to cool for approximately 40 min with the suction system on.

4.6. Kjeldhal distillation procedure

4.6.1. Switch on the Kjeldahl distiller. Allow **Self-tests** to run until **Selection** appears on the screen. Switch to **Manual** mode, insert an empty tube, and close the sliding door.

4.6.2. Purge the titrant burette (0.02 N H_2SO_4) (lift the cover) by pressing it at the bottom and top several times, and eliminate air bubbles from the pipes by squeezing the tube of the H_2SO_4 bottle. Close the hood.

4.6.3. Purge the H_3BO_3 receiving solution 3x.

4.6.4. Add water 3x, and switch to **Active steam** (10 min). Switch to the **Kjeldahl 1 analysis** program. Enter **Blanco** using the arrows at the **Result line** level.

4.6.5. Insert the tube. Start with the four blanks, and calculate their averages. Enter the value in the **Blanco** line.

NOTE: After the tube is inserted, the device automatically and successively adds 30 mL of H_2O, 30 mL of H_3BO_3, and 40 mL of 10 N NaOH.

4.6.6. Switch to **mL of titrant** at the **result line**. Insert the tube, and note the amount of H_2SO_4 used.

NOTE: To test the Kjeldahl distiller, consider that 50 mg of glycerin correspond to 18.60% ± 5% of % N. At the end of each titration, the device automatically empties and cleans the tube.

4.6.7. Calculate the percentage of N.

$$\% N = \frac{\mathrm{V\,s.a\,(mL)} \times \mathrm{Ts.a\,(N)} \times 0.014}{\mathrm{S\,(g)}} \times 100$$

V s.a : Volume of sulfuric acid
T s.a : 0.02 N H_2SO_4
S: sample mass

5. Ash content in extracted lignin

5.1 Dry the ceramic crucibles for 1 h at 105 °C. Leave them to cool in a desiccator.

5.2 Weigh a crucible, and note its number. Add approximately 1 g of the sample powder. Place the crucible in the muffle furnace with the following program: a 2 h ramp up to 575 °C; a plateau of 4 h at 575 °C.

5.3 Allow the oven to cool to 100 °C. Remove the crucibles, place them in the desiccator, and weigh them.

6. Carbohydrate content

6.1. Preparation of sodium borohydride ($NaBH_4$)/dimethylsulfoxide (DMSO) solution

6.1.1. Place 2 g of $NaBH_4$ in a 100 mL volumetric flask, and fill to the mark with DMSO. Heat to 100 °C in a Mayor's bath, and stir the solution until completely dissolved.

6.2. Preparation of MIX solution

6.2.1. Place 20 mg each of xylose, arabinose, rhamnose, glucose, galactose, mannose, and 2-deoxyglucose in a 100 mL volumetric flask, and fill up to the mark of 100 mL with deionized water.

6.3. Hydrolysis of the sample

6.3.1. Weigh a 50 mg sample of lignin in a borosilicate glass tube, add 3 mL of 1 M H_2SO_4, and heat the mixture for 3 h at 100 °C.

6.3.2. Cool the sample, add 1 mL of 15 M ammonium hydroxide (NH_4OH), and check the pH to ensure that it is neutral or alkaline. Add exactly 1 mL of internal standard (2-deoxyglucose) to each sample.

NOTE: The 2-deoxyglucose added as an internal standard makes it possible to quantify the quantity of each dose present in the sample.

6.4. Reduction and acetylation of monosaccharides into alditol acetate

6.4.1. Take 400 µL of the solution from step 6.3.2, and place it in special tubes. Take 400 µL of the control MIX solution, and place it in special tubes.

NOTE: Using the MIX solution facilitates the calculation of response factors (RFs) and monosaccharide percentages.

6.4.2. Add 2 mL of the $NaBH_4$/DMSO solution prepared in section 6.1. Close the tube, and incubate for 90 min at 40 °C in a water bath. Remove the tube from the water bath, and add 0.6 mL of glacial acetic acid.

NOTE: As this is an exothermic reaction, bubbles and smoke will appear.

6.4.3. Add approximately 0.4 mL of 1-methylimidazole and approximately 4 mL of acetic anhydride. After 15 min, add 10 mL of distilled water, cool, and add ~3 mL of dichloromethane (CH_2Cl_2).

6.4.4. After at least 2 h, collect ~1 mL of the lower (organic) phase, and inject it into a gas chromatograph equipped with a flame ionization detector capillary column, HP1-

methylsiloxane (30 m (length) x 320 µm (internal diameter), 0.25 µm (film thickness)). Analyze the data.

6.4.5. Use the following formula to calculate the response factor (RF).

$$RF = \frac{\mathrm{A\ m.p\ \times\ M\ a.h\ of\ 2\ deoxy\ glucose}}{\mathrm{A\ 2dg.p\ \ \times\ M\ a.h\ of\ the\ monosaccharide}}$$

$\mathrm{A\ m.p}$: Average of the area of the monosaccharide peak in the MIX solution
$\mathrm{M\ a.h\ of\ 2 - deoxy\ glucose}$: Mass of 2-deoxyglucose after hydrolysis
$\mathrm{A\ 2dg.p}$: Average of the area of the 2-deoxyglucose peak in the MIX solution
$\mathrm{M\ a.h\ of\ the\ monosaccharide}$: Mass of the monosaccharide after hydrolysis

NOTE: Anhydro correction is 0.8 for rhamnose, 0.88 for arabinose and xylose, and 0.9 for mannose, glucose, and galactose. Mass after hydrolysis = anhydro correction x mass (g) of the monosaccharide used in the MIX solution.

6.4.6. Use the following formula to calculate the monosaccharide mass.

$$Monosaccharide\ mass = \frac{\mathrm{AP.M\ \times\ M.IS}}{\mathrm{AP.2\ \ \times\ RF}}$$

AP.M: Monosaccharide peak area in the analyzed sample
M. IS: Mass of internal standard added; here, C SI=1 mg/mL
AP.2: Peak area of 2-deoxyglucose in the sample
RF: response factor

6.4.7. Calculate the percentage of each monosaccharide using the following formula.

$$Monosaccharide\ (\%) = \frac{Monosaccharide\ mass}{Initial\ sample\ (g)} \times 100$$

7. Chemical functions in extracted lignin (Fourier-transformed infrared)

7.1. To identify the chemical functional groups in extracted lignin, use an FT-IR spectrometer equipped with an attenuated total reflectance (ATR) module. Open the spectroscopy software, and adjust the parameters: resolution 4 cm^{-1}, sample scan time 32, background scan time 16, save data from 4000 to 400 cm^{-1}, result spectrum transmittance.

7.2. Do not add any sample; press **background single channel**. Now place 1 mg of the sample on the crystal, and press **sample single channel**. Process the obtained spectra.

8. Molecular weight of extracted lignin (gel permeation chromatography)

8.1. Prepare a solution of dimethylformamide (DMF) with 0.5% lithium chloride (LiCl). Take 5 g of LiCl in a 1 L volumetric flask, add DMF up to the gauge line, and mix the contents until a homogenous liquid is obtained.

8.2. Dissolve 3 mg of the lignin sample in 3 mL of DMF with 0.5% LiCl. Centrifuge in a 10 mL centrifuge tube, and separate the soluble fraction into a vial.

8.3. Dissolve 3 mg of polystyrene standard 1 kDa, 2 kDa, 3 kDa, 10 kDa, 20 kDa, and 30 kDa in the solution of DMF with 0.5% LiCl. Centrifuge in 10 mL borosilicate glass tubes, and transfer the soluble fraction to a vial.

8.4. Prepare a high-performance liquid chromatography-ultraviolet (UV) system.

8.4.1. Open the data system, and check the UV detector.

8.4.2. Purge the system with distilled water. Install the plunger in the eluent (DMF with 0.5% LiCl). Open the purging valve, and purge the line with a flow rate of 1 mL/min for 15 min. Stop the flow and close the purging valve.

8.4.3. Set the flow rate to 1 mL/min for 10 min to clean the eluent pathway to the detector. Stop the flow rate.

8.4.4. Install the column preceded by a guard column (see the **Table of Materials**). Switch on the column heater at 45 °C, switch on the UV detector, and set the flow rate gradually until a flow rate of 0.6 mL/min is reached.

8.5. Inject 30 µL of each sample for 40 min at a wavelength of 270 nm. Process the obtained data, and calculate the mass distribution using the calibration line.

8.6. Calculate the number average molecular weight (Mn), weight average molecular weight (Mw), and polydispersity index (PDI).

$$Mn = \frac{\sum \mathrm{NiMi}}{\sum \mathrm{Ni}}$$

$$\mathrm{Mw} = \frac{\sum \mathrm{NiMi}^2}{\sum \mathrm{NiMi}}$$

$$PDI = \frac{\mathrm{Mw}}{\mathrm{Mn}}$$

Mi: molecular weight of a chain
Ni: number of chains for that molecular weight

9. Data treatment and statistical analyses

9.1. Perform all analytical experiments in triplicate and express the results as % of dry matter.

9.2. Perform one-way analysis of variance (ANOVA), and compare the means using Tukey's multiple comparison test.

9.3. Perform principal component analysis (PCA).

REPRESENTATIVE RESULTS:

Figure 2A–C depict the lignin yield of extraction from the six feedstocks, shown in **Figure 1A–F**, after the combined microwave–DES pretreatment**.** The results show that the lignin yield obtained with DES1 (ChCl–oxalic acid) (**Figure 2A**) was lower than the yields obtained with

DES2 (ChCl–lactic acid) and DES3 (ChCl–urea) (**Figure 2B,C**). In addition, lignin yields from pinecones (PC) and olive pomace (OP) were higher at 32.31% and 26.04% for DES1 treatment and 48.72% and 43.76 for DES3, respectively. Lignin yield from alfa leaves (A) was significantly higher than the yields of all other lignins extracted with DES2. **Figure 3A–C** show that lignin purity exceeded 70% for the three pretreatments of the biomasses, except for DES3 pretreatment of alfa leaves (A), aegagropile (Ag), and almond shells (AS) in the DES3 (ChCl–urea) treatment, which gave a lignin purity of 65%. The highest lignin purity (> 90%) was obtained with the DES1 treatment: alfa leaves (A) 94%, almond shells (AS) 93%, pinecones (PC) 90%, Posidonia leaves (PL) 92%, and olive pomace (OP) 91%.

Lignin purity and yield data were subjected to principal component analysis (PCA) by considering two parameters (yield and purity) and 18 treatments. **Figure 4** shows that the correlation circle explained 100% of the total variation. The first component, PCA1, explained 58.09%, and the second component, PCA2, explained 41.91% of the total variation. Lignin purity was positively correlated with DES1 (Ox) treatment. The Pearson correlation coefficients (R) were alfa (A Ox) 0.32, olive pomace (OP Ox) 0.27, pinecones (PC Ox) 0.2, Posidonia leaves (PL Ox) 0.35, almond shells (AS Ox) 0.32, and aegagropile (Ag Ox) 0.05, respectively. However, DES3 treatment was negatively correlated with lignin yield with R-values that oscillated between −0.37 and −0.05. Thus, PCA results confirmed that lignin extracted with DES1 was the purest with the lowest yield.

Lignin was characterized for its sugar, nitrogen, and ash contents **(Figure 5A–C)**. The total sugar content was determined by gas chromatography (GC). The carbohydrate content in lignin was extracted using DES3 (ChCl–urea) was the highest (6–15%). This was followed by lignin extracted using DES2 (ChCl–lactic acid), which had a carbohydrate content of 3–12%. However, the lowest carbohydrate content (1%) was reported for lignin extracted using DES1 (ChCl–oxalic acid). The type of sugars identified differed significantly (**Figure 6A–C**); D-xylose and D-glucose were the most abundant monosaccharides. These results indicate that DES1 was extremely selective in its extraction of lignin compared with the other two DESs, which extracted not only lignin, but also carbohydrates. In other words, lignin purity was lower after extraction with the lactic acid and urea DESs.

The high selectivity of DES1 to fractionate the lignocellulosic matrix and extract pure lignin is probably because of the high acidity of its hydrogen bonds (alpha = 1.3). Choline chloride contains chloride ions that break the intramolecular interactions of hydrogen bonds, and the carboxylate groups in oxalic acid contribute to dissolve the lignin polymers. Similarly, the nitrogen content of lignin extracted using DES1 was lower than the nitrogen content of lignin extracted using DES2 and DES3, reaching up to 3% (**Figure 5A–C**). Lignin extracted from alfa leaves had the highest nitrogen content: 2.70, 3.84, and 3.40 for DES1, DES2, and DES3, respectively. These results prove that nitrogenous compounds were extracted and co-precipitated with lignin. Furthermore, lignin calcination in all the samples indicated that lignin extracted using DES2 and DES3 contained a higher inorganic component than lignin extracted using DES1.

These results indicate that DES1 promoted the extraction of lignin with high purity, but with low nitrogen, carbohydrate, and ash contents. In other words, lignin extracted using DES1 (ChCl–oxalic acid) was purer than that extracted using DES2 (ChCl–lactic acid) and DES3 (ChCl–

urea), which possesses lower purity and high nitrogen, carbohydrate, and ash contents. **Table 1** summarizes the molecular mass distribution of lignin, as analyzed by gel permeation chromatography (GPC) and represented by the number-average molecular weight (Mn), weight-average molecular weight (Mw) and polydispersity index (PDI). The M_w values ranged from 48,123 to 147,233 g mol^{-1}. The lignin extracted by DES2 from alfa leaves, almond shells, and aegagropile had a lower PDI than the lignin extracted by DES1, DES3, and alkali, as well as raw lignin. In contrast, lignin extracted by DES2 from pinecones, olive pomace, and Posidonia leaves showed higher PDI. The lower PDI of the lignin extracted from aegagropile indicates that its molecular weight is more homogenous than that of the lignins extracted from the other biomasses.

The chemical functional groups present in extracted lignin were investigated by FTIR spectroscopy (**Figure 7A–F**). The strong, broad band between 3,441 and 3,198 cm^{-1} was attributed to OH stretching vibrations of the alcoholic and phenolic hydroxyl groups involved in hydrogen bonding. The signals in the wavenumber range 2,963–2,852 cm^{-1} were assigned to alkyl C-H stretching vibrations. Olive pomace, alfa leaves, and almond shells showed more intense bands than the other biomasses. No bands were observed from 2,800 to 1,800 cm^{-1}. The lignin obtained by DES1 and DES2 treatment, had a rising band at 1,708 cm^{-1}, which indicated the presence of unconjugated C=O groups. However, this signal was absent in the solvent spectra (**Figure 8B**). Lactic and oxalic acid spectra were characterized by a band in the 1,737–1,723 cm^{-1} range, which indicated the presence of unconjugated C=O groups, whereas the urea spectrum was characterized by two signals in the wavenumber range of 1,660 cm^{-1} and 1,604 cm^{-1} attributed to amide groups. The bands at 1,606–1,618 cm^{-1}were observed in lignin extracted by DES1 and DES2 treatment, associated with ring-conjugated C=C stretch.

The signal at 1,640 cm^{-1} in lignin extracted by DES3 indicated the presence of C=O stretching vibration in conjugated carbonyl groups of lignin. The signal at 1516 cm^{-1} arose from the vibrations of the aromatic rings present in lignin, while the band at 1200 cm^{-1} indicated the presence of ether groups. Bands in the wavenumber range of 1,250–1,200 cm^{-1} were assigned to C-O stretching of nonaromatic alcohols. The band at 953 cm^{-1} was assigned to methyl substituents. The results indicate that DES-lignin fractions spectra showed signals at 1,730–1,702 cm^{-1} and 1,643–1,635 cm^{-1}, assigned to the stretching vibration of unconjugated and conjugated carbonyl groups, respectively. However, these band ranges were absent in three commercial lignins: raw, soda-processed, and alkali-extracted lignins (**Figure 8A**). This observation indicates that during its extraction and solubilization, some functional groups of lignin were conjugated with oxalic and lactic acid.

Figure 1: Mediterranean biomasses studied. (**A**) Almond shells, (**B**) Olive pomace, (**C**) Cone pines, (**D**) Aegagropile (Posidonia balls), (**E**) Posidonia leaves, (**F**) Alfa leaves.

Figure 2: Lignin yield. (**A**) Choline chloride + Oxalic acid (DES1), (**B**) Choline chloride + Lactic acid (DES2), (**C**) Choline chloride + Urea (DES3). Significant differences were determined with one-way ANOVA and Fisher's post-hoc test (*P < 0.05; **P < 0.01; ***P < 0.001). Abbreviations: A = Alfa leaves, AS = Almond shells, PC = Pinecones, PL = Posidonia leaves, OP = Olive pomace, Ag = Aegagropile; ns = not significant.

Figure 3: Lignin (%). (**A**) Choline chloride + Oxalic acid (DES1), (**B**) Choline chloride + Lactic acid (DES2), (**C**) Choline chloride + Urea (DES3). Significant differences were determined with one-way ANOVA and Fisher's post hoc test (*P < 0.05; **P < 0.01; ***P < 0.001). Abbreviations: A = Alfa leaves, AS = Almond shells, PC = Pinecones, PL = Posidonia leaves, OP = Olive pomace, Ag = Aegagropile; ns = not significant.

Figure 4: Principal component analysis of yield and purity of lignin extracted from Mediterranean biomasses. Hydrogen-bond acceptor (HBA) is choline chloride (ChCl) and hydrogen-bond donors (HBD) are Ox = oxalic acid, Lac : lactic acid, and Urea. PCA = principal component analysis; A = Alfa leaves, AS = Almond shells, PC = Pinecones, PL = Posidonia leaves, OP = Olive pomace, Ag = Aegagropile.

Figure 5: Carbohydrate (%), nitrogen (%), and ash content (%) in lignin samples. (**A**) Choline chloride + Oxalic acid (DES1), (**B**) Choline chloride + Lactic acid (DES2), (**C**) Choline chloride + Urea (DES3). Significant differences were determined with one-way ANOVA and Fisher's post-hoc test (*P < 0.05; **P < 0.01; ***P < 0.001). Abbreviations: A = Alfa leaves, AS = Almond shells, PC = Pinecones, PL = Posidonia leaves, OP = Olive pomace, Ag = Aegagropile; ns = not significant.

Figure 6: Identification of monosaccharides in lignin samples (%).(**A**) Choline chloride + Oxalic acid (DES1), (**B**) Choline chloride + Lactic acid (DES2), (**C**) Choline chloride + Urea (DES3). Significant differences were determined with one-way ANOVA and Fisher's post-hoc test (*P < 0.05; **P < 0.01; ***P < 0.001). Abbreviations: A = Alfa leaves, AS = Almond shells, PC = Pinecones, PL = Posidonia leaves, OP = Olive pomace, Ag = Aegagropile; ns = not significant.

Figure 7: Fourier-transform infrared spectra of lignin samples. (**A**) Alfa leaves, (**B**) Almond shells, (**C**) Pinecones, (**D**) Posidonia leaves, (**E**) Olive pomace, (**F**) Aegagropile. Abbreviations: DES1 = Choline chloride + Oxalic acid, DES2 = Choline chloride + Lactic acid, DES3 = Choline chloride + Urea.

Figure 8: Fourier-transform infrared spectra. (**A**) Lignin controls, (**B**) hydrogen bond donors.

Table 1: Molecular weights of the lignins. Abbreviations: A = Alfa leaves, AS = Almond shells, PC = Pinecones, PL = Posidonia leaves, OP = Olive pomace, Ag = Aegagropile; Mn = number-average molecular weight; Mw = weight-averaged molecular weight; PDI = polydispersity index; Ox =oxalic acid; Lac = lactic acid.

Figure S1: Lignin.

Figure S2: Samples after being autoclaved (30 mg of lignin + 1 mL of 72% sulfuric acid + 28 mL of distilled water).

Figure S3: Lignin pellets.

Figure S4: Solid residue washed four times to recover maximum lignin content.

Figure S5: Gel permeation chromatograms of lignin controls, raw and alkali-extracted lignins.

Figure S6: Gel permeation chromatograms of lignin samples. Abbreviations: A = Alfa leaves, AS = Almond shells, PC = Pinecones, PL = Posidonia leaves, OP = Olive pomace, Ag = Aegagropile; DES1 = Choline chloride + Oxalic acid, DES2 = Choline chloride + Lactic acid, DES3 = Choline chloride + Urea.

Figure S7: Flowsheet of the deep eutectic solvent (DES)–microwave process for lignin extraction.

DISCUSSION:

This study had many objectives; the first of which was to prepare and use low-cost green solvents with the characteristics of both ionic liquids and organic solvents. The second objective was to fractionate the biomass and extract lignin in a single step, without requiring preliminary steps such as the extraction of extractables using Soxhlet or hemicellulose using alkaline solvents, basic, or thermophysical techniques. The third aim was to recover lignin by simple filtration after the treatment, without adjustment of pH, but simply by adding distilled water. The results of the ultrafast extraction of lignin from six different sources using the microwave-assisted, DES-based process using three different DESs indicate that the extraction yield can vary depending on the biomass and nature of the DES. For example, the highest yield of lignin extraction among all three DESs was from olive pomace. This was followed by the yields from alfa leaves, pinecones, and almond shells. The extraction yields were lower for the leaves and balls of *Posidonia oceanica*.

The purity of lignin was evaluated using the Klason, Kjeldahl (nitrogen), carbohydrate (GC), and ash methods. As depicted in **Figure 3** and **Figure 5A–C**, the purity of lignin decreased owing to the co-precipitation of nitrogen, carbohydrate, and ash components with lignin. The conditions for lignin extraction with DES1 ensured high purity, but a low yield, indicating that process improvements are necessary for the positive correlation between the yield and purity of lignin. The lignin yield can be improved if the duration of the treatment is longer, the microwave power is increased from 800 W to 1200 W, or the ratio of solid:solvent (1:10) is reduced. Lignin molecular weight data provide an insight into the dissociation or repolymerization of lignin fragments after treatment. An increase in the Mw of lignin for the biomasses was observed after the extraction using microwave–DES, as is evident, for example, in the case of Posidonia leaves (the Mw is 50093 for DES3 and is 70726 for DES1), which demonstrates that depolymerization occurred during the extraction of lignin and was followed by a rapid repolymerization of the carbon–carbon interunit under the action of the DES. This requires the use of a capturing agent, such as formaldehyde, to stabilize the deployment.

In DES pretreatment, lignin dissociation and condensation are the two competing reactions. The PDI of the extracted lignins is lower than that of beech lignin extracted by organic solvents (ethanol/water/H_2SO_4) reported in the literature[17]. This indicates that DES treatment improves molecular weight homogeneity in lignin compared to treatment with organic solvents. The FTIR spectra indicate that lignin functional groups are influenced by the DES solvent used. Spectra show signals at 1,730–1,702 cm^{-1} assigned to the stretching vibration of unconjugated carbonyl groups, whereas peaks at 1,643–1,635 cm^{-1} indicate the stretching vibration of conjugated carbonyl groups. These results demonstrate the possibility of extracting value-added lignin of high purity from Mediterranean biomasses (which is presently undervalued and used either as feed or as soil amendment) and can help determine the optimal DES solvent while ensuring the purity of lignin. For example, DES1 demonstrated the purest extraction of lignin, though with a lower yield than that observed using the other two DESs.

The proposed method can be applied easily because of the inexpensive and green ChCl–oxalic acid deep eutectic solvent system. Choline chloride is an organic salt and oxalic acid is available as a natural product of plants, which are abundant with low cost. This technique (an ultrafast protocol, which in one step provides biomass fractionation and high-purity lignin recovery) is applicable to any type of lignocellulosic biomass that has a chemical composition similar to that studied here on the laboratory scale using the microwave–DES process or at the pilot scale using the DES–ultrasound process or by convectional heating.

ACKNOWLEDGMENTS:

MK and TB thanks Haitham Ayeb for statistical analyses and figure preparation, Walloon Region (European Regional Development-VERDIR) and Minister of Higher Education and Scientific Research (Taoufik Bettaieb) for funding.

DISCLOSURES:

The authors report no conflict of interest.

REFERENCES:

1. Kammoun, M. et al. Hydrothermal dehydration of monosaccharides promoted by seawater : fundamentals on the catalytic role of inorganic salts. *Frontiers in Chemistry.* **7**, 132 (2019).
2. Kammoun, M., Ayeb, H., Bettaieb, T., Richel, A. Chemical characterisation and technical assessment of agri-food residues, marine matrices, and wild grasses in the South Mediterranean area: A considerable inflow for biorefineries. *Waste Management.* **118**, 247–257 (2020).
3. Zhang, C. W., Xia, S.Q., Ma, P. Facile pretreatment of lignocellulosic biomass using deep eutectic solvents. *Bioresource Technology.* **219**, 1–5 (2016).
4. Mora-Pale, M., Meli, L., Doherty, T. V., Linhardt, R. J., Dordick, J. S. Room temperature ionic liquids as emerging solvents for the pretreatment of lignocellulosic biomass. *Biotechnology and Bioengineering*. **108** (6), 1229–1245 (2011).
5. Chen, Z., Wan, C. Ultrafast fractionation of lignocellulosic biomass by microwave-assisted deep eutectic solvent pretreatment. *Bioresource Technologie.* **250**, 532–537 (2018).
6. Francisco, M., Van Den Bruinhorst, A., Kroon, M. C. New natural and renewable low transition temperature mixtures (LTTMs): screening as solvents for lignocellulosic biomass processing. *Green Chemistry*. **14** (8), 2153–2157 (2012).
7. Liu, Y. C. et al. Efficient cleavage of lignin – carbohydrate complexes and ultrafast extraction of lignin oligomers from wood biomass by microwave-assisted treatment with deep eutectic solvent. *Chem sus chem.* **10**, 1692–1700 (2017).

8. Xu, G. C., Ding, J. C., Han, R. Z., Dong, J. J. Ni, Y. Enhancing cellulose accessibility of corn stover by deep eutectic solvent pretreatment for butanol fermentation. *Bioresource Technologie.* **203**, 364–369 (2016).

9. Jablonský, M., Andrea, Š., Kamenská, L., Vrška, M., Šima, J. Deep eutectic solvents : fractionation of wheat straw deep eutectic solvents : fractionation of wheat straw. *Bioresources.* **10** (4), 8039–8047 (2015).

10. Shen, X. J. et al. Facile fractionation of lignocelluloses by biomass-derived deep eutectic solvent (DES) pretreatment for cellulose enzymatic hydrolysis and lignin valorization. *Green Chemistry.* **21**, 275–283 (2019).

11. Alvarez-Vasco, C. et al. Unique low-molecular-weight lignin with high purity extracted from wood by deep eutectic solvents (DES): a source of lignin for valorization. *Green Chemistry.* **18**, 5133–5141 (2016).

12. Banu, J. R. et al. A review on biopolymer production via lignin valorization. *Bioresource Technologie.* **290**, 121790 (2019).

13. Gordobil, O., Olaizola, P., Banales, J. M., Labidi, J. Lignins from agroindustrial by-products as natural ingredients for cosmetics : chemical structure and in vitro sunscreen and cytotoxic activities. *Molecules.* **25** (5), 1131 (2020).

14. Lee, C. S., Thu Tran, T. M., Weon Choi, J., Won, K. Lignin for white natural sunscreens. *International Journal of Biological Macromolecules.* **122**, 549–554 (2019).

15. Widsten, P. Lignin-based sunscreens—state-of-the-art, prospects and challenges. *Cosmetics.* **7**, 85 (2020).

16. Qian, Y., Qiu, X., Zhu, S. Lignin: a nature-inspired sun blocker for broad-spectrum sunscreens. *Royal Society of Chemistry*. **17**, 320–324 (2015).

17. Zijlstra, D. S. et al. Extraction of lignin with high β-O-4 content by mild ethanol extraction and its effect on the depolymerization yield. *Journal of Visualized Experiments.* **143**, 58575 (2019).

Figure 1

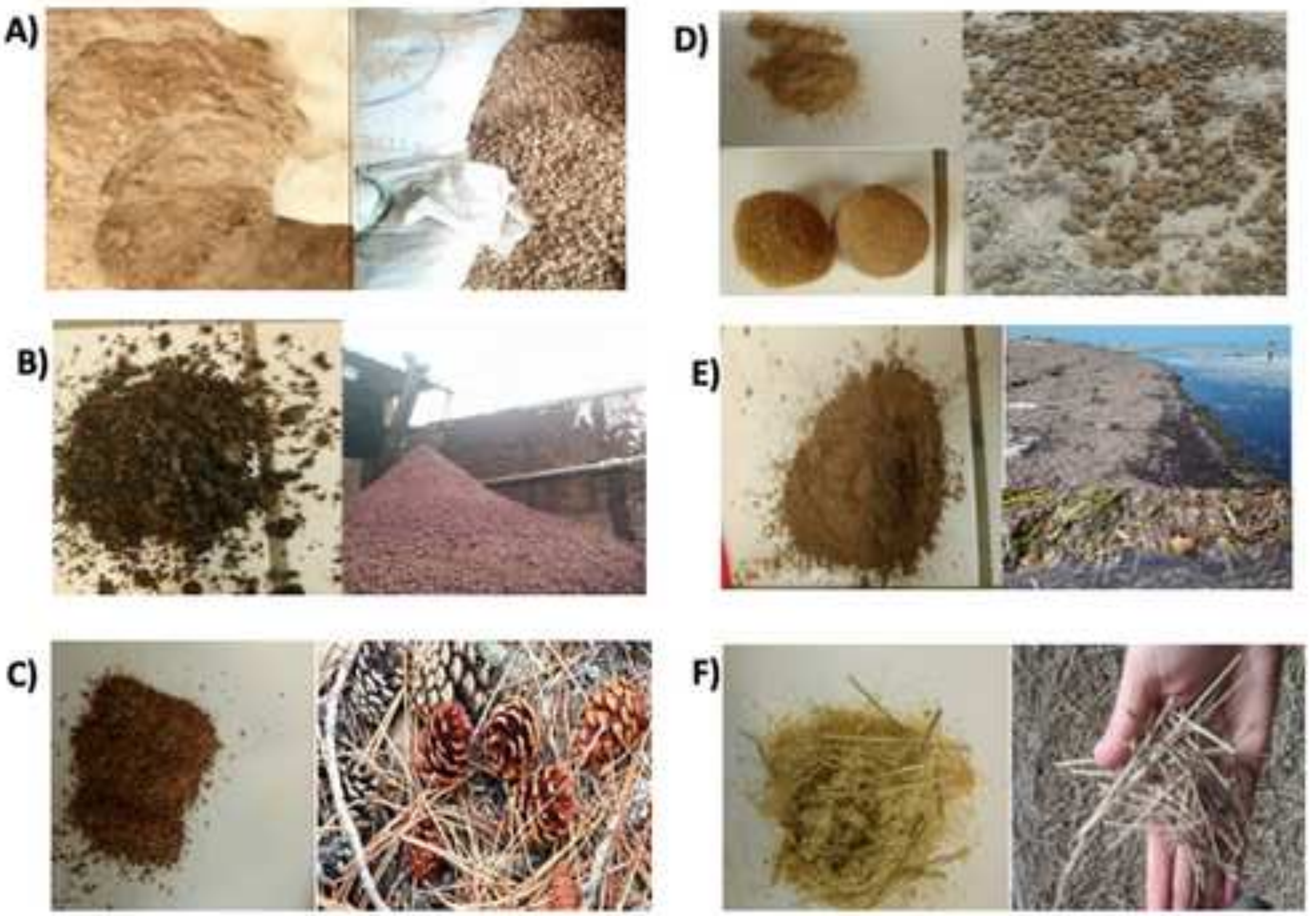


Figure 2

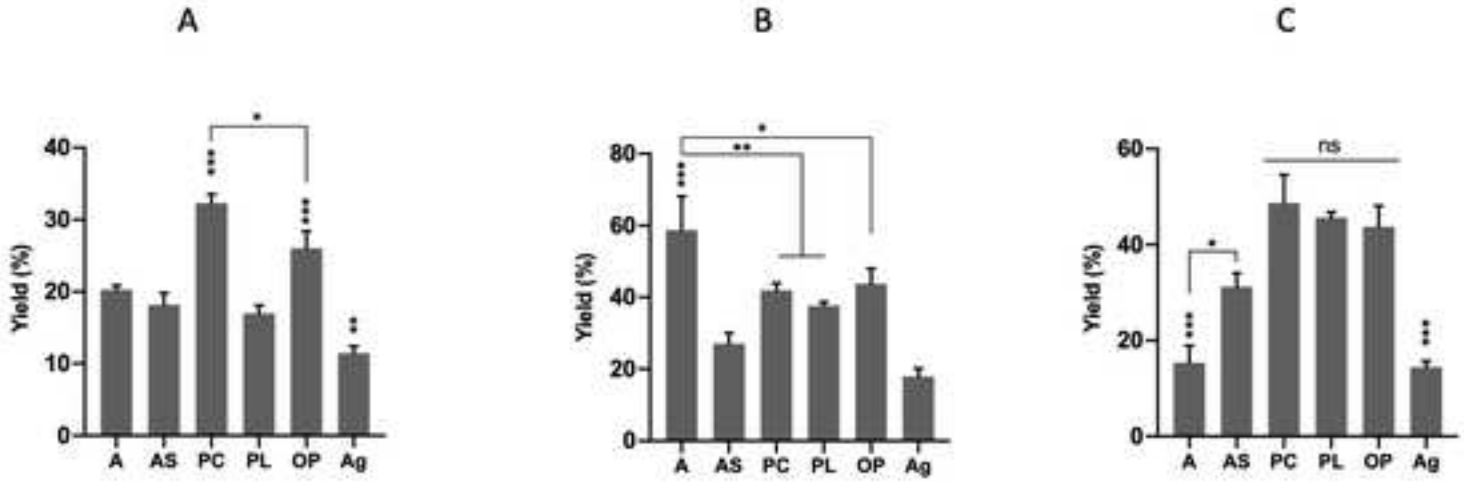
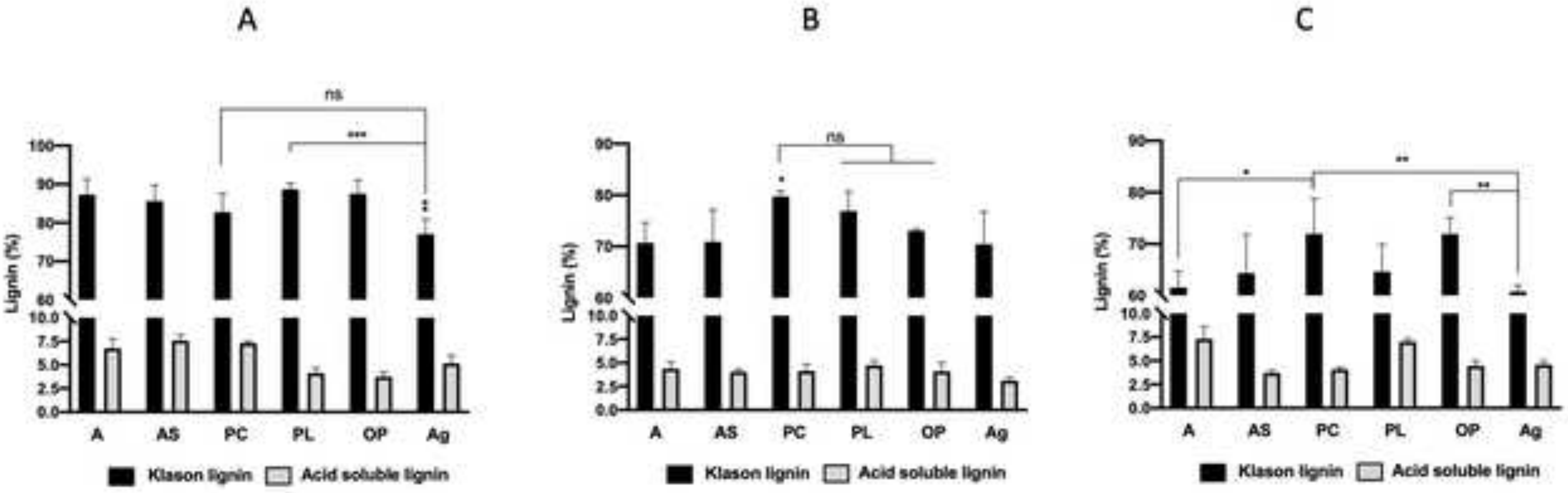
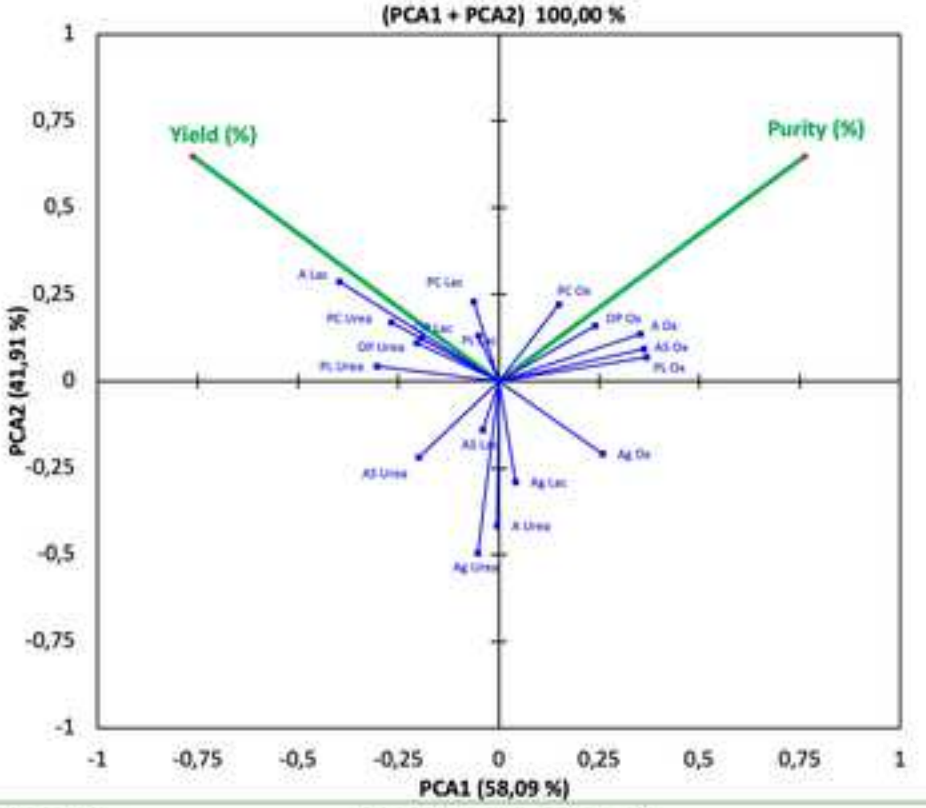
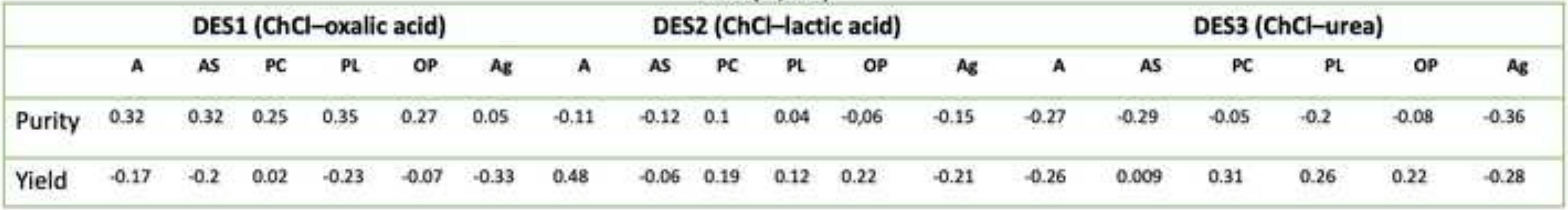

A
Yield (%)
0
10
20
30
40
A
AS
PC
PL
OP
Ag
*
***
***
**
B
Yield (%)
0
20
40
60
80
A
AS
PC
PL
OP
Ag
*
**
***
C
Yield (%)
0
20
40
60
A
AS
PC
PL
OP
Ag
ns
*
***
***

Figure 3

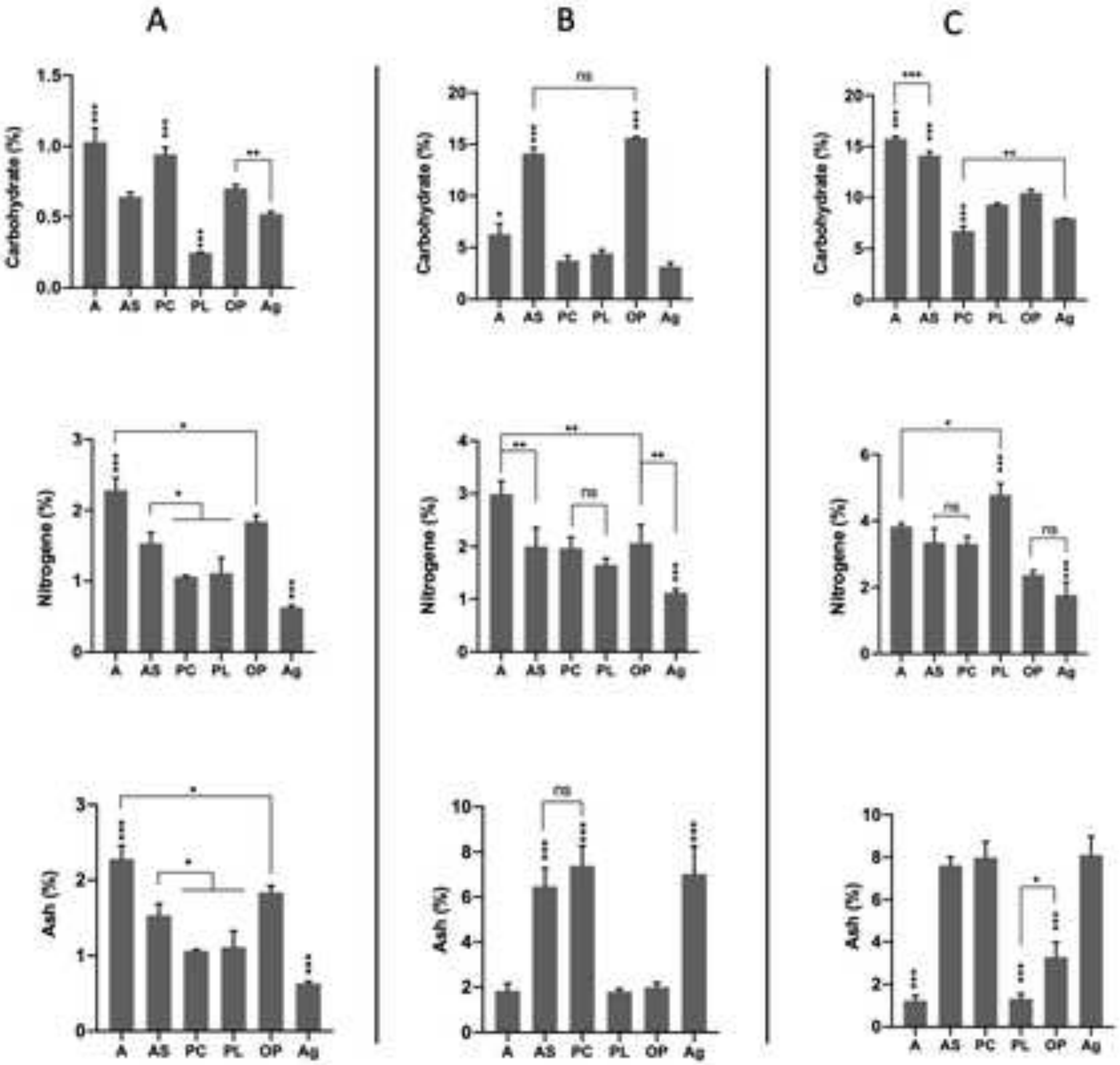

A
B
C
Lignin (%)
100
90
80
70
60
10.0
7.5
5.0
2.5
0.0
ns
***
**
*
A
AS
PC
PL
OP
Ag
■ Klason lignin
☐ Acid soluble lignin





	DES1 (ChCl–oxalic acid)						DES2 (ChCl–lactic acid)						DES3 (ChCl–urea)					
	A	AS	PC	PL	OP	Ag	A	AS	PC	PL	OP	Ag	A	AS	PC	PL	OP	Ag
Purity	0.32	0.32	0.25	0.35	0.27	0.05	-0.11	-0.12	0.1	0.04	-0,06	-0.15	-0.27	-0.29	-0.05	-0.2	-0.08	-0.36
Yield	-0.17	-0.2	0.02	-0.23	-0.07	-0.33	0.48	-0.06	0.19	0.12	0.22	-0.21	-0.26	0.009	0.31	0.26	0.22	-0.28

Figure 5


A
B
C
Carbohydrate (%)
Nitrogene (%)
Ash (%)
A
AS
PC
PL
OP
Ag
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0.5
1.0
1.5
0
5
10
15
20
0
1
2
3
4
6
8
10
ns
*
**
***

Figure 6

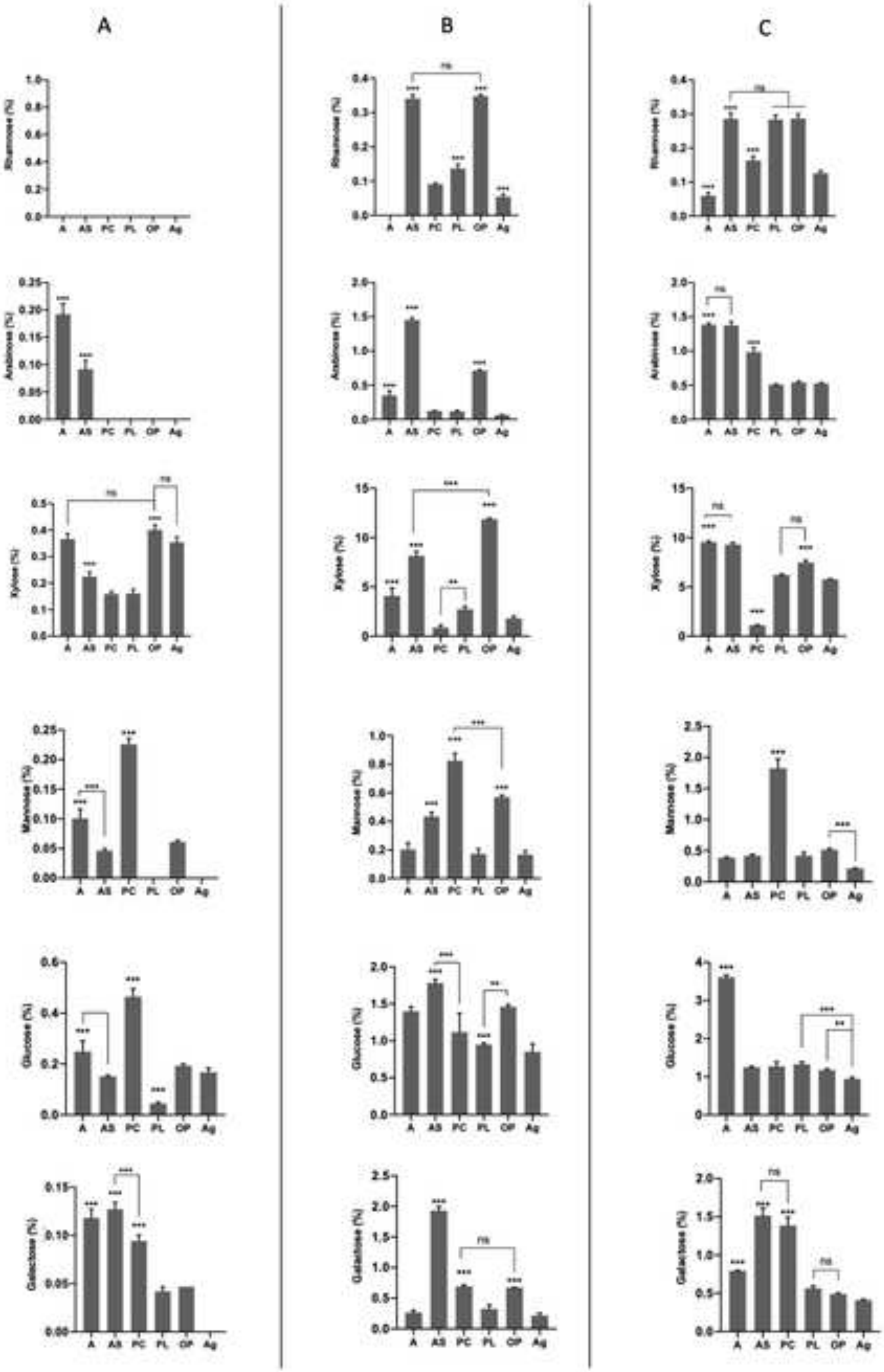
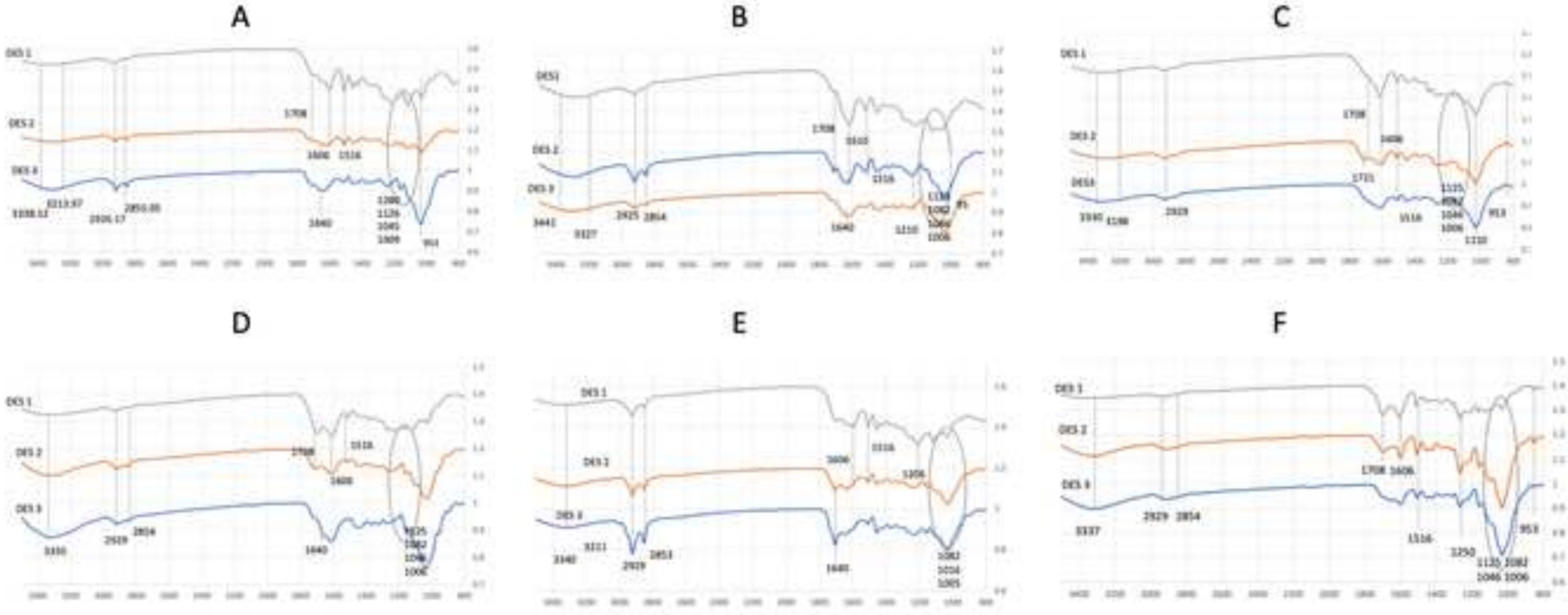

A
B
C
Rhamnose (%)
Arabinose (%)
Xylose (%)
Mannose (%)
Glucose (%)
Galactose (%)
A
AS
PC
PL
OP
Ag
ns
***
**

Figure 7


A
DES 1
DES 2
DES 3
1708
1606
1516
3338.12
3213.97
2926.17
2850.00
1640
1200
1126
1041
1009
951
B
DES1
DES 2
DES 3
1708
1610
1516
3441
3327
2925
2854
1640
1210
1130
1082
1060
1006
C
DES 1
DES 2
DES3
1708
1606
1721
3340
3198
2929
1516
1125
1046
1006
1110
913
D
DES 1
DES 2
DES 3
1708
1516
1606
3330
2929
2854
1640
1125
1082
1046
1006
E
DES 1
DES 2
DES 3
1606
1516
1206
3340
3211
2929
2853
1640
1082
1016
1005
F
DES 1
DES 2
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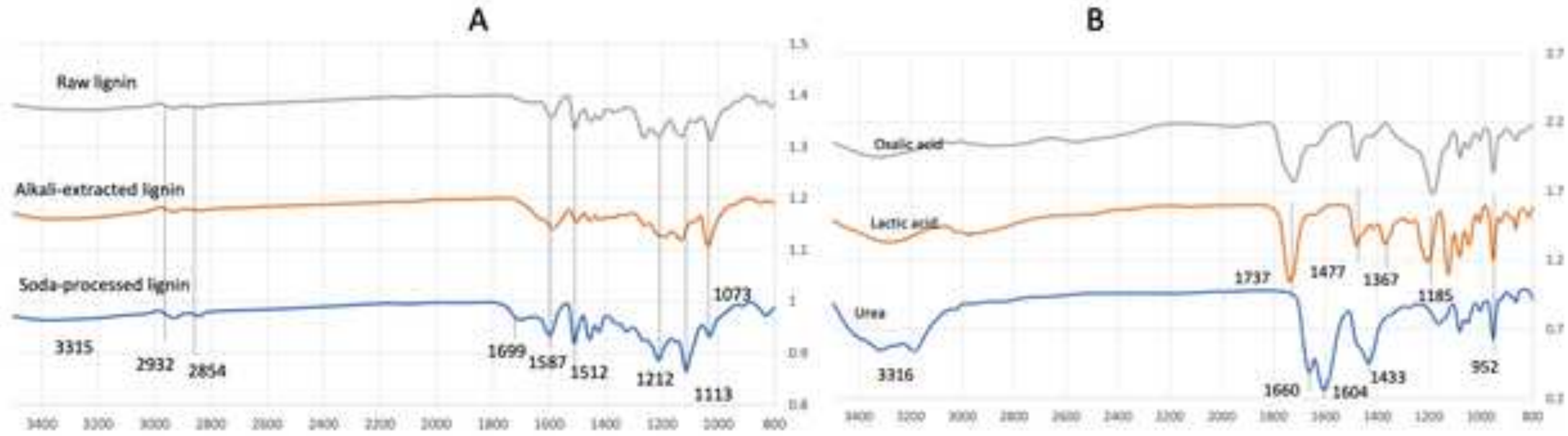
1082
1046
1006
953

Figure 8


A
Raw lignin
Alkali-extracted lignin
Soda-processed lignin
3315
2932
2854
1699
1587
1512
1212
1113
1073
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
B
Oxalic acid
Lactic acid
Urea
3316
1737
1477
1367
1185
1660
1604
1433
952
2.7
2.2
1.7
1.2
0.7
0.2
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800

Sample	Treatment	Mn	Mw	PDI
A	Urea	47558	120141	2.5
	Lac	35241	73665	2.1
	Ox	35793	84312	2.4
AS	Urea	50181	105817	2.1
	Lac	60409	104915	1.7
	Ox	83112	147233	1.8
PC	Urea	34013	65181	1.9
	Lac	55513	145963	2.6
	Ox	46409	102298	2.2
PL	Urea	25696	50093	1.9
	Lac	45530	122900	2.7
	Ox	28427	70726	2.5
OP	Urea	29669	70424	2.4
	Lac	26735	66743	2.5
	Ox	34161	75509	2.2
Ag	Urea	30184	48123	1.6
	Lac	33835	52123	1.5
	Ox	30025	49808	1.7
Control	Raw lignin	23275.3	36496.5	1.6
	Alkali-extracted lignin	22792.6	43014.3	1.9

Name of Material/ Equipment	Company	Catalog Number
HPLC Gel Permeation Chromatography	Agilent 1200 series	
1 methylimadazole	Acros organics	
2-deoxy-D-glucose (internal standard)	Sigma Aldrich (St. Louis, USA)	
Acetic acid	Sigma Aldrich (St. Louis, USA)	
Acetic anhydride	Sigma Aldrich (St. Louis, USA)	
Adjustables pipettors		
Alkali		
Arabinose (99%)	Sigma Aldrich (St. Louis, USA)	
Autoclave	CERTO CLAV (Model CV-22-VAC-Pro)	
Water Bath at 70 °C		
Boric acid	Sigma Aldrich (St. Louis, USA)	
Bromocresol	Sigma Aldrich (St. Louis, USA)	
Catalyst	CTQ (coded A22) (1.5 g K_2SO_4 + 0.045 g $CuSO_4.5\ H_2O$ + 0.045 g TiO_2)	Merck
Centrifugation container		
Centrifuge	BECKMAN COULTER	Avanti J-E centrifuge
Ceramic crucibles		
Choline chloride 99%	Acros organics	
Column	Agilent PLGel Mixed C (alpha 3,000 (4.6 × 250 mm, 5 µm) preceded by a guard column (TSK gel alpha guard column 4.6 mm × 50 mm, 5 µm)	
Column	HP1-methylsisoxane (30 m 0.32 mm, 0.25 mm)	
Crucible porosity N°4 (Filtering crucible)	Shott Duran Germany	boro 3.3
Deonized water		
Dessicator		
Dimethylformamide	VWR BDH Chemicals	
Dimethylsulfoxide	Acros organics	
Erlenmeyer flask		
Ethanol	Merck (Darmstadtt, Germany)	

Filtering crucibles, procelain		
Filtration flasks		
Fourrier Transformed Inra- Red	Vertex 70 Bruker apparatus equipped with an attenuated total reflectance (ATR) module. Spectra were recorded in the 4,000–400 cm^{-1} range with 32 scans at a resolution of 4.0 cm^{-1}	
Galactose (98%	Sigma Aldrich (St. Louis, USA)	
Gaz Chromatography	Agilent (7890 series)	
Glass bottle 100 mL		
Glass tubes (borosilicate) with teflon caps 10 mL		
Glucose (98%	Sigma Aldrich (St. Louis, USA)	
Golves		
Graduated cylinder 50 mL /100 mL		
H_2SO_4 Titrisol (0.1 N)	Merck (Darmstadtt, Germany)	
H_2SO_4 (95-98%)	Sigma Aldrich (St. Louis, USA)	BUCHI R-114)
Hummer cutter equiped with 1 mm and 0.5 mm sieve	Mill Ttecator (Sweden)	Cyclotec 1093
Indulin		
Kjeldahl distiller	Kjeltec 2300 (Foss)	
Kjeldahl tube	FOSS	
Kjeldhal rack		
Kjeldhal digester	Kjeltec 2300 (Foss)	
Kjeldhal suction system		
Lab Chem station Software		
Lactic acid	Merck (Darmstadtt, Germany)	
Lithium chloride LiCl	Sigma Aldrich (St. Louis, USA)	
Mannose (98%)	Sigma Aldrich (St. Louis, USA)	
Methyl red		

Microwave	START SYNTH MILESTONE Microwave laboratory system	
Microwave temperature probe		
Microwave container		
Muffle Furnace		
NaOH	Merck (Darmstadtt, Germany)	
Nitrogen free- paper		
Opus		
Oven	GmbH Memmert SNB100	Memmert SNB100
Oxalic acid	VWR BDH Chemicals	
P 1000		
pH paper		
precision balance		
Infrared spectroscopy		
Quatz cuvette		
Rhamnose (98%)	Sigma Aldrich (St. Louis, USA)	
Rotary vacuum evaporator	Bucher	
Round-bottom flask 500 mL		
sodium borohydride $NaBH_4$		
Schott bottle		
Sovirel tubes	sovirel	
Spatule		
Special tube		
Spectophotometer	UV-1800 Shimadzu	
Sterilization indicator tape		
Stir bar in teflon		
Stirring plate		
Syringes		
Sodium borohydride	Sigma Aldrich (St. Louis, USA)	
Titrisol	Merck	Merck 109984
Urea	VWR BDH Chemicals	
Vials		

VolumetriC flask 2.5 L /5 L	Bucher
Vortex	
Xylose (98%)	Sigma Aldrich (St. Louis, USA)

Comments

alkali-extracted lignin

Raw lignin control

GC data analysis

spectroscopy software

Soda-processed lignin

glass bottle
Borosilicate glass tubes

0.1 N H_2SO_4

Gembloux, 19th January 2021

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Journal of Visualized Experiments

Dear Editor,

We are pleased to submit the revised copy of the manuscript ‘Ultrafast lignin extraction from unusual Mediterranean lignocellulosic residues’ by M. Kammoun, T.Berchem and A.Richel. We thank you very much for the corrections and comments made by the editors. We have made all the corrections requested.

In this study, we reported an ultrafast approach for lignin-carbohydrate cleavage, assisted by Deep Eutectic Solvent-Microwave combined pretreatment of Mediterranean biomasses, using Three different solvents on six different feedstocks.

The objectives of this method were to test low-cost green solvent, to evaluate the effect of this combined pretreatment on feedstock fractionation, to investigate its influence on lignin purity and yield, and to study its effect on the molecular weights and chemical functional groups in the extracted lignin.

Results reported that the process deep eutectic solvent-Microwave is efficient to extract lignin with high purity (>90°) in 1 min.

Hoping you will consider our work with interest.

Kind regards,

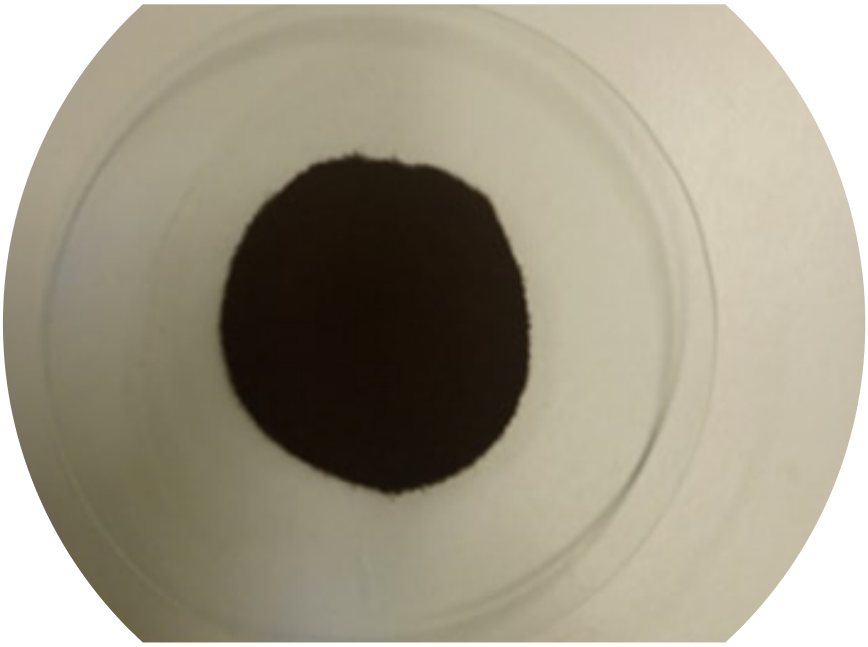

Figure S1: Lignin.

Figure S2: Samples after being autoclaved
(30 mg of lignin + 1 mL of 72% sulfuric acid + 28 mL of distilled water).

Figure S3: Lignin pellets.

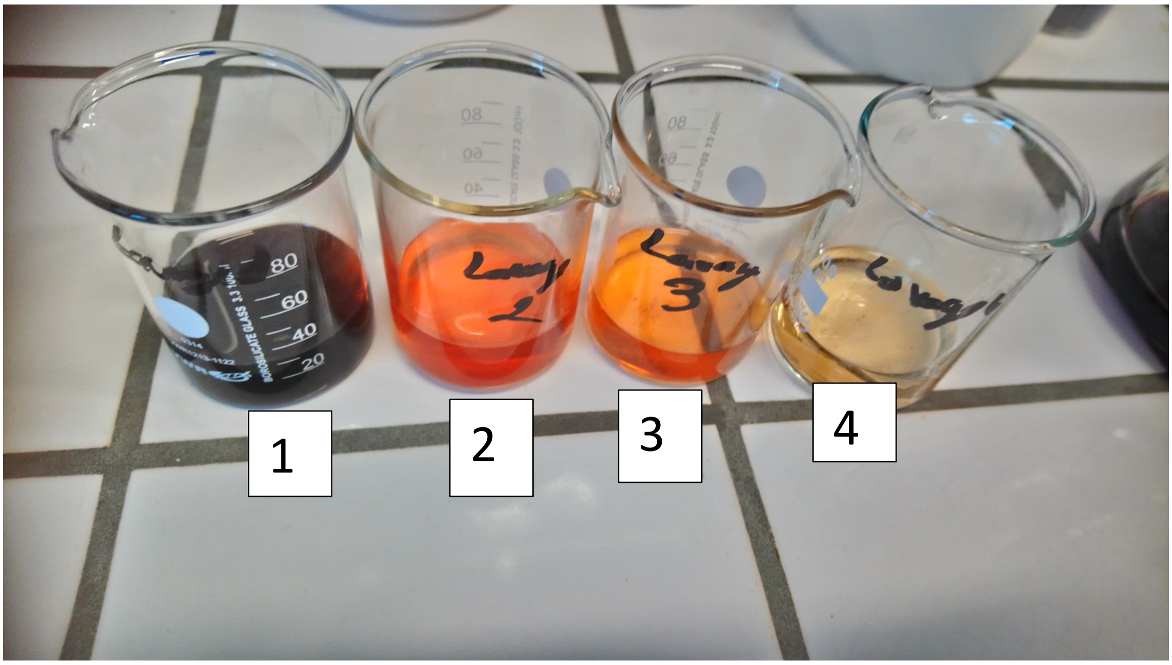

Figure S4: Solid residue washed four times to recover maximum lignin content.

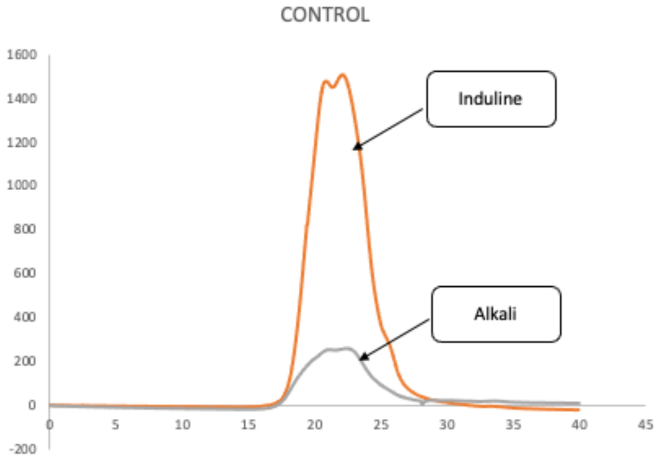

Figure S5: Gel permeation chromatograms of lignin controls, raw and alkali-extracted lignins.

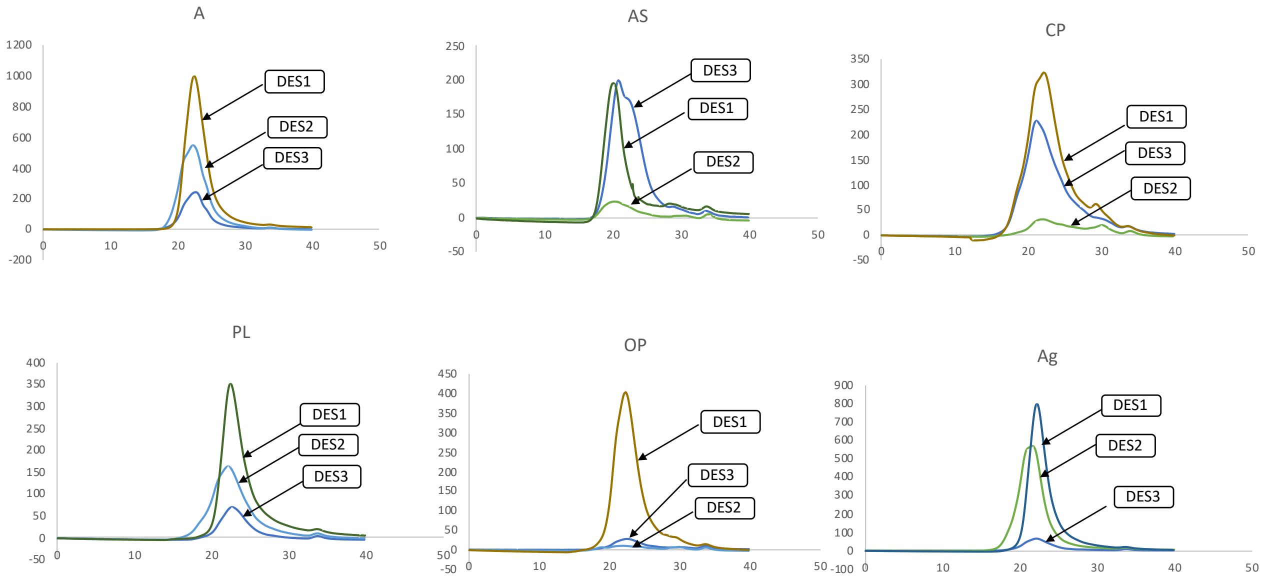

Figure S6: Gel permeation chromatograms of lignin samples.

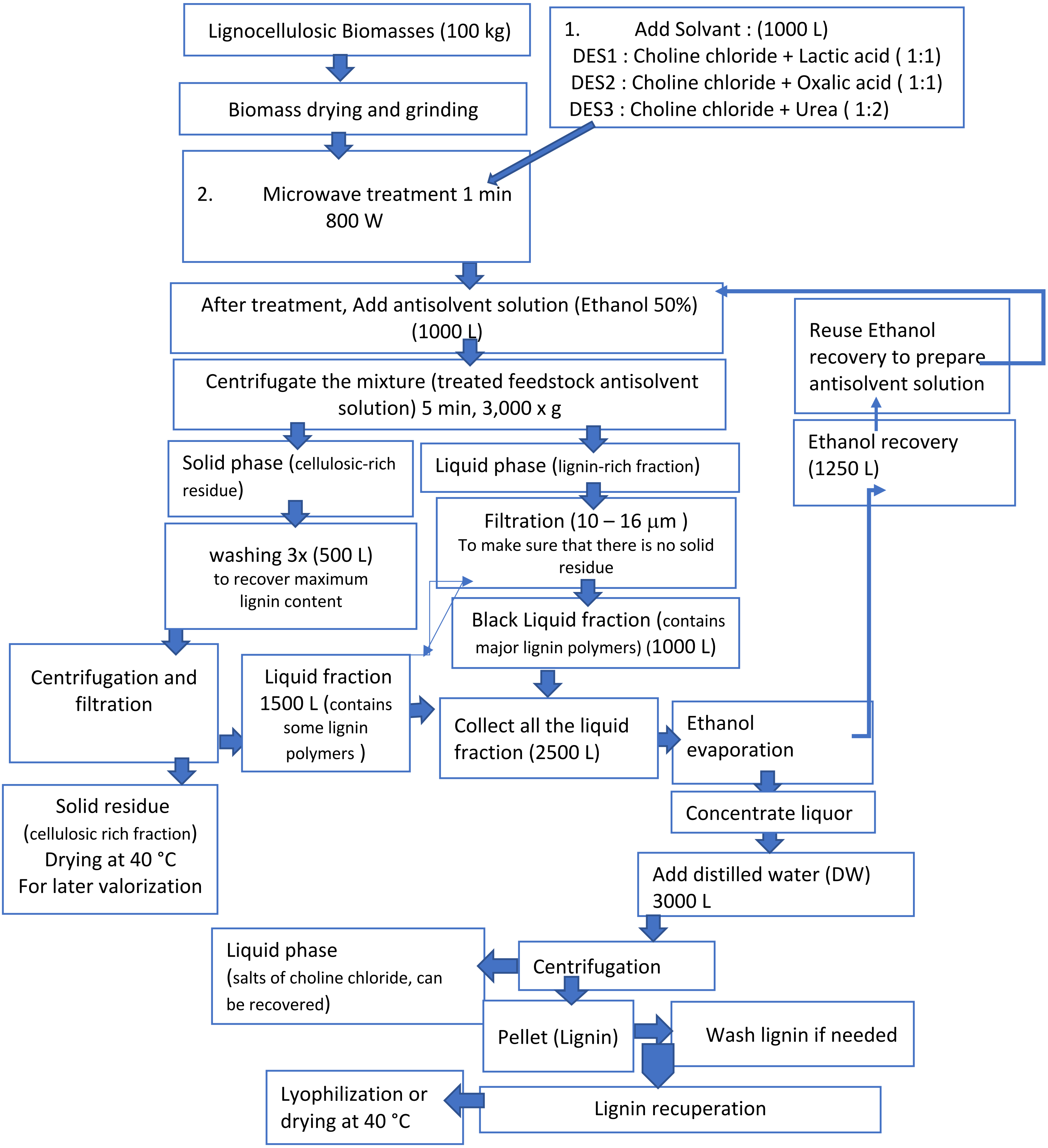

Figure S7 : Flowsheet of the deep eutectic solvent (DES)-microwave process for lignin extraction.