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# Enhanced extraction of phenolic compounds using choline chloride based deep eutectic solvents from *Juglans regia* L.

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ABSTRACT

The extraction of phenolic compounds from walnut leaves (*Juglans regia* L.) was optimized using heat-assisted extraction and deep eutectic solvents based on choline chloride and carboxylic acids. A preliminary solvent screening was performed using a selected group of carboxylic acids as hydrogen bond donors, showing that the highest extraction yield of phenolic compounds was obtained using choline chloride mixtures with butyric or phenylpropionic acid at a mole ratio 1:2, with 20% of water (w/w). The extraction conditions (time, temperature and water proportion) were then optimized by an experimental design, assisted by response surface methodology. To evaluate the response, the three most abundant compounds identified by HPLC (neochlorogenic acid, quercetin 3-*O*-glucoside and quercetin *O*-pentoside) were quantified. Additionally, the solid/liquid ratio effect at the optimal conditions, in dose-response format, was studied in view of its upscale, not showing any significant decrease until 140 g/L. The results here presented provide valuable information towards the design of a process in a pre-industrial form for the extraction of phenolic compounds from *J. regia* leaves using deep eutectic solvents.

## 1. Introduction

Deep eutectic solvents (DES) were first introduced in 2003 by Abbott and co-workers (Abbott et al., 2003) reporting mixtures of urea and quaternary ammonium salts, in particular choline chloride (CC). Since then many other DES have been reported and their use proposed for a wide range of applications (Francisco et al., 2012; Smith et al., 2014). A DES is a mixture between two (or more) starting materials (hydrogen bond acceptor – HBA and hydrogen bond donor – HBD) where the eutectic temperature of the mixture is considerably lower than that of either of the constituents (Abbott et al., 2004). Some authors prefer to call them low transition temperature mixtures (Francisco et al., 2013; Jancheva et al., 2017).

The replacement of volatile and toxic organic solvents by greener and more performant solvents is one the most important challenges of our days for the chemical industry in general, and biorefineries in particular (Pena-Pereira and Namieśnik, 2014). Choi et al. (2011) introduced the DES as an alternative media to extract secondary metabolites, such as phenolic compounds, from natural matrices instead of conventional organic solvents. Nowadays, this idea is being expanded to create designer solvents, using several combinations of HBA and HBD, with tunable properties to selectively dissolve and extract natural and bioactive compounds from plants, oils or biomass, valorizing natural products or wastes as a source of valuable compounds (Dai et al., 2016; Nam et al., 2015; Paradiso et al., 2016). A very recent review on the application of deep eutectic solvents for the extraction of phenolic compounds can be found in literature, describing the possibility of using DES both as solvent and formulation media with potential cosmetics, pharmaceutical, or food applications (Ruesgas-Ramón et al., 2017).

This new generation of solvents entails several characteristics, which support their insertion in different industries. First, they are simple to prepare from cheap starting materials (Dai et al., 2016). Then, the HBA and HBD can be selected to be less toxic than organic volatile solvents and also biodegradable (Mbous et al., 2017). Moreover, in general, these solvents have low volatility and flammability (Dai et al., 2016). The addition of water to DES, for extraction purposes, is a well-established procedure to reduce the viscosity of the solvents and

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improve the mass transfer of bioactive molecules from the natural matrices (García et al., 2016; Gu et al., 2014). Regarding temperature, its increase leads to a lower viscosity and, consequently, to higher extraction yields (Dai et al., 2016; Jancheva et al., 2017).

In the present study, the performance of choline chloride (CC) based DES combined with carboxylic acids (CA) is investigated for the extraction of phytochemical compounds from the leaves of walnut trees. Walnut leaves were chosen as a plant model as they stand out as a significant source of bioactive compounds such as phenolic acids and flavonols (Santos et al., 2013; Vieira et al., 2017). These leaves are well known in traditional medicine due to their health benefits arising from their antioxidant, antitumor (Santos et al., 2013), antiproliferative (Carvalho et al., 2010), anti-inflammatory and antinociceptive (Erdemoglu et al., 2003) activities. A DES screening of mixtures of CC and CA is carried by heat-assisted extraction (HAE). Fifteen acids were selected to assess the effect of the alkyl chain length, the number of carboxylic acid groups, and the additional presence of hydroxyl and/or phenyl groups over the extraction performance. The best two DES selected will be used to optimize the extraction conditions of time (*t*), temperature (*T*) and water content (*S*) by response surface methodology (RSM). The work here presented can be better comprehended as a continuation of a previous work from other authors (Vieira et al., 2017), in which they optimized the extraction of bioactive compounds from walnut leaves using hydro-alcoholic mixtures.

