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ORIGINAL ARTICLE

Choline chloride derivative-based deep eutectic liquids as novel green alternative solvents for extraction of phenolic compounds from olive leaf

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Abstract In the presented study, a new methodology based on the use of deep eutectic solvents (DESs) and microwave-assisted extraction (MAE) and subsequent analysis by HPLC-DAD-ESI-TOF-MS was proposed for the extraction of phenolic compounds from olive leaf. Nine different DESs, using choline chloride as hydrogen bond acceptor in combination with different hydrogen bond donors (four polyalcohols, three organic acids, one sugar and urea), were firstly scanned. A total of 48 phenolic compounds were identified in the olive leaf using HPLC-DAD-ESI-TOF-MS. Experimental results and multivariate data analysis pointed to choline chloride-ethyleneglycol as being the most effective within the tested DESs, showing extraction yields similar to those exhibited by conventional solvents. A Box-Behnken Design and response surface methodology were applied with the aim to optimize the main parameters involved in the extraction process. The optimal extraction conditions were 79.6 °C of temperature, 43.3% of water and 16.7 min of irradiation time. Correlation coefficients ($R^2 > 0.98$) indicated a good relationship between experimental data and the fitted quadratic term models. Results indicated that DESs could be a sustainable alternative to traditional solvents for the extraction of bioactive compounds among many other applications.

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1. Introduction

Due to the increasing demand for environmentally friendly analytical methods, there have been different attempts to improve the extraction procedures during the past few years, which have led to the development of more sustainable, efficient and profitable strategies. Conventional organic solvents such as hexane, ethyl acetate, chloroform, acetone or methanol among others are widely used to perform the extraction process due to their dissolution ability and extraction power. However, the use of these solvents entails certain limitations according to the principles of green chemistry (American Chemical Society and Green Chemistry, 2014). To overcome these constraints, new type of solvents, named deep eutectic solvents (DESs), have recently emerged. These new solvents have been widely acknowledged as a greener replacement to conventional solvents (Zhang et al., 2012).

Deep eutectic solvents (DESs) are defined as a mixture of two or more components, a hydrogen bond acceptor and a hydrogen bond donor, in solid or liquid state, which, at a particular molar ratio and at room temperature, become liquids (Paiva et al., 2014). The newly formed liquids have significantly lower melting points than either of the individual components. When the DES are composed of primary metabolites such as, aminoacids, organic acids, sugars or choline derivatives, they are also called natural deep eutectic solvents (NADES) (Choi et al., 2011; Dai et al., 2013a). DESs present a variety of useful properties such as low volatility, adjustable viscosity and water miscibility. Consequently, they are regarded as effective solvents for the dissolution and extraction of a wide range of non-polar and polar compounds. In addition to their lower environmental and economic impact, they present other advantages as solvents, for example, biodegradability, low cost, simple preparation methods and the fact that the precursors used are renewable, non-toxic and natural compounds (García et al., 2016; Paiva et al., 2014; Zhang et al., 2012).

DESs are increasingly generating interest within the scientific community, who are trying to understand the specific characteristics of these fluids. In recent years, DESs have been employed in many applications such as chemical catalysis, organic synthesis, electrodeposition and enzymatic reactions (Dai et al., 2013a; Zhang et al., 2012). Despite increasing interest in the different separation processes, there is still a lack of information on practical issues related to their application as extraction solvents, i.e. their efficiency and optimal physical properties such as viscosity and polarity. However, few studies have been focused on the application of DESs in the extraction of bioactive compounds from plant materials (Paiva et al., 2014; Wei et al., 2015).

The olive leaf (*Olea europaea*) is one of the by-products of olive farming, which is accumulated during the pruning of the olive trees and account for up to 10% of the total weight of the olives at olive-oil mills (Tabera et al., 2004). Although the olive leaf has been used in traditional medicine to make infusions for a wide range of ailments (Hashmi et al., 2015), recent scientific evidences have demonstrated their potential health benefits as antihypertensive, anticarcinogenic, anti-inflammatory, hypoglycemic, antimicrobial, antiviral, anti-tumor, antithrombotic and their hypocholesterolemic effect (Barrajón-Catalán et al., 2015; Fu et al., 2010; Jemai et al., 2008; Lee and Lee, 2010; Micol et al., 2005; Susalit et al., 2011; Taamalli et al., 2012a). These health benefits seem to be attributable to the presence of a high number of phenolic compounds of different nature and structure. Among them, it is notable the occurrence of several secoiridoids, phenylethanoids and flavonoids with biological properties (Hashmi et al., 2015; Taamalli et al., 2012a). As a result, the olive leaf has been postulated as a good source of natural bioactive compounds.

Recently, DESs have proven to be an excellent solvent to isolate phenolic compounds with a wide range of polarities from olive oil (García et al., 2016). However, to the best of our knowledge, no extraction procedures based on DESs have been reported for the extraction of phenolic compounds from by-products of olive farming such as olive leaf. Therefore, the aim of this study is to propose an eco-friendly media to carry out the extraction of valuable compounds from olive leaf. For this purpose, an experimental design was implemented with a view to optimize the main variables involved in the extraction process in order to maximize the extraction of phenolic compounds from olive leaf which were subsequently characterized by using HPLC-DAD-ESI-TOF-MS. The issues of the methodology proposed were compared with the rest of methodologies reported previously in bibliography with the aim of (i) evaluating the green aspects of the DESs for extraction purposes and (ii) proposing a greenness analytical methodology alternative to the use of toxic organic solvents.

2. Materials and methods

2.1. Plant material: Olive leaf

Olive leaves from *Hojiblanca, a* variety grown in Seville (Spain), were randomly collected from different parts of a number of trees with no phytosanitary treatment. After collection, the fresh leaves were immediately transferred to the laboratory, washed with distilled water and dried indoors under controlled temperature, at 22 °C, until their weight remained constant. The collected samples were ground and pooled into a unique sample which was stored at −20 °C until it was used.

2.2. Chemical and reagents

Choline chloride, lactic acid, oxalic acid, tartaric acid, 1,4-butanediol, ethylene glycol, xylitol, 1,2-propanediol, maltose and urea were purchase from Sigma-Aldrich (St. Louis, MO, USA). Water was purified through a Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade methanol and acetonitrile were purchased from Panreac (Barcelona, Spain) and Labscan (Dublin, Ireland) respectively. Acetic acid of analytical grade (assay >99.5%) was sourced from Fluka (Switzerland). Standard compounds such as hydroxytyrosol (>98%), tyrosol, luteolin (>98%) and apigenin (>95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and oleuropein (>98%) was provided by Extrasythese (Lyon, France). Folin Ciocalteu's phenol reagent, gallic acid and Na_2CO_3 were supplied by Merck (Darmstadt, Germany).

2.3. DESs preparation

The DESs were prepared by heating choline chloride as the hydrogen bond acceptor (HBAs) with different hydrogen bond

donors (HBDs) and according to a scheduled molar ratio. The two components of each mixture were placed in a capped flask at 80 °C under constant stirring until a homogeneous colourless liquid was obtained.

2.4. Screening of DESs for extraction of phenolic compounds from olive leaf

In closed-vessels, an accurately quantified 200 mg of olive leaf were extracted with 1.5 mL of DES using a microwave extractor (Anton Paar GmbH, Graz, Austria). The solvent choice is of fundamental importance in any extraction process. Therefore, to select the best DES to extract phenolic compounds from olive leaf, the extractions were performed under the following initial conditions: the DESs were mixed with 25% of water and extractions were performed for an irradiation time of 20 min at 65 °C. In parallel, with the aim to compare the extraction efficiency exhibited by the DES, the same procedure was carried out using a conventional solvent as methanol:water (80:20 v/v). The applied analytical procedure is illustrated schematically in Fig. 1. Once the DES was selected, an experimental design was performed to optimize the MAE parameters involved in the extraction process.

2.5. Experimental design

A three-level (−1, 0, +1) three-factor Box-Behnken design (BBD) combined with response surface methodology (RSM) was conducted to perform an experimental design. Three major influence factors such as temperature, irradiation time and percentage of water were considered as independent variables. The extraction temperature was evaluated within the range of 40–80 °C, the irradiation time covered a range from 10 to 40 min and the percentage of water evaluated was within 0–70%. The selected variables were both, the yield of the sum of the selected compounds and their individual yields, as well as, the total index of polyphenols, measured using the Folin-Ciocalteu method. The BBD test resulted in a total of 15 experiments with different combinations of three factors, twelve factorial design points and three replicates at center point in a randomized run order. For the description of the response sample extraction, a second-order polynomial model was fitted to the data:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{j=1}^{k} \beta_{ij} X_i X_j$$

X_i and X_j are the levels of the independent variables which influence the response variable Y. β_0 represents intercept, β_j represents the linear, β_{jj} quadratic and β_{ij} interaction effect of the factors.

Statistical analysis and optimization were performed using the software Stat-Ease Design-Expert 10. The same software was used to draw contour and 3D response surface plots.

2.6. Total phenol index by Folin Ciocalteu method

The total phenol index (TPI) of extracts was determined following the Folin-Ciocalteu procedure (Singleton and Rossi, 1965). Thus, 10 µL of properly diluted extracts were mixed with 600 µL of deionized water and 50 µL of Folin-Ciocalteu reagent. After 10 min, 150 µL of Na_2CO_3 solution 20% (w/v) and 190 µL of deionized water were added. After 2 h of incu-

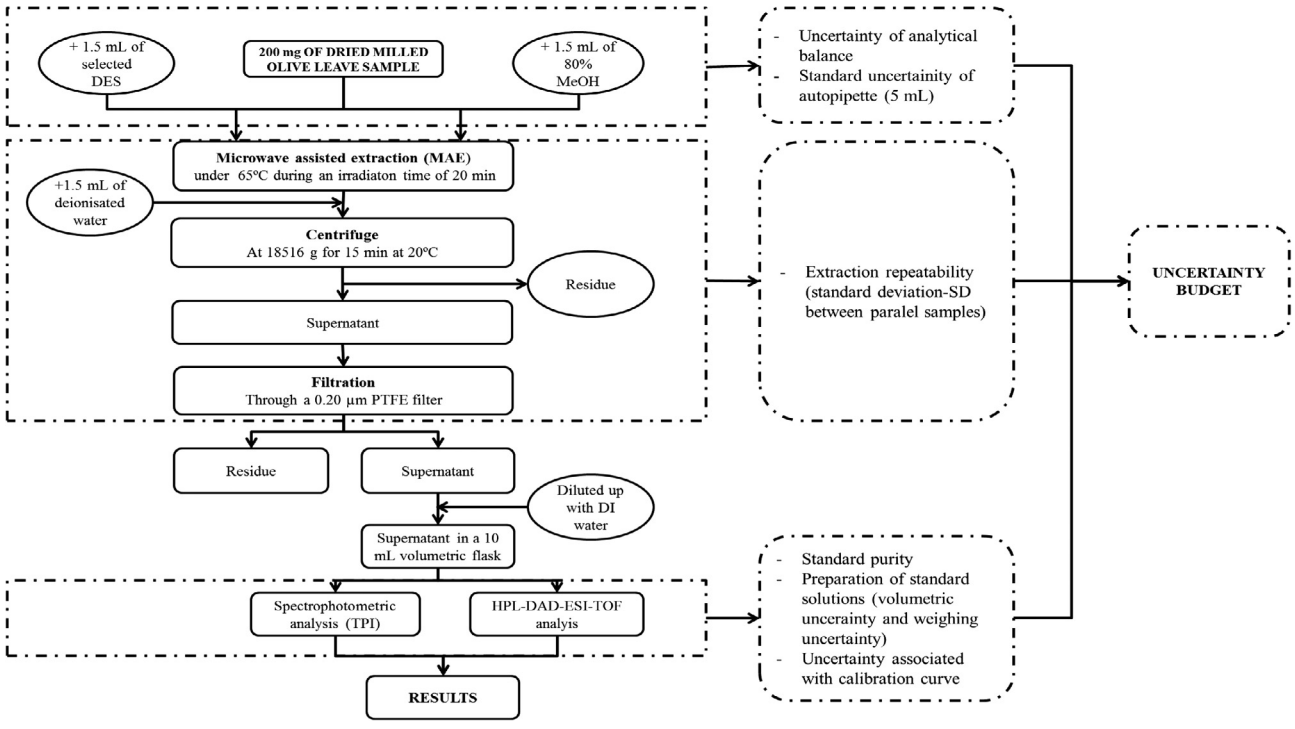

Fig. 1 Flow diagram of determination of phenolic compounds from olive leaves by MAE-DES-HPLC-DAD-ESI-TOF-MS method. Interrupted line represented possible experimental errors (uncertainties) of the proposed analytical procedure.

bation in the dark and at room temperature, 200 μL were transferred into a microplate with 96-wells. The absorbance was then measured at 760 nm in a BioTec spectrophotometer microplate reader (Winooski, Vermont, EEUU). The results were expressed in milligrams of gallic acid equivalents (GAE) per gram of dry weight using a gallic acid standard curve (50–200 μg mL^{-1}). The analysis were performed in triplicate.

2.7. Determination of phenolic compounds by HPLC-DAD-ESI-TOF-MS

Analysis of the phenolic fraction of olive leaf were performed on an Agilent 1200 series Rapid Resolution Liquid Chromatograph (Agilent Technologies, CA, USA) coupled to a micrOTOF (Bruker Datonics, Bremen, Germany), which is an orthogonal-accelerated TOF mass spectrometer, and using an electrospray interface (modelG1607A from Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation was carried out on a Poroshell 120 EC-C18 analytical column (4.6 × 100 mm, 2.7 μm) supplied by Agilent Technologies. The mobile phases used were water with acetic acid 1% (phase A) and acetonitrile (phase B). The linear gradient for solvent B was as follows: 0 min, 5% B; 4 min, 9% B; 7 min, 12% B; 8 min, 15% B; 9 min, 16% B; 14 min, 20% B; 15 min, 22% B; 18 min, 38% B; 19 min, 30% B; 20 min, 31% B; 21.50 min, 32% B; 23 min, 34% B; 24 min, 35% B; 25.5 min, 40% B; 27 min, 50% B; 30 min, 100% B; 35 min, 100% B; 37 min, 5% B (Talhaoui et al., 2014).

The flow rate was set at 0.8 mL min^{-1}. The column temperature was set at 25 °C and a sample volume of 10 μL was injected. The effluent from the HPLC column was split using a T type phase separator before introducing it into the mass spectrometer (split ration 1:3). The final flow that arrived to the ESI-TOF-MS detector was 0.2 mL min^{-1}. The UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240 and 280 nm. The parameters for ESI-TOF-MS were set using a negative ion mode with spectra acquired over a mass range from m/z 50 to 1500. The optimum values of the ESI-TOF-MS parameters were as follows: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L min^{-1}; and nebulizing gas pressure, 2 bar (Gómez-Caravaca et al., 2011). The accurate mass data of the molecular ions were processed through the software Data Analysis 3.4 (Bruker Daltonik GmgH, Bremen, Germany), which provided a list of possible elemental formulas by using the Generate Molecular Formula (GMF) Editor. The Generate Molecular Formula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value) for increased confidence in the suggested molecular formula (Bruker Daltonics Technical Note 008, Molecular formula determination under automation). Prior to compound characterization, all of the spectra were calibrated by using a sodium acetate cluster containing 5 mM of sodium hydroxide and 0.2% of acetic acid in water:isopropanol (1:1, v/v) which was injected at the beginning of each run with a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL) directly connected to the interface.

2.8. Method validation parameters and metrological characteristics of the analytical procedure

The HPLC-DAD-ESI-TOF-MS method for the determination of phenolic compounds in olive leaf was validated for linearity, precision as repeatability, limit of detection (LOD) and limit of quantitation (LOQ).

For that purpose, standard phenolic compounds were dissolved in 80% MeOH and diluted to appropriate concentration ranges (0.1–100 mg L^{-1}) for establishing calibration curves. Oleuropein was used as standard compound for quantitative determination of oleuropein and its derivative, while luteolin was selected as standard compound for quantitative determination of luteoline and its corresponding derivative. The calibration curves were constructed by plotting the average of peak areas versus the concentrations of phenolic compounds selected. For linearity determination, calibration curves were fitted to linear least squares regression (r^2). Additionally, the calibration functions ($\hat{y}_i = ax_i + b$) were calculated from the measured values. The regression parameters α and β were estimated by the last squares estimators a and b considering the quantities that minimize the residual sum of squares, $\sum_{i=1}^{n}(y_i - \hat{y}_i)^2$, where $\hat{y}_i$ is predicted depended variable given by the estimated regression, x_i the known concentration, b the estimated of intercept ($b = \bar{y} - a\bar{x}$), and a is the estimate of slope $\left(a = (\sum(x_i - \bar{x})(y_i - \bar{y}))/\sum(x_i - \bar{x})^2\right)$.

Precision was evaluated through intraday and interday repeatability expressed as the relative standard deviation in percentage (RSD %). The LOD was determined as the minimal concentration of analyte required to obtain a signal-to-noise ratio of 3, and the LOQ was determined as the minimal concentration of analyte required to give a signal-to-noise ratio of 10.

Finally, the phenolic compounds measurement uncertainties were calculated by the bottom-up method from the validation data afforded during each step of the analytical procedure (Konieczka and Namieśnik, 2010). Uncertainty components (Fig. 1) arising from the standard compounds, balances, volumetric measuring devices, calibration curves and repeatability were individually estimated and combined together as an expanded uncertainty using a coverage factor (k = 2) at the 95% confidence level. The detailed calculation for measurement uncertainties are presented in supplementary material (Table S1).

In order to check the significant differences between the extraction yields with conventional and different DESs, a one-way ANOVA test at a confidence level of 95% was applied to the chemical data. The Student–Newman–Keuls's t test was also applied to discriminate among the means of chemical data. All statistical analysis were carried out by using the IBM SPSS statistics 19 for Windows statistical package.

3. Results and discussion

3.1. Screening of DESs

The different components of DESs had significant influence on their physicochemical properties such as polarity, viscosity and solubilisation ability which affected the extraction efficiency of target compounds (Dai et al., 2013a). With the aim to select

the most suitable DES for extraction of phenolic compounds from olive leaf, nine types of DESs were prepared and evaluated in this study. All DESs were based on choline chloride in combination with three different organic acids, four polyalcohols, one sugar and urea at an appropriate mixing molar ratio. The different types of DESs prepared and tested in this study, their abbreviated names and the molar ratios of their components are shown in Table 1.

The viscosity of DESs differed significantly according to their composition. This is their main constraint, since it hinders the handling and efficiency as extraction solvents compared to conventional ones. Thus, the addition of water could adjust the properties of DES and decrease significantly the viscosity and the surface tension. The temperature could also decrease DESs viscosity and improve the extraction yield. For those reasons, a percentage of 25% of water (w/w), 65 °C of temperature and 20 min were set up as initial conditions for the screening extraction of the DESs evaluated. MAE technique was chosen since it is a rapid and efficient extraction technology whose mechanical agitation enhances the efficiency of the extraction. In order to compare the extraction efficiency of DESs to that displayed by a traditional solvent, a conventional extraction with a mixture of methanol/water (80:20, v/v) was performed due to the excellent performance of methanol in previous studies (Taamalli et al., 2012a; Talhaoui et al., 2014) under the same conditions.

The chemical characterization was carried out by means of extracts analysis by HPLC-DAD-ESI-TOF-MS. The tentative identification was based on, the interpretation of their mass spectra determined by TOF-MS, and, the data reported in bibliography (Quirantes-Pine et al., 2013; Talhaoui et al., 2014). Thus, a total of 48 compounds were characterized and quantified in all the extracts. Table 2 shows the compilation of spectral and chromatographic data related to the phenolic compounds characterization process in olive leaf extract. The quantification process was done according to the HPLC-DAD data using calibration curves from several commercial standards. Oleuropein and other secoiridoids were quantified with the calibration curve of oleuropein at $\lambda = 240$. Apigenin and its derivatives were quantified based on a calibration curve of apigenin recorded at $\lambda = 240$; luteolin and its derivatives were quantified with a calibration curve of luteolin at $\lambda = 240$; hydroxytyrosol, its derivatives and verbascoside were quantified based on a calibration curve of hydroxytyrosol recorded at $\lambda = 280$. The calibration plots indicated good correlations between peak areas and analyte concentrations, showing regression coefficients higher than 0.994 in all cases.

Table 1 Composition of deep eutectic solvents used.

Abbreviation	Hydrogen bond acceptors	Hydrogen bond donors	Mole ratio
CCLac	Choline chloride	Lactic acid	1:2
CCOx	Choline chloride	Oxalic acid	1:1
CCTart	Choline chloride	Tartaric acid	2:1
CCBut	Choline chloride	1,4-Butanediol	1:6
CCEtg	Choline chloride	Ethylene glycol	1:2
CCXy	Choline chloride	Xylitol	2:1
CCProp	Choline chloride	1,2-Propanediol	1:1
CCMalt	Choline chloride	Maltose	3:1
CCU	Choline chloride	Urea	1:2

The limit of detection (LOD) was determined as signal-to-noise ratio of 3:1 and the limit of quantification (LOQ) was determined as the signal-to-noise ratio of 10:1. LOD was found to range between 0.03 and 0.64 $\mu g\ mL^{-1}$ while LOQ ranged between 0.12 and 2.03 $\mu g\ mL^{-1}$. An intraday and interday precision test was performed to assess the repeatability of the method. The same sample was injected several times on the same day (intraday precision, n = 6) and also for 3 consecutive days (interday precision, n = 3). The highest intraday repeatability observed was 2.54% RSD, meanwhile the highest interday repeatability was 5.91% RSD.

The HPLC-DAD-TOF-MS analysis of the olive leaf extracts revealed the presence of a wide range of compounds from different families: simple and glycosylated phenols (hydroxytyrosol, hydroxytyrosol glucoside and caffeoylglucoside), iridoids (epiloganic acid), secoiridoids (oleoside/secologanoside, elenolic acid glucoside, oleuropein aglycone, hydroxyl-oleuropein, 2″-methoxyoleuropein, oleuropein, oleuropein glucoside and ligstroside), flavonoids in aglycone form (luteolin and apigenin), glycosylated flavonoids (luteolin rutinoside, apigenin glucoside, rutin, luteolin glucoside, apigenin rutinoside and diosmetin glucoside), phenylpropanoids (verbascoside) and phenethylalcohols (phenethyl primeveroside). Among them, oleuropein was confirmed as the most abundant phenolic compounds in olive leaf. According to the results reported by other authors, the concentration of oleuropein can reach up to 140 $mg\ g^{-1}$ of dry matter in young olives and 60–90 $mg\ g^{-1}$ of dry matter in the olive leaf (Tayoub et al., 2012). Oleuropein is the most important secoiridoid compound due to its pharmacological effects including antioxidant, anti-inflammatory, anticancer, antiviral, antimicrobial, and antiatherogenic (Hamdi and Castellon, 2005; Jemai et al., 2008).

The sum of the total phenolic compounds as well as the yield of the major compounds detected such as oleuropein, oleoside, elenolic acid, luteolin glucoside and ligstroside in extracts, were used for the evaluation of DES extraction efficiency which was defined as mg of phenolic compounds per g of dried plant material (Fig. 2). Calculated values of relative standard uncertainties combined standard uncertainties and expanded uncertainties for the quantitative determination of the phenolic compounds are compiled in Table 3. Metrological parameters were tried to compare with other protocols described in the literature with the same purpose. Unfortunately, no original papers were found in which metrological characterization of analytical procedures were pointed out. In most cases, estimating the uncertainty is limited to calculating the standard deviation (SD) or relative standard deviation (RSD). The same limitation was also reported previously (Konieczka and Namieśnik, 2010).

The results showed significant differences regarding to the yields extracted for the DESs tested. In relation to the organic acid-based DESs, three different acids (lactic acid, oxalic acid and tartaric acid) were used at a proper molar ratio to form eutectic mixtures with choline chloride. Among them, significant higher extraction yields were achieved with choline chloride-lactic acid DES (CCLac). This fact could be attributable to the high viscosity of oxalic and tartaric acid based DES, which hindered the efficiency as extraction solvents due to their low mass transport. Indeed, among all tested DESs, the worst recovery results for the extraction of phenolic compounds from olive leaf were observed in choline chloride-tartaric acid

Table 2 Spectral and chromatographic data regarding to the phenolic compounds identified in olive leaf extract by HPLC-ESI-TOF.

Peak	RT (min)	λ (nm)	*m/z* experimental	*m/z* calculated	Tolerance (ppm)	Error (ppm)	mSigma	Molecular formula	Proposed Compound
1	3.26	240	375.1303	375.1297	10	−0.6	15.3	$C_{16}H_{24}O_{10}$	(Epi)loganic acid isomer 1
2	3.43	240	389.1081	389.1089	10	2.2	24.4	$C_{16}H_{21}O_{11}$	Oleoside /Secologanoside isomer 1
3	3.61	240	389.1085	389.1089	10	1.2	22.5	$C_{16}H_{21}O_{11}$	Oleoside /Secologanoside isomer 2
4	3.96	240	389.1095	389.1086	10	−1.3	22.3	$C_{16}H_{21}O_{11}$	Oleoside /Secologanoside isomer 3
5	4.52	280	315.1075	315.1085	10	3.2	18.7	$C_{14}H_{20}O_{8}$	Hydroxytyrosol-glucoside isomer 1
6	4.78	280	315.1073	315.1085	10	3.9	9.4	$C_{14}H_{20}O_{8}$	Hydroxytyrosol-glucoside isomer 2
7	5.00	280	153.0550	153.0557	10	4.7	15.7	$C_{8}H_{10}O_{3}$	Hydroxytyrosol
8	5.10	240	389.1100	389.1089	10	−2.9	21.1	$C_{16}H_{22}O_{11}$	Oleoside/Secologanoside isomer 4
9	5.55	240	375.1288	375.1297	10	2.4	17.8	$C_{16}H_{24}O_{10}$	(Epi)loganic acid isomer 2
10	7.36	280	339.0722	339.0722	10	0.0	55.6	$C_{15}H_{16}O_{9}$	Esculin
11	8.01	280	341.0866	341.0878	10	3.5	30.9	$C_{15}H_{18}O_{9}$	Caffeoylglucoside
12	9.28	240	389.1095	389.1086	10	−1.3	22.3	$C_{16}H_{22}O_{11}$	Oleoside/Secologanoside isomer 5
13	10.05	240	389.1139	389.1089	20	−12.8	27.2	$C_{16}H_{22}O_{11}$	Olesoide/Secologanoside isomer 6
14	11.29	240	403.1267	403.1246	10	−5.2	8.9	$C_{17}H_{24}O_{11}$	Elenolic acid glucoside isomer 1
15	12.32	240	377.1466	377.1453	10	−3.4	14.1	$C_{16}H_{26}O_{10}$	Oleuropein/oleuroside aglycone
16	13.15	240	609.1479	609.1461	10	−2.9	28.0	$C_{27}H_{30}O_{16}$	Glucosyl rhamnosylquercetin (rutin) isomer 1
17	16.30	240	403.1254	403.1246	10	−2.0	3.1	$C_{17}H_{24}O_{11}$	Elenolic acid glucoside isomer 2
18	13.68	240	415.1612	415.1610	10	−0.6	5.8	$C_{19}H_{28}O_{10}$	Phenethyl primeveroside isomer 1
19	13.88	240	415.1607	415.1610	10	0.7	7.3	$C_{19}H_{28}O_{10}$	Phenethyl primeveroside isomer 2
20	14.58	240	403.1973	403.1974	10	0.0	3.3	$C_{19}H_{32}O_{9}$	Ethyl-glucopyranosyloxy-oxopropyl-cyclohexaneacetic acid
21	15.35	240	555.1804	555.1719	20	−15.3	58.7	$C_{25}H_{32}O_{14}$	Hydroxyoleuropein/hydroxyoleuroside isomer 1
22	15.52	240	555.1810	555.1719	20	−16.3	55.3	$C_{25}H_{32}O_{14}$	Hydroxyoleuropein/hydroxyoleuroside isomer 2
23	15.69	240	609.1485	609.1461	10	−3.9	27.7	$C_{27}H_{30}O_{16}$	Glucosyl rhamnosylquercetin (rutin) isomer 2
24	15.82	240	593.1551	593.1512	10	−6.5	36.3	$C_{27}H_{29}O_{15}$	Luteolin rutinoside
25	16.50	240	447.1014	447.0933	20	−18.1	66.6	$C_{21}H_{20}O_{11}$	Luteolin glucoside isomer 1
26	16.72	240	623.1979	623.1981	10	0.3	11.2	$C_{29}H_{36}O_{15}$	Verbascoside
27	16.86	240	555.1727	555.1719	10	−1.4	4.3	$C_{25}H_{32}O_{14}$	Hydroxyoleuropein/hydroxyoleuroside isomer 3
28	18.06	240	577.1604	577.1563	10	−7.2	12.0	$C_{27}H_{30}O_{14}$	Apigenin rutinoside
29	18.23	240	701.2301	701.2298	10	−0.3	9.9	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 1
30	18.50	240	701.2307	701.2298	10	−1.2	9.2	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 2
31	18.88	240	701.2336	701.2298	10	−5.4	30.4	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 3
32	18.98	240	431.1017	431.0984	10	−7.7	15.0	C21H20O10	Apigenin glucoside
33	19.11	240	447.0974	447.0933	10	−9.2	30.5	C21H20O11	Luteolin glucoside isomer 2
34	19.45	240	461.1129	461.1089	10	−8.7	15.3	C22H22O11	Diosmetin glucoside
35	19.80	240	701.2307	701.2298	10	−1.3	12.3	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 4
36	20.35	240	701.2345	701.2298	10	−6.7	39.7	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 5
37	20.07	240	569.1930	569.1876	10	−9.5	34.6	$C_{26}H_{34}O_{14}$	2″-methoxyoleuropein isomer 1
38	20.25	240	569.1941	569.1876	20	−11.5	52.5	$C_{26}H_{34}O_{14}$	2″-methoxyoleuropein isomer 2
39	20.60	240	539.1931	539.1770	30	−29.8	13.9	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside isomer 1
40	21.21	240	539.1791	539.1770	10	−3.8	22.1	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside isomer 2
41	21.57	240	539.1888	539.1770	30	−21.9	59.4	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside isomer 3
42	21.87	240	557.2253	557.2240	10	−2.5	8.3	$C_{26}H_{38}O_{13}$	(dimethyl hydroxy octenoyloxi) secologanoside isomer 1
43	22.44	240	523.1892	523.1821	20	−13.5	32.8	$C_{25}H_{32}O_{12}$	Ligstroside
44	23.00	240	285.0412	285.0405	10	−2.8	0.7	$C_{15}H_{10}O_{6}$	Luteolin
45	23.30	240	557.2243	557.2240	10	−0.6	17.3	$C_{26}H_{38}O_{13}$	(dimethyl hydroxy octenoyloxi) secologanoside isomer 2
46	23.50	240	553.1927	553.1927	10	−0.1	14.6	$C_{26}H_{34}O_{13}$	Oleuropein/oleuroside methyl ether
47	23.85	240	539.1778	539.1770	10	−1.4	17.2	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside isomer 4
48	25.45	240	269.0457	269.0455	10	−0.5	92.8	$C_{15}H_{10}O_{5}$	Apigenin

