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A tunable deep eutectic solvent-based processing for valorization of chestnut wood fiber as a source of ellagic acid and lignin

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ABSTRACT

Three different deep eutectic solvent (DES)-based extraction procedures were rationally designed and optimized for the recovery of antioxidants from chestnut wood fiber (CWF), a clean and largely available solid waste of the tannin industry. First, a mild protocol was developed using a choline chloride (ChCl)/tartaric acid DES at 50 °C, for 90 min. Ellagic acid (EA) was identified as the only low molecular weight phenolic component of the extract. In other experiments, harsher conditions were explored involving treatment of CWF with ChCl-based DESs at 120 °C for 8 h, which afforded a solid sample characterized by high phenolic content (up to 1.0 mg of gallic acid equivalents/mg of sample) and antioxidant properties (EC_{50} <0.025 mg/mL in the 2,2-diphenyl-1-picrylhydrazyl assay), and containing guaiacyl-syringyl lignin along with EA. Based on these results, a sequential two-step DES-based treatment of CWF was eventually designed, allowing to selectively obtain both an EA-enriched and an EA-free, lignin-enriched sample, with an overall 50% w/w of the starting CWF dissolved. In particular, a 2.3% w/w yield of EA was achieved, which is significantly higher than those reported in the case of DES-based processing of other agricultural wastes. The proposed tunable, straightforward, and eco-friendly approach may allow to fully exploit CWF as a green, cheap, and easily accessible source of high-value products.

1. Introduction

Agri-food industry is responsible for the generation of up to 140 billion tons *per* year of organic wastes/by-products [1–4] which can be considered as a largely available, low-cost source of value-added compounds such as polyphenols, including mainly flavonoids and phenolic acids [5–11]. These latter are well-known for their health beneficial effects [6,12], ascribed in part to their efficient antioxidant properties [13] that have also recently prompted their use as additives for implementation of functional materials. This applies in particular to phenolic polymers, mainly tannins and lignin, which have been exploited in a variety of sectors [14]. As an example, tannins are largely used to implement antioxidant functional coatings [15–17] or as antimicrobial agents for the control of bacteria growth in food packaging [18], whereas lignin has been widely exploited as a green additive to improve the mechanical and functional properties of polymeric matrices for tissue engineering [19], drug delivery [20], diagnostics [21], food packaging [22], and environmental applications [23].

Among the agri-food industry by-products, wood wastes represent one of the cheapest and most abundant natural sources of lignin. Recently, it has been estimated that 50 million cubic meters of wood wastes are generated each year in the European Union [24,25]. These include also wastes from tannin industry such as exhausted woods, that is the residual biomasses from hot water extraction of wood tannins [26,27]. In particular, chestnut wood fiber (CWF), deriving from exhausted chestnut wood dried under a hot air flux in a continuous bed dryer and subsequently ground in an industrial knife mill, has been recently described as an easily available material with good antioxidant

Abbreviations: CCD, central composite design; ChCl, choline chloride; ChCl:LA2, 1:2 mol/mol ChCl/lactic acid; ChCl:LA9, 1:9 mol/mol ChCl/lactic acid; ChCl:TA2, 2:1 mol/mol ChCl/tartaric acid; CT, chestnut tannins; CWF, chestnut wood fiber; DES, deep eutectic solvent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EA, ellagic acid; EPR, electron paramagnetic resonance; eqs, equivalents; FRAP, ferric reducing/antioxidant power; HBA, hydrogen bond acceptors; HBD, hydrogen bond donors; HTS, harsh treatment sample; MTS, mild treatment sample; TPC, total phenolic content.

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properties in several chemical assays and able to efficiently adsorb pollutants such as toxic gases, organic dyes and heavy metals [27]. The main phenolic components of CWF are residual, non-extracted, hydrolyzable ellagitannins [27], lignin, and low molecular weight phenolic compounds, mainly ellagic acid (EA) [28], which is well known for its potent anticancer, anti-inflammatory and antimicrobial properties [29]. The possibility to practically exploit CWF as a source of high-value, antioxidant phenolic compounds has however still remained unexplored, despite the many advantages offered by this material including the constant composition, the continuous production along the year without seasonal influences, the large availability (>10000 tons *per* year), and the cleanness of the manufacturing process.

In this scenario, and in a green chemistry perspective, the present study was carried out to establish environmentally friendly extraction protocols for CWF phenolic compounds, based on the use of deep eutectic solvents (DESs) [30]. These are emerging as an efficient, cost-effective and green solution for fractionation and processing of lignocellulosic biomasses, including wood-based materials [31,32], and plant sources in general, for the recovery of lignin and antioxidant compounds [2,33–39].

The approach employed (Fig. 1) included a first screening of different DESs and eutectic mixtures to select the best one in terms of antioxidant properties and total phenolic content (TPC) of the obtained extract. Results from all eutectic mixture-based protocols were compared with those obtained using conventional extraction solvents i.e. water, ethanol, and methanol. An optimization of the extraction conditions was then performed in terms of percentage of added water, operating temperature, solid-to-solvent ratio, and extraction time (path A in Fig. 1). Harsher DES-based extraction conditions were also assayed for recovery of lignin (path B in Fig. 1), yielding a sample endowed with very efficient antioxidant properties in model assays. The identification and quantitation of the main phenolic components present in the extracts were carried out based on HPLC, UV-Vis, NMR, ATR-FTIR, and electron paramagnetic resonance (EPR) spectroscopy, as well as chemical degradation experiments. Finally, based on the previous results, a sequential two-step DES-based extraction protocol was developed, selectively affording an EA-enriched and an EA-free, lignin-enriched sample (path C in Fig. 1).

2. Material and methods

2.1. Materials

CWF and chestnut tannins (CT) were provided by Silvateam (S. Michele Mondovì, Cuneo, Italy). CWF was obtained from exhausted chestnut wood after drying in an oven overnight at 60 °C followed by milling to obtain < 250 μm particles. Choline chloride (ChCl) (≥99%), tartaric acid (≥99.5%), lactic acid (≥85%), sodium acetate (≥99%), glycerol (≥99%), Na-K tartrate (≥99%), ethylene glycol (≥99%), glycolic acid (≥99%), oxalic acid dihydrate (≥99%), urea (≥99%), malic acid (≥98%), maleic acid (≥99%), malonic acid (≥99%), fructose (≥99%), glucose (≥99.5%), sorbitol (≥98%), hydrogen peroxide 30%, 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron(III) chloride (97%), 2,4,6-tris(2-pirydyl)-s-triazine (≥98%), Folin-Ciocalteu reagent, (±)−6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (97%), gallic acid (≥97.5%), EA (≥95%), vanillic acid (≥97%), and syringic acid (≥95%) were purchased from Sigma-Aldrich and used without further purification.

2.2. Methods

UV-Vis spectra were recorded using a HewlettPackard 8453 Agilent spectrophotometer.

ATR-FTIR spectra were recorded on a Nicolet 5700 Thermo Fisher Scientific instrument. Spectra were recorded as an average of 128 scans in the range 4000 to 450 cm^{-1} (resolution of 4 cm^{-1}).

^1H NMR spectra were recorded in DMSO-d_6 at 400 MHz on a Bruker instrument.

EPR measurements were performed using a Bruker Elexys E-500 spectrometer equipped with a superhigh sensitivity probe head. The samples were transferred to flame-sealed glass capillaries, which in turn were coaxially inserted in a standard 4 mm quartz sample tube. Measurements were performed at room temperature. The instrumental settings were as follows: sweep width, 140 G; resolution, 1024 points; modulation amplitude, 1.0 G; conversion time 20.5 ms; time constant 10.24 ms. The amplitude of the field modulation was preventively checked to be low enough to avoid detectable signal overmodulation. The number of scans and microwave power were optimized to avoid

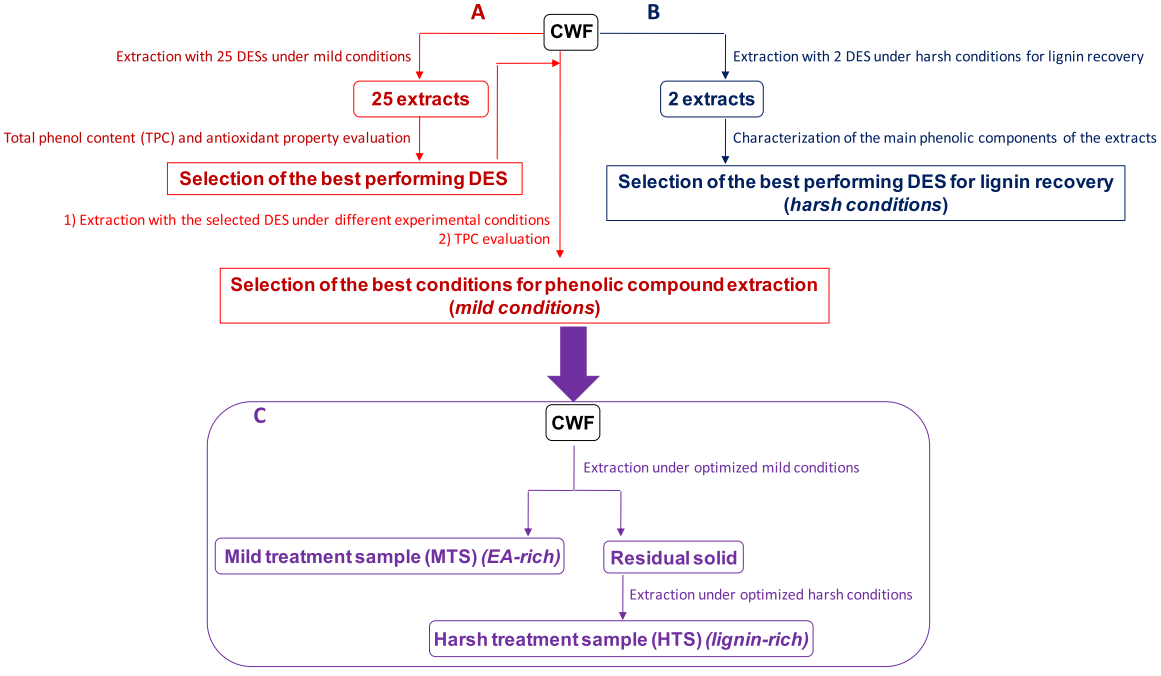

Fig. 1. Approach employed for the recovery of phenolic compounds from CWF.

microwave saturation of resonance absorption curve. For power saturation experiments, the microwave power was gradually incremented from 0.001 to 127 mW. The g value and the spin density were evaluated by means of an internal standard, Mn^{2+}-doped MgO, prepared by a synthesis protocol reported in the literature [40]. Since sample hydration was not controlled during the measurements, spin density values have to be considered as order of magnitude estimates [41]. A 10% error in the radical concentration mainly derives from sample positioning in the cavity, while an error of g of about 3×10^{-4} is related to the linewidth. The EPR spectra of the DMSO soluble samples (20 μL in a flame-sealed glass capillary) were acquired at a microwave power equal to 7.93 mW, which was preventively checked to be a non-saturating condition. For these measurements, a TEMPO solution in DMSO (10^{-5} mol/kg) was used as an external standard in order to estimate the sample spin density.

HPLC analysis were performed with an Agilent instrument equipped with a UV-Vis detector; a Phenomenex Sphereclone ODS column (250 ×4.60 mm, 5 μm) was used, at a flow rate of 1.0 mL/min. A gradient elution using 0.1% formic acid in water (solvent A) and methanol (solvent B) was performed as follows: 5% B, 0–10 min; from 5% to 80% B, 10–57.5 min. The volume of injection was 10 μL and the detection wavelength was 254 nm for all the sample. Analyses were performed at room temperature. The retention times of the standard compounds EA, vanillic acid and syringic acid were 35, 25 and 27 min, in that order.

The quantitative determination of EA was performed according to the HPLC peak area measurements. The calibration curve was built using standard solutions of EA in the concentration range 30–100 μg/mL. Each sample was analyzed three times.

2.3. Eutectic mixture preparation

Different DESs and eutectic mixtures were prepared as shown in Table A.1, following reported procedures [42]. Briefly, different hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD) were mixed at appropriate ratios and heated under stirring at proper temperatures until a homogeneous liquid was formed. All the solvents were stored at ambient temperature. No crystal precipitation was observed over the period of use.

2.4. Eutectic mixture screening for extraction of antioxidant compounds from CWF

0.2 g of CWF was added at 66.7 g/Kg solid-to-solvent ratio to the different solvents containing 30% w/w of water in a capped pyrex bottle. After stirring in a water bath for 60 min at 40 °C, the mixtures were centrifuged at 5000 rpm for 10 min. Extracts were diluted 1:5 v/v in methanol before further analysis. Control experiments were performed using water, ethanol, or methanol as conventional solvents.

2.5. Optimization of the extraction conditions using independent variables

Treatment of CWF with 2:1 mol/mol ChCl/tartaric acid (ChCl:TA2) was repeated by sequentially varying the solid-to-solvent ratio (33.3–200 g/Kg), the extraction time (60–180 min), the extraction temperature (40–90 °C), and the percentage of water (20–50% w/w).

2.6. Experimental design for optimization of the extraction conditions

A central composite design (CCD) was used to investigate the effects of extraction time, temperature, solid-to-liquid ratio, and the percentage of added water on the recovery of antioxidants from CWF. The CCD consisted of a full two-level factorial design (24 points), eight axial points at a distance ± α from the central point and six replicates of the central point. The value of α was taken as $(2^4)^{1/4} = 2$ to ensure the orthogonality and rotatability of the design. Factor levels were chosen based on the results of preliminary experiments and literature studies. They are reported in Table A.2 in actual (X_i) and coded (x_i) values, the latter being calculated as:

$$x_i = \frac{X_i - X_{i,0}}{\Delta X_i} \quad (1)$$

where $X_{i,0}$ is the value of the i-th factor at the center-point level and ΔX_i is the step change value for that factor.

The yield of antioxidant extraction, expressed as g of gallic acid equivalents (eqs) *per* 100 g of the starting material, was taken as the response variable. Overall, the experimental design consisted of 30 runs (Table A.3), which were performed in random order to minimize the effects of uncontrolled factors. The statistical design and analysis of experiments were performed using the Design-Expert® software (version 7.0.0, Stat-Ease, Inc., Minneapolis, MN, USA).

2.7. Lignin recovery with DESs under harsh conditions [43,44]

2 g of CWF was added to 20 g of ChCl:TA2 or 1:2 mol/mol ChCl/lactic acid (ChCl:LA2), both containing 20% w/w of water, and the mixture was taken under stirring for 8 h in a capped pyrex glass bottle placed in an oil bath at 120 °C. After cooling, 15 mL of ethanol was added and the suspension was vacuum filtered. Subsequently, the solid residue was washed twice with 50 mL of ethanol. The liquid phases collected from initial filtration and washing of the solid were combined and taken to a rotary evaporator to remove the organic solvent. Then, two different protocols were applied to precipitate lignin: (a) The dark brown liquid was poured into 200 mL of 7:3 v/v acetone/water and stirred for 2 h; acetone was then removed in a rotary evaporator at 60 °C, after that water was added until precipitation of a brown solid was observed; the precipitate was collected by filtration, washed three times with 1:9 v/v ethanol/water, and lyophilized to give 200 mg of a brown powder (10% w/w yield with respect to starting CWF). (b) The dark brown liquid was poured into 130 mL of 0.01 M HCl and the solid that precipitated was collected as above (203 mg, 10% w/w yield with respect to starting CWF).

When required, the brown powder obtained was suspended in DMSO (10 mg/mL) and after 72 h the mixture was centrifuged (7000 rpm, 15 min): the supernatant was collected and stored until further analysis, whereas the precipitate was washed three times with 0.01 M HCl and recovered by lyophilization (44–52% w/w).

2.8. EA- and lignin-enriched sample recovery by sequential two-step DES-based treatment of CWF

1 g of CWF was added to 10 g of ChCl:TA2 containing 20% w/w water, and the mixture was taken under stirring in a pyrex glass bottle at 50 °C for 90 min (mild treatment). Then, the residual solid CWF was separated from the supernatant by centrifugation (7000 rpm, 20 min), and the latter was poured into 100 mL of 1% KCl aqueous solution and kept at room temperature for 4 h. The precipitate that separated was recovered by centrifugation (7000 rpm, 20 min, 4 °C), washed three times with 1% KCl and lyophilized (mild treatment sample (MTS), 75 mg, 7.5% or 28% w/w yield with respect to starting CWF or dissolved CWF, respectively). The residual solid CWF was instead added to 10 g of ChCl:LA2, containing 20% w/w water, and the mixtures were taken under stirring in a pyrex glass bottle at 120 °C for 8 h (harsh treatment). The dark brown liquids collected by centrifugation (7000 rpm, 20 min, 4 °C) were poured into 100 mL of 1% KCl aqueous solution or 0.01 M HCl and kept at 4 °C for 24 h. The formed precipitates were then recovered by centrifugation (7000 rpm, 20 min, 4 °C), washed three times with 1% KCl or 0.01 M HCl and lyophilized (harsh treatment sample (HTS), 50 mg, ca. 5% or 10% w/w yield with respect to starting CWF or dissolved CWF for both precipitation protocols). In other experiments harsh treatment of residual solid CWF was performed with

1:9 mol/mol ChCl/lactic acid (ChCl:LA9), resulting in the recovery of ca. 54 mg of HTS (ca. 5% w/w yield with respect to starting CWF).

2.9. Recovery and reuse of ChCl:LA2

After precipitation of the lignin, the water in the ChCl:LA2 used in the sequential two-step DES-based treatment of CWF was removed under vacuum rotary evaporation [37]. The recovered DES was reused as such for a new lignin recovery process from MTS as described in section 2.6, affording HTS in ca. 4% w/w yield with respect to starting CWF.

2.10. TPC assay [45]

Diluted extracts (10–200 μL) were added to 2.1 mL of water followed by 0.15 mL of Folin & Ciocalteu's reagent and 0.45 mL of a 75 g/L Na_2CO_3 solution. After 30 min incubation at 40 °C, absorbance at 765 nm was measured. For solid samples, these were added at final doses of 0.0025–0.1 mg/mL to the same solutions as above. Gallic acid was used as reference compound. Experiments were run in triplicate.

2.11. DPPH assay [46,47]

Diluted extracts (15–375 μL) were added to 3 mL of a 0.2 mM ethanolic solution of DPPH, and after 10 min under stirring at room temperature the absorbance at 515 nm was measured. In the case of solid samples, these were added at 0.0025–0.8 mg/mL to the DPPH solution and the mixtures were analyzed as above. Trolox was used as a reference antioxidant. Experiments were run in triplicate.

2.12. Ferric reducing/antioxidant power (FRAP) assay [48]

To 3 mL of 0.3 M acetate buffer (pH 3.6) containing 1.7 mM $FeCl_3$ and 0.83 mM 2,4,6-tris(2-pyridyl)-s-triazine, 0.75–30 μL of diluted extracts were added, and after 10 min under stirring at room temperature the absorbance of the solutions at 593 nm was measured. In the case of the solid samples, these were added at final doses of 0.000625–0.1 mg/mL to the FRAP solution and the mixtures were analyzed as above. Results were expressed as Trolox eqs. Experiments were run in triplicate.

2.13. Alkaline hydrogen peroxide degradation [49]

10 mg of CT, MTS, or HTS were suspended in 1 M NaOH (1 mL) and 50 μL of 30% H_2O_2 was added. The mixture was kept at room temperature under vigorous stirring and after 24 h treated with 5% $Na_2S_2O_5$ in water, taken to pH 3 with 6 M HCl, filtered on a 0.45 μm PVDF filter, and analyzed by HPLC.

3.14. Acid degradation [50]

50 mg of CT, MTS, or HTS were treated in a pyrex tube with 5 mL of 4 M HCl at 90 °C for 24 h. The mixtures were then allowed to cool to room temperature, taken to pH 2.5 by addition of 6 M NaOH, and centrifuged (7000 rpm, 10 min). The supernatants were recovered, taken to 10 mL by addition of water, and analyzed by HPLC after filtration on a 0.45 μm PVDF filter. The solid residues were dissolved in 10 mL of DMSO/methanol 1:1 v/v and analyzed by HPLC as well.

3. Results and discussion

3.1. Eutectic mixture screening for extraction of antioxidant compounds from CWF

A first series of experiments was directed to a systematic screening of different eutectic mixtures for the recovery of phenolic compounds from CWF. Twenty-five eutectic mixtures differing in HBA and HBD components and molar ratios were prepared as reported in Table A.1 and in Section 2.1.

Extraction was initially carried out at 40 °C for 60 min with a 66.7 g/Kg solid-to-solvent ratio. Due to the high viscosity of some of them, the eutectic mixtures were used as 70% w/w aqueous solution. Actually, addition of water in specific amounts reduces the viscosity of DESs, allowing for higher mass transfer rates and, consequently, higher extraction yields [33,35,51,52]. However, since hydrogen bonding plays a fundamental role in DES formation and properties [53], hydration of the solvent may have an important impact on the eutectic mixture system [54]. Actually, several research papers aimed at investigating the molecular behavior of various DES-water systems have demonstrated that the eutectic mixture system is preserved by addition of water up to 50% w/w. On this basis, in the present work a 30% w/w water content in the eutectic mixture was used.

The efficiency of the different eutectic mixtures in extracting antioxidant compounds from CWF was evaluated based on validated chemical assays, that is TPC, and DPPH and FRAP assays, which measure the efficiency of electron transfer processes from the sample to a stable organic radical or to iron(III) ions, respectively [55] (Fig. A.1). Most of the extracts obtained with the eutectic mixtures showed a TPC higher and more efficient antioxidant properties than that obtained using conventional solvents, that is water, methanol, and ethanol. Overall, ChCl-based solvents proved to be the most efficient systems, together with LA:GLU, LA:FRU, LA:SA3, and LA:SA5. In particular, the ChCl-tartaric acid based DESs ChCl:TA1 and ChCl:TA2 afforded extracts endowed with very high iron(III)-reducing properties, which were 2.6- and 3.3-fold higher than that exhibited by the ethanol or methanol extract under the same conditions, respectively. These results are in line with several articles, which have reported the use of ChCl as component of DESs for an efficient treatment of lignocellulosic biomasses [33].

By making a compromise between the results of the TPC, DPPH, and FRAP assays, ChCl:TA2 was selected as the most promising DES for the recovery of antioxidant phenolic compounds from CWF. Indeed, the extract obtained using this solvent exhibited the highest TPC (Fig. A.1a) and the highest iron(III) reducing properties (Fig. A.1c) and an intermediate EC_{50} value in the DPPH assay (Fig. A.1b), when compared to the other DES extracts.

3.2. Optimization of the experimental conditions for the recovery of antioxidant phenolic compounds from CWF with ChCl:TA2

As reported in Section 3.1, ChCl:TA2 was selected as the most promising DES for the recovery of antioxidant phenolic compounds from CWF based on the overall results from the TPC, DPPH, and FRAP assays. However, several other factors may affect the efficacy of a DES-based extraction, such as the solid-to-solvent ratio, the extraction time, the extraction temperature and the water content. Therefore, a subsequent series of experiments was directed to optimize the experimental conditions for the extraction of phenolic compounds from CWF using the selected solvent. TPC was initially chosen as the parameter to compare the extraction efficacy. As far as the solid-to-solvent ratio is concerned, 100, 66.7, 50 and 33.3 g/Kg values were adopted, keeping the other operating conditions fixed (that is, 60 min, 40 °C, and 30% w/w water content). It was not possible to carry out the extraction at higher solid-to-solvent ratios (e.g. 200 g/Kg), because the high viscosity of the DES made it impossible to separate the supernatant from the residual solid under these conditions [52]. The results (Fig. A.2a) showed that the extraction efficiency linearly improved ($R^2 = 0.97$) (Fig. A.2b) with the increase of the solid-to-solvent ratio, indicating that no solvent saturation occurred. Based on these results, a solid-to-solvent ratio of 100 g/Kg was chosen for further experiments.

Extraction times between 60 and 180 min were then investigated, keeping fixed the other operating conditions (that is, 100 g/Kg solid-to-solvent ratio, 40 °C, 30% w/w water content). Although no statistically significant differences were observed, the results shown in Fig. A.3

indicated that the highest TPC (7.3 ± 0.5 mg/mL gallic acid eqs) was obtained with an extraction time of 90 min, whereas with prolonging of extraction time up to 180 min the phenolic content decreased slightly (6.5 ± 0.3 mg/mL gallic acid eqs). This could be explained by Fick's second law of diffusion which states that the final equilibrium between solid and extraction solvent is reached after a given time, after which no more extraction is possible [56]. The slight decrease of TPC values could therefore be likely due to decomposition or chemical modification processes occurring at the expense of phenolic compounds during the exceeding long time extraction treatment. Thus, 90 min was chosen as the optimal extraction time.

Temperature is well-known to affect DES viscosity and compound solubility, and hence the extraction efficiency. As shown in Fig. A.4, the TPC content increased by increasing the extraction temperature from 40 °C to 50 °C (7.3 ± 0.5 vs 10.8 ± 0.4 mg/mL gallic acid eqs), probably because the highest temperature decreases the viscosity of the DES, allowing for a more efficient contact of the sample with the extraction solvent. However, a decrease of TPC was observed at higher temperatures (e.g. 9.0 ± 0.1 mg/mL gallic acid eqs at 70 °C), likely again as a result of phenolic compound oxidation/degradation. On this basis, 50 °C was selected as the optimal extraction temperature.

In a last series of experiments the water content in the DES was varied from 20% to 50% w/w. Results shown in Fig. A.5a indicated that high DES dilution in water actually limited the interaction between the phenolic compounds and the extraction solvent, thus decreasing the DES efficacy. In particular, TPC linearly decreased with increasing proportions of water (Fig. A.5b). 20% w/w was therefore chosen as the optimal water content.

Finally, a rigorous procedure based on the CCD described in section 2.4 was used to systematically investigate the effects of extraction time, temperature, solid-to-liquid ratio, and the percentage of added water on the recovery of antioxidants from CWF. This procedure allows a quantitative determination of the effects of the tested factors and their interactions, as well as an estimation of optimal extraction conditions.

The experimental data were analyzed using different polynomial models (linear, two-factor interaction, quadratic and cubic). The best result was obtained with the quadratic model:

$$y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=1, i<j}^{4} \beta_{ij} x_i x_j \quad (2)$$

where y is the process response, x_i are the coded independent variables, β_0 is the intercept and β_i, β_{ii} and β_{ij} are the linear, pure quadratic and interaction coefficients, respectively.

A stepwise method, with entrance and removal levels of 0.1, was used to estimate the statistically significant terms. By this procedure, the following reduced model was derived:

$$y = \beta_0 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{33} x_3^2 \quad (3)$$

The estimated coefficients, the standard errors and the p-values are reported in Table A.4. Analysis of variance (ANOVA) indicated that the model was statistically significant ($p < 0.0001$) while the lack-of-fit was not ($p = 0.9403$). Moreover, internally studentized residuals were randomly scattered between −3 and +3 (Fig. A.6), with no outliers detected.

From an examination of the model coefficients, the following considerations can be made:

(a) under the conditions tested, the recovery of antioxidants was influenced by temperature (T), solid-to-liquid ratio (R) and the percentage of added water (W);
(b) the extraction yield was affected linearly by T and W, whereas the effect of R involved both a linear and a quadratic term;
(c) T and W had a positive effect on antioxidant recovery and the contribution of the latter was greater;
(d) all the interaction coefficients were not significant, indicating that each factor exerted its effect independently of the others.

The reduced model was used to optimize the extraction conditions. Maximization of the response variable was performed numerically using the gradient descent method. The extraction time (E), which was found to be not significant under the conditions of the study, was set to its center-point value (90 min). The following results were obtained: T = 70 °C; R = 40 g/kg; W = 50% w/w. The corresponding extraction yield (y = g of gallic acid eqs per 100 g of the starting material) was 23.17 g/100 g.

The developed model was validated by performing new experiments under the optimum conditions and in two points inside and outside the factorial region of the CCD. The results in Table 1 show that the experimentally determined values (y_{exp}) were very close to the model predictions (y_{pred}) and all included in their 95%-prediction intervals (PI 95%). This clearly attests the good predictive ability of the model and the effectiveness of the optimization procedure.

The experimental conditions optimized by the CCD model (90 min, 70 °C, 40 g/Kg solid-to-solvent ratio, and 50% w/w of water) provided a total phenolic compound extraction yield of 25.8 g/100 g of starting CWF, which is about 1.6-fold higher than that determined for the extract deriving from the protocol optimized using independent variables (15.7 g/100 g of starting CWF). With the aim of further comparing the two extraction protocols, the two extracts were analyzed by UV-Vis spectroscopy and HPLC after proper dilution. The UV-Vis spectra (see Fig. A.7a for a representative sample) showed a broad maximum at 340–370 nm, suggestive of the presence of EA [57]. This hypothesis was confirmed by HPLC analysis (Fig. A.7b), showing a main peak eluted at ca. 35 min, identified as EA based on the comparison of the chromatographic properties with those of an authentic standard. Notably, EA yields of 3.3% w/w and 0.16% w/w with respect to starting CWF were determined for the independent variables- and CCD-optimized extraction, respectively. This could be a consequence of the higher water content envisaged by the CCD-optimized protocol (50% w/w vs 20% w/w in the case of the independent variables-optimized protocol), limiting the extractability of water insoluble compounds like EA. On this basis, being the recovery of EA one of the main objectives of this study, in further experiments only the extract obtained through the independent variables-optimized protocol was taken into consideration.

Overall, based on the experiments reported in this section, solid-to-solvent ratio and water content were found to be the dominant factors affecting the extraction performance in both the independent variables and CCD model optimization approach. However, the extent of the effect of these two parameters must not be regarded as absolute, since it may depend on the target compound. As a remarkable example, a high water content was found to positively affect the TPC of the extract, whereas an opposite effect was observed on EA extraction yield.

3.3. Antioxidant properties of ChCl:TA2 CWF extract obtained under the selected conditions

Based on the results reported and discussed in Section <ins></ins>2.3, it was concluded that the most suitable conditions for the

Table 1
Results of validation experiments. y_{exp} and y_{pred} are the experimental and predicted (by Eq. 3) extraction yields, with the associated 95%-prediction intervals (PI 95%).

Point	E (min)	T (°C)	R (g/kg)	W (% w/w)	y_{exp} (g/100 g)	y_{pred} (g/100 g)	PI 95% (g/100 g)
Optimum	90	70	40	50	25.76	23.17	19.12–27.22
Internal	90	60	50	35	17.09	16.13	12.25–20.01
External	90	45	90	25	8.98	7.64	3.21–12.07

extraction of EA and other phenolic compounds from CWF are a water content of 20% w/w, a temperature of 50 °C, an extraction time of 90 min, and a solid-to-solvent ratio of 100 g/Kg. Under these optimized conditions ca. 27% of the starting CWF was dissolved. The antioxidant properties of the CWF extract obtained using the aforementioned optimized protocol were then investigated by the TPC, DPPH and FRAP assays protocol in comparison to those obtained for extracts prepared using water, ethanol and methanol under the same experimental conditions. The results are reported in Fig. 2.

Extraction with the DES afforded a TPC content significantly higher than that obtained with pure water and ethanol or methanol (ca. 7-fold and 2-fold higher, respectively) (Fig. 2a). Notably, also in the DPPH assay the DES extract exhibited very satisfactory antioxidant properties, being characterized by an EC_{50} value almost 5-fold lower than that of the water extract and not significantly different from that of methanol extract (Fig. 2b). A similar trend was observed in the FRAP assay, with the ChCl:TA2 extract exhibiting a number of Trolox equivalents significantly higher (4.8, 2.3 and 1.8-fold in that order) than those determined for the water, ethanol, and methanol extract, in that order (Fig. 2c).

These findings clearly demonstrated the higher efficiency of the ChCl:TA2 DES compared to conventional solvents in yielding CWF extracts with high TPC and superior antioxidant properties.

3.4. Characterization of the main phenolic compounds present in the ChCl:TA2 CWF extract

As stated above (Section <ins></ins>2.3), EA was identified as the main low molecular weight phenolic component in the ChCl:TA2 CWF extract (3.3% w/w yield with respect to starting CWF, ca. 12% w/w with respect to the solubilized material). Notably, the yield of EA obtained under the selected conditions was comparable to that obtained using DMSO, chosen as a reference extraction solvent for this polyphenol [58]. On the other hand, lower EA yields were instead obtained with conventional solvents (ca. 0.03% w/w and 0.6% w/w with respect to starting CWF in the case of water and methanol, respectively). These results are in accordance with recent studies reporting the high capability of DESs to extract EA from e.g. chestnut shell [37,59]. As stated in Section 1, CWF contains also residual, non-extracted ellagitannins and, most interestingly, lignin, which could account in part for the remaining 88% w/w of the ChCl:TA2 extract. However, all the attempts to recover this latter as a solid sample, e.g. by precipitation further to the addition of acidic water [60], failed. Therefore, in further experiments different extraction conditions were investigated as detailed in Section 3.5.

3.5. CWF lignin extraction by treatment with DESs under harsh conditions

Based on the encouraging results previously discussed, the possibility to exploit ChCl:TA2 for a DES-based, green lignin extraction from CWF was explored. To this aim, harsher experimental conditions, involving higher operating temperatures and longer extraction times, were adopted, following literature reported procedures [43,44]. For comparison, the efficacy of ChCl:LA2 was also tested, having this latter being reported to exhibit a strong selective dissolving ability toward lignin from different biomasses [61,62]. Treatment of CWF with the two DESs was performed at 120 °C for 8 h. The dark brown liquid obtained was then subjected to two different precipitation protocols, involving addition of 0.01 M HCl or of an acetone/water mixture [44], both leading to a brown solid in 10% w/w yield (although 43–48% w/w of the starting CWF was dissolved).

The antioxidant properties and the TPC of the samples obtained under the different experimental conditions are reported in Table 2, together with those of starting CWF and pure EA for comparison.

Notably, all the samples exhibited at least 4.5-fold stronger antioxidant properties and 2.5-fold higher TPC than the starting CWF. The ChCl:TA2 samples were found to be on average 1.3-fold more active than those recovered with ChCl:LA2, whereas no statistically significant effect of the precipitation protocol was observed. In particular, it has to be noticed that the ChCl:TA2 samples were characterized by EC_{50} values comparable to those exhibited by the reference antioxidant Trolox (0.011 ± 0.001 mg/mL) in the DPPH assay, and very high TPC values.

To gain information on the phenolic composition of the recovered solid samples, these were dissolved in DMSO and analyzed by UV-Vis spectroscopy and HPLC after proper dilution in methanol. For simplicity, only the samples recovered by addition of acetone/water mixtures were analyzed. The UV-Vis spectrum of both the ChCl:TA2 and

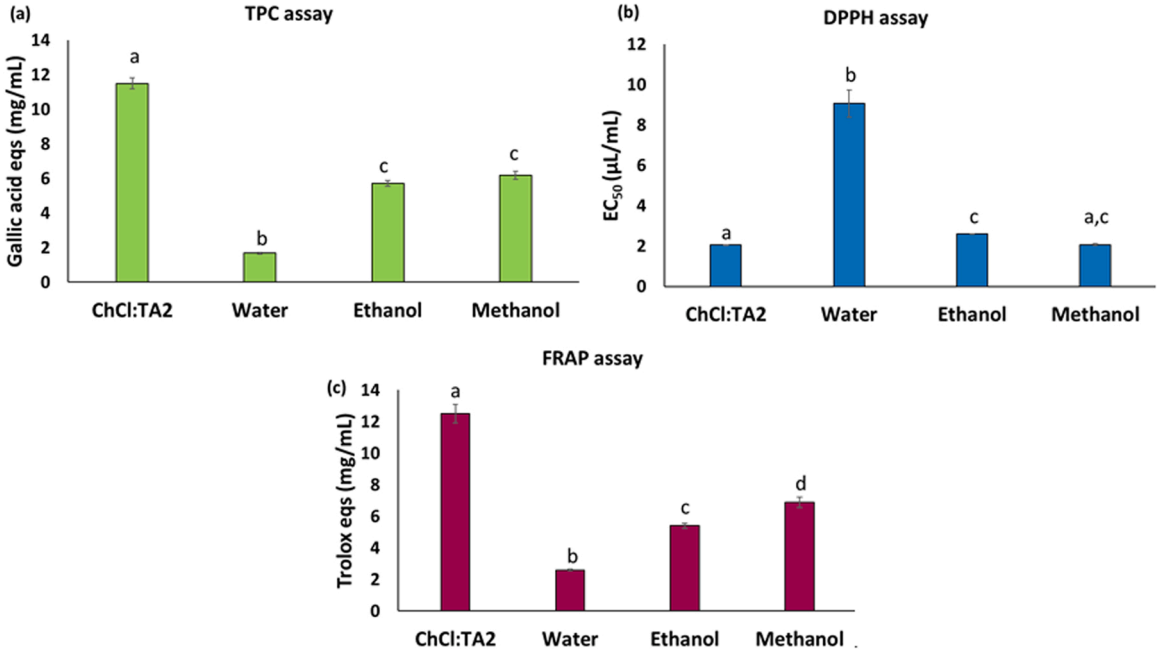

Fig. 2. (a) TPC, (b) DPPH and (c) FRAP assay results for CWF extracts prepared with ChCl:TA2 and conventional solvents under optimized experimental conditions (100 g/Kg solid-to-solvent ratio, 50 °C, 90 min, 20% w/w of water). Reported are the mean ± SD values of at least three experiments. Values without a common letter are significantly different ($p < 0.05$).

Table 2
Antioxidant properties of samples recovered from CWF by treatment with ChCl:TA2 or ChCl:LA2, at 120 °C, for 8 h.[a].

Sample	EC_{50} (mg/mL) (DPPH assay)	Trolox eqs (mg of Trolox/mg of sample) (FRAP assay)	Gallic acid eqs (mg of gallic acid/mg of sample) (TPC assay)
ChCl:TA2 (+ 0.01 M HCl)	0.018 ± 0.001^a	0.52 ± 0.02^a	1.0 ± 0.1^a
ChCl:TA2 (+ acetone/water)	0.0193 ± 0.0001^a	0.50 ± 0.05^a	1.01 ± 0.06^a
ChCl:LA2 (+ 0.01 M HCl)	0.0237 ± 0.0009^b	0.35 ± 0.01^b	0.72 ± 0.04^b
ChCl:LA2 (+ acetone/water)	0.0244 ± 0.0001^b	0.38 ± 0.01^b	0.73 ± 0.01^b
EA	0.0051 ± 0.0004^c	1.04 ± 0.02^c	2.5 ± 0.1^c
CWF	0.11 ± 0.01^d	0.037 ± 0.007^d	0.281 ± 0.004^d

[a] Reported are the mean ± SD values of at least three experiments. Values in the same column without a common italic letter (*a-d*) are significantly different ($p < 0.05$).

ChCl:LA2 samples showed an absorption maximum at around 367 nm (Fig. A.8a), suggesting as above the presence of EA, which was confirmed by HPLC analysis (Fig. A.8b). However, the amount of EA present in the two samples was found to be different, being 27 ± 3% w/w and 16 ± 2% w/w in the ChCl:TA2 and ChCl:LA2 sample, respectively. On this basis, it can be argued that the stronger antioxidant properties determined for the ChCl:TA2 samples are due to the higher concentration of EA.

Actually, based on the amount of EA calculated for the recovered samples and on the data reported for pure EA in Table 2, the antioxidant properties of the CWF-derived samples shall not be attributed solely to EA, but probably also to non-chromatographable phenolic species, such as lignin. As an example, the ChCl:LA2 sample exhibited Trolox and gallic acid equivalent concentrations 2.2- and 1.8-fold higher than that expected for a 16% w/w EA content. Moreover, being the recovered yields of the ChCl:TA2 and ChCl:LA2 samples comparable, the additional antioxidant components should be particularly abundant in the ChCl:LA2 sample, in agreement with the reported high selectivity of ChCl:LA2 for lignin extraction.

To gain structural information on these additional antioxidant components, ^{1}H NMR spectra were recorded in DMSO-d_6 (Fig. A.9). For both samples, a singlet at 7.48 ppm due to EA was observed, together with a broad signal centered at ca. 10.67 ppm due to the protons of the phenolic hydroxyl groups [63]. Furthermore, a very broad signal in the region 6.0–7.4 ppm, indicative of the presence of a heterogeneous phenolic polymer such as lignin [64] was particularly evident, as expected, in the spectrum of the ChCl:LA2 sample. Interestingly, a progressive sedimentation of a brown solid was observed in the NMR tubes, likely as a result of a slow lignin precipitation from the organic solvent. With the aim to investigate the possibility to exploit this observation for the recovery a lignin-enriched sample, in another series of experiments the solids obtained from treatment of CWF with the two DESs at 120 °C for 8 h were solubilized in DMSO at 10 mg/mL, and the solutions were left to settle at room temperature for 72 h. The precipitates were then recovered by centrifugation and lyophilization in ca. 44% and 51% yield for the ChCl:TA2 and ChCl:LA2 sample, respectively. As expected, based on the high solubility of EA in DMSO, HPLC analysis of the supernatants indicated the complete solubilization of this compound, whereas no detectable amount of EA was present in the precipitates, as demonstrated by ^{1}H NMR analysis of these latter after immediate dissolution in DMSO-d_6 (data not shown).

The DMSO washing procedure was therefore exploited to confirm the presence of lignin in the CWF extracts obtained under the harsh experimental conditions. In Fig. A.10 the ATR-FTIR spectra of the ChCl:TA2 sample before (black trace) and after (red trace) washing with DMSO are shown, together with those of a commercial EA sample (blue trace). As expected, no traces of this latter were present in the DMSO-washed sample, which was instead characterized by two sharp peaks in the 2950–2850 cm^{-1} region (evident also in the spectrum of the unwashed sample), typically associated to the C−H stretching vibration of lignins [1,8]. On the other hand, the spectrum of the starting ChCl:TA2 sample was almost superimposable to that of EA, particularly in the region between 1700 and 500 cm^{-1} (Fig. A.10b). Similar results were obtained for the ChCl:LA2 sample, although in this case the lignin signals in the 2950–2850 cm^{-1} region were more intense (Fig. A.10c), whereas a significantly lower intensity was evident for the EA signals (Fig. A.10d), as expected.

For further characterization of the lignin component, EPR spectra of CWF samples at different steps of purification using ChCl:LA2 as solvent, given the higher efficiency of this latter for lignin recovery, were recorded (Fig. A.11). It is well-assessed that lignin isolated from biomasses contains a significant amount of stable organic radicals, which are responsible for an easily detectable EPR signal [65,66]. The lignin radical species are recognized to be oxygen-centered substituted *o*-semiquinone radicals [67]. A possible further contribution comes from the unpaired electron delocalization in the polyphenolic matrix, as reported for melanins [41] and synthetic phenolic polymers [68], which would lead to the formation of carbon-centered radicals. The EPR spectrum of the untreated CWF sample showed a singlet at a g value of 2.0036, similar to those observed for other lignin-rich samples derived from wood [69]. The CWF signal was quite broad (ΔB= 5.7 G) and presented a significant Gaussian contribution to the lineshape (around 50%). Both evidences demonstrated the chemical heterogeneity of the material, the signal arising from a mixture of different species in different environments (supramolecular organization). The sample spin density, somehow representative of the lignin content, was about 10^{17} spin g^{-1}, in line with the literature [69]. The spectrum of the sample recovered from ChCl:LA2 extraction was slightly narrower and, at the same time, the Gaussian contribution to the lineshape decreased, thus suggesting that the extraction procedure enriched the sample in selected components, reducing its heterogeneity. The observed g value also changed, decreasing to 2.0031. The g value has been reported to be determined by the protonation state of the phenolic groups, a low value being expected for lignins treated with acidic solutions, while it increases for samples exposed to an alkaline environment [66]. The observed g decrease is consistent with the presence of lactic acid in the DES used for the lignin recovery and the use of 0.01 M HCl for the sample precipitation.

Interestingly, the spin density of the ChCl:LA2 sample decreased by one order of magnitude with respect to the pristine sample. This quite unexpected result is in line with the fact that components other than lignin, such as EA, were extracted in the DES. On the other hand, a higher weight normalized intensity was determined for the ChCl:LA2 sample after washing with DMSO, in agreement with an enrichment in the lignin component. Normalized power saturation curves (Fig. A.11b) confirmed a lower degree of variety of the free-radical population in the DES-recovered samples: in fact, the intensity decrease at high microwave power indicated a more homogeneous relaxation behavior.

Based on all these data, it could be concluded that the DMSO-washing works efficiently in providing a lignin-enriched sample from CWF. However, when the antioxidant properties of the DMSO-washed ChCl:LA2 sample were evaluated by the DPPH assay, an EC_{50} value of 0.72 ± 0.01 mg/mL was found, which was more than 30-fold higher than that of the pristine ChCl:LA2 sample, indicating that DMSO washing had the effect of removing not only EA but also other low molecular weight lignin components endowed with potent antioxidant

properties. This was confirmed by the EPR spectrum of the DMSO soluble fraction (Fig. A.11a), which showed a weak but clearly detectable signal attributable to lignin related species (note that EA is EPR silent).

On this basis, along with all the evidence collected from these and previous experiments, an ad hoc treatment of CWF aimed to selectively obtain an EA- and a lignin-enriched sample was finally designed.

3.6. EA- and lignin-enriched sample recovery by sequential two-step DES-based treatment of CWF

As stated in Section 3.5, in a last series of experiments the possibility to apply a sequential two-step DES-based treatment of CWF to selectively obtain an EA- and a lignin-enriched sample was explored. Firstly, a mild treatment of CWF with ChCl:TA2 was performed using the optimized "mild" protocol initially developed (100 g/Kg solid-to-solvent ratio, 50 °C, 90 min, 20% w/w of water). In order to separate the recovered sample from the DES, salting-out effects were exploited by addition of 1% KCl aqueous solution, which led to the precipitation of a light brown solid (indicated as mild treatment sample, MTS) that was recovered by centrifugation. Subsequently, the residual, undissolved CWF was treated with ChCl:LA2 under the harsh conditions previously reported (120 °C, 8 h), and the dark brown liquid thus obtained was added with 1% KCl or 0.01 M HCl to give a fine brown precipitate in comparable yields (harsh treatment sample, HTS).

The antioxidant properties as well as the TPC of MTS and HTS are reported in Table A.5 and Fig. 3.

MTS exhibited antioxidant properties significantly stronger than HTS, which was some 50% less active in the DPPH assay. In any case, all the samples exhibited more efficient antioxidant properties and a higher TPC than the starting CWF (see Table 2).

To gain information on the phenolic composition of the samples, these were dissolved in DMSO and analyzed by HPLC after proper dilution in methanol. As expected, the UV-Vis spectrum (Fig. A.12a) and the chromatographic profile (Fig. A.12b) of MTS showed a maximum at 367 nm and a single peak eluted at ca. 35 min, respectively, indicative of the presence of EA. Quantitative analysis indicated an EA content of 31 ± 4% w/w, which was higher than that determined for all the samples prepared under the previously described conditions. On the other hand, HTS contained only 0.4% w/w EA, highlighting the selectivity of the two-step treatment, affording both an EA- rich sample and a EA-free, lignin-rich (see below) sample. The yield of recovery of EA achieved in the present study in the case of MTS (ca. 2.3% w/w with respect to starting CWF) is, at best of our knowledge, the highest ever reported for DES-based extraction of agricultural wastes such as pomegranate peels (ca. 0.7% w/w) [70], chestnut shells (up to 0.3% w/w) [37,59], or strawberry waste (0.01% w/w) [71]. This result is of particular relevance, given the well-recognized health beneficial properties [59] of this polyphenol.

Based on the EA content and on the antioxidant properties of standard EA reported in Table 2, it can be concluded again that the antioxidant properties of MTS and especially HTS are due to phenolic compounds different from EA. Actually, the ATR-FTIR spectrum of MTS (Fig. 4) was almost superimposable to that of standard EA, although the two sharp peaks in the 2950–2850 cm^{-1} region associated to the C–H stretching vibration of lignins [1,8] were also present. On the other hand, the peculiar signals of EA at 3558 cm^{-1} and in the 1700–500 cm^{-1} region were not present in the spectrum of HTS, in agreement with the low content of EA determined by HPLC analysis.

EPR analysis (Fig. 5) confirmed the efficacy of the two-step treatment in providing HTS as a lignin fraction with good purity, as evident from the relatively high spin density and the low ΔB values, indicating, together with the power saturation profile, a homogenous free radical population. The relatively low g value is ascribable to the acidic environments to which the material was subjected during the extraction procedure, as discussed in Section 3.5. Interestingly, MTS also presented a well-detectable EPR signal, likely due to low-molecular weight lignins extracted during the initial mild treatment. This observation is in agreement with the ATR-FTIR results. However, this lignin fraction appears more heterogeneous, as highlighted by the power saturation trend, which reached a plateau at high incident power (Fig. 5b).

To rule out the possible contribution of other phenolic polymers, mainly residual tannins still present in CWF, to the HTS signal, EPR analysis of a reference CT sample was performed. As shown in Fig. A.13, CT exhibited spectral characteristics quite different from HTS, particularly a normalized power saturation curve with just a slop change in a monotonously increasing trend, with no evident maximum.

To further characterize HTS, chemical degradation experiments were performed. These included alkaline hydrogen peroxide and acid degradation: the first is commonly employed to analyze insoluble and structurally complex phenolic polymers such as melanin pigments and lignins, and is based on the identification of chromatographable, low-molecular weight markers, deriving from oxidative breakdown of the polymer [49]; as to the acid degradation method, this has been proposed as a validated approach for the characterization of extractable and nonextractable ellagitannins in plant sources [50]. The HPLC profile of the alkaline hydrogen peroxide degradation mixture of HTS showed, among others, two main peaks eluted at 25.0 and 26.7 min which were identified as vanillic and syringic acid, respectively (Fig. A.14a). These data are indicative of the presence of guaiacyl and syringyl units in HTS. On the other hand, no detectable amounts of EA were observed in the HPLC profile of the supernatant from the acid degradation mixture (Fig. A.14b), confirming the absence of significant amounts of CT in HTS. The low intensity peak due to EA found in the solid residue from the acid degradation mixture (Fig. A.14c) is attributable to EA present in HTS. For comparison, chemical degradation experiments were performed also on CT and MTS: for both samples no vanillic or syringic acids were observed in the elutographic profile of the alkaline hydrogen peroxide degradation mixtures, whereas EA was observed, as expected, in the supernatant from the acid degradation mixture of CT (data not shown).

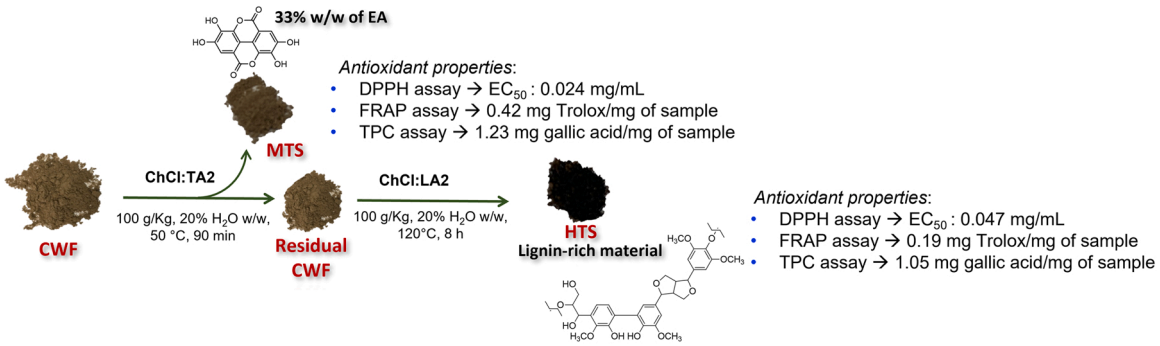

Fig. 3. Schematic representation of the sequential two-step DES based treatment of CWF developed in the present work and antioxidant properties of MTS and HTS.

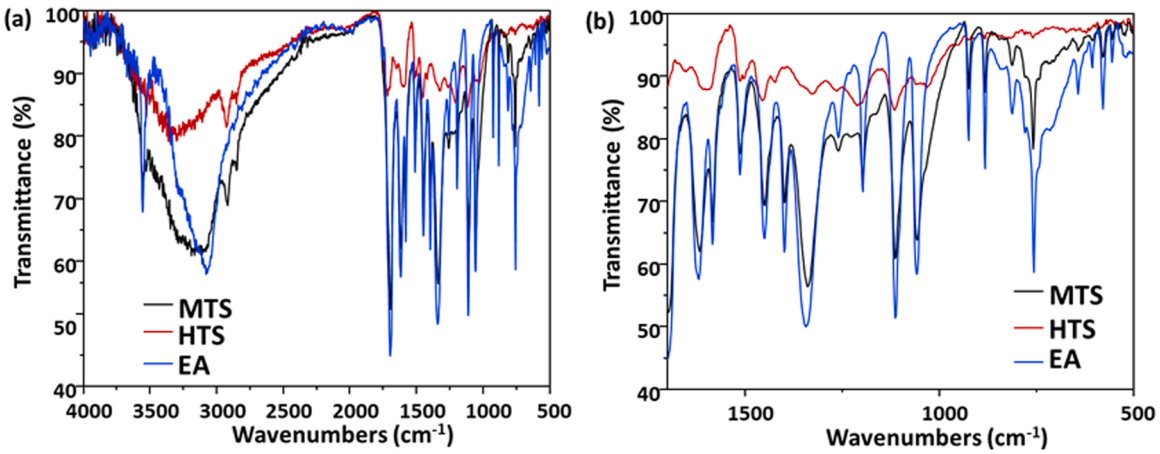

Fig. 4. (a) ATR-FTIR spectra of MTS (black trace), HTS (red trace) and standard EA (blue trace). (b) Expanded plot (1700–500 cm^{-1} region).

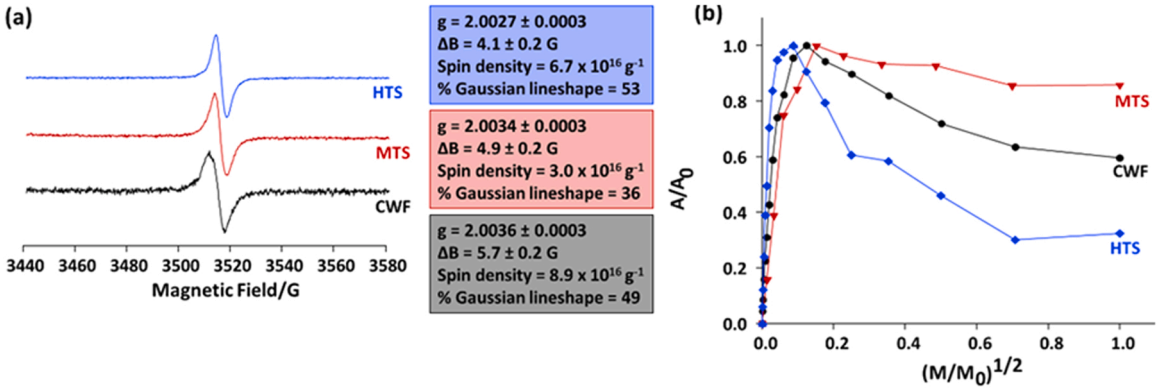

Fig. 5. (a) Solid state EPR spectra and (b) power saturation profiles of starting CWF (black trace), MTS (red trace) and HTS (blue trace).

Taken together, all the collected experimental evidence confirmed the high purity and homogeneity of the lignin-rich sample HTS form the sequential two-step DES based treatment of CWF. The possibility to recover a lignin-rich fraction endowed with potent antioxidant properties is an important outcome of this work, since lignin is finding increasing applications also in the health sector, e.g. in diabetes treatment, obesity control, and tissue therapy, and most of these therapeutic effects seem to be dependent on its antioxidant activity [72,73].

In a second series of experiments, the possibility to use ChCl:LA9 as DES in the harsh treatment step was investigated, since it is well known from the literature that lignin solubility in ChCl:lactic acid mixtures increases with the increase of lactic acid ratio [74]. Actually, ChCl:LA9 provided a solid in comparable yields and containing a comparable amount of EA with respect to ChCl:LA2. However, a slightly lower TPC was determined (0.91 vs 1.05 mg of gallic acid/mg of sample) for the ChCl:LA9 sample. On this basis, ChCl:LA2 was confirmed as the optimal solvent for the lignin-enriched sample recovery in the two-step DES--based treatment of CWF.

Finally, the possibility to recover and reuse the ChCl:LA2 DES after the lignin-rich fraction precipitation was evaluated, as this aspect represents a major issue in the proposal of a green and sustainable extraction process. The DES was recovered from the supernatant by removing water at a rotary evaporator and reused in the two-step treatment of CWF, leading only to a slight decrease in the extraction yields of HTS (4 vs 5% w/w), which exhibited a comparable amount of EA with respect to the sample obtained with the fresh DES (HPLC evidence).

4. Conclusions

In conclusion, a straightforward, low cost, and smart green protocol has been developed based on a two-step DES-based treatment of CWF, a clean industrial by-product of wood tannin extraction, for the selective recovery of an EA-rich and a lignin-rich material. In particular, treatment of CWF with ChCl:TA2 afforded EA in higher yields compared to those reported for DES-based extraction protocols applied to other agricultural wastes, whereas subsequent treatment of residual CWF with ChCl:LA2 allowed to obtain an extract containing mainly a structurally homogeneous guaiacyl-syringyl lignin, as demonstrated by EPR and chemical degradation analysis. In addition, both extracts were characterized by high total phenol content and potent antioxidant properties.

Although we are aware that several issues still need to be addressed, including e.g. the improvement of the recovery yields, the possibility to implement the proposed protocol on a large scale, and a detailed cost-benefit analysis, the disclosed approach looks amenable in view of a full exploitation and valorization not only of CWF, but also of other agri-food by-products for the selective recovery of both low- and high-molecular weight phenolic compounds for application as antioxidant additives in biomedicine, food and/or cosmetic sector.

CRediT authorship contribution statement

Federica Moccia: Conceptualization, Data Curation, Investigation, Methodology, Writing – original draft. **Noemi GallucSci**: Investigation. **Samuele Giovando:** Resources, Writing – review & editing. **Antonio Zuorro:** Conceptualization, Data curation, Methodology, Writing – review & editing. **Roberto Lavecchia**: Conceptualization, Data Curation,

Methodology, Writing – review & editing. **Gerardino D'Errico**: Data curation, Validation, Writing – review & editing. **Lucia Panzella:** Conceptualization, Supervision, Validation, Writing – review & editing. **Alessandra Napolitano:** Resources, Writing – review & editing.

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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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2022.107773.

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