## 2. Material and methods

### 2.1. Standards and reagents

HPLC-grade acetonitrile and anhydrous citric acid were from Fisher Scientific (Lisbon, Portugal). The phenolic compounds standards 5-*O*-caffeoylquinc acid (Extrasynthèse, Genay, France) and quercetin 3-*O*-glucoside, as well as glutaric acid (99 wt%) and glycolic acid (99 wt%) were purchased from Sigma-Aldrich. Butyric acid (99 wt%), lactic acid (88–92 wt%) and valeric acid (99 wt%) were purchased from Riedel-de Haen. Acetic acid (99.5 wt%) was obtained from Labsolve JMGS, propionic acid (99 wt%) from Merck, DL-malic acid (99.5 wt%) from Panreac, malonic acid (98 wt%) from Fluka, phenyl-acetic acid (99 wt%) from Alfa Aesar. Choline chloride (98 wt%), 3-phenylpropionic acid (99 wt%), 4-phenylbutyric acid (99 wt%) and 5-phenylvaleric acid (99 wt%) from ACROS Organics. All the other chemicals were of analytical grade and purchased from common sources.

### 2.2. Plant material

*Juglans regia* L. (walnut) dried leaves were purchased from Soria Natural, S.A., Spain. According to the distributor, the leaves were collected in Soria (Spain) in June 2014 and naturally dried in a room with controlled humidity. The samples were reduced to a fine dried powder (60–20 mesh) and stored in a desiccator protected from light for subsequent assays.

### 2.3. Screening analysis of DES

#### 2.3.1. Preparation of DES

DES were prepared using the heating-stirring method proposed by Abbot and co-workers (Abbott et al., 2004) with slight modifications. Firstly, the water amount present in the starting materials was measured using a Metrohm 831 Karl Fisher coulometer (data not shown). Then, each component was accurately weighed ($\pm 10^{-4}$ g) to a round-bottom flask. The mixtures were stirred in a water bath (from 50 to 80 °C) at 600 rpm until a homogenous solvent was obtained. The resulting DES are listed in Table 1.

#### 2.3.2. Conditions for the screening extraction by heat-assisted extraction

For the screening extractions, DES were prepared with 20% (w/w) of water except for phenylbutyric and phenylvaleric acids that were prepared with 5% (w/w) of water due to their low solubility in water. For comparison purposes, the DES CC:phenylpropionic acid was also prepared with 5% of water.

The solid-liquid heat assisted extractions (HAE) were performed using a Carousel 12 Plus Reaction Station™ (Radleys Tech). This equipment allows stirring and temperature control within ± 0.5 °C, with protection from light. It is also coupled to a condensation system, avoiding the loss of solvent. The powdered samples (0.15 g) were extracted with 5 mL of each solvent, during 60 min at 50 °C and 600 rpm. A control extraction using the conventional solvent ethanol:water (1:1, *v/v*) was also performed. After extraction, the mixtures were centrifuged at 6000 rpm during 10 min at room temperature and the supernatant was filtered through a Whatman n°4 for further analysis. For the extracts obtained with choline chloride:aromatic acids, a previous dilution was made with methanol due to thermal and physical stability.

### 2.4. Chromatographic analysis of the main phenolic compounds

Extract solutions were two-fold diluted with water and filtered through 0.2 μm disposable LC filter disks (30 mm, regenerated cellulose). The DES composed by CC and aromatic acids were diluted with methanol to avoid precipitation at room temperature. The samples were analysed as previously applied (Vieira et al., 2017) using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan) with a quaternary pump and a photodiode array detector (PDA) coupled to an LC solution software data-processing station using a Waters Spherisorb S3 ODS-2$C_{18}$, (3 μm, 4.6 mm × 150 mm) column operating at 35 °C for separation. Double online detection was carried with a diode array detector (DAD) operating at 280 and 370 nm as preferred wavelengths and the target phenolic compounds were identified according to their UV spectra and retention time (Barros et al., 2013). The results were expressed in mg per g of dry weight (mg/g dw).

### 2.5. HAE optimization by RSM of the selected DES, experimental design, model analysis and statistical evaluation

An illustrative diagram of the different steps carried out to obtain an optimal phenolic extract from *J. regia* L. is presented in Figure A1 . The solid-liquid extractions were performed with the HAE using the same equipment above described for the DES screening.

#### 2.5.1. Experimental design

The study of the impact of all independent variables was carried using one-factor-at-a-time, to identify the most influent, and to determine the initial range of the processing variables. Through the analysis of this experimental results (data not shown), $X_1$ (*t*, min), $X_2$ (*T*, in °C) and $X_3$ (*S*, in%) were chosen as variables for the RSM design. Therefore, the combined effect of these variables on the extraction of the three main phenolic compounds present in *J. regia* (maximizing responses individually or globally) was studied using *circumscribed central composite design* (*CCCD*) with 5 levels of each factor. The experimental design is based on 20 independent combinations, 6 of which are replicas at the central point of the experiment Box and Hunter (1957). The mathematical expressions used to calculate the design distribution, code and decode the tested variables can be found all detailed in the supplemental section (supplementary material). Once the optimal conditions ($X_1$, $X_2$ and $X_3$) were optimized, the study was advanced furthermore with the study of the *S/L* condition ($X_4$, in g/L).

#### 2.5.2. Mathematical model

The response surface models were fitted by means of least-squares calculations using the following second-order polynomial equation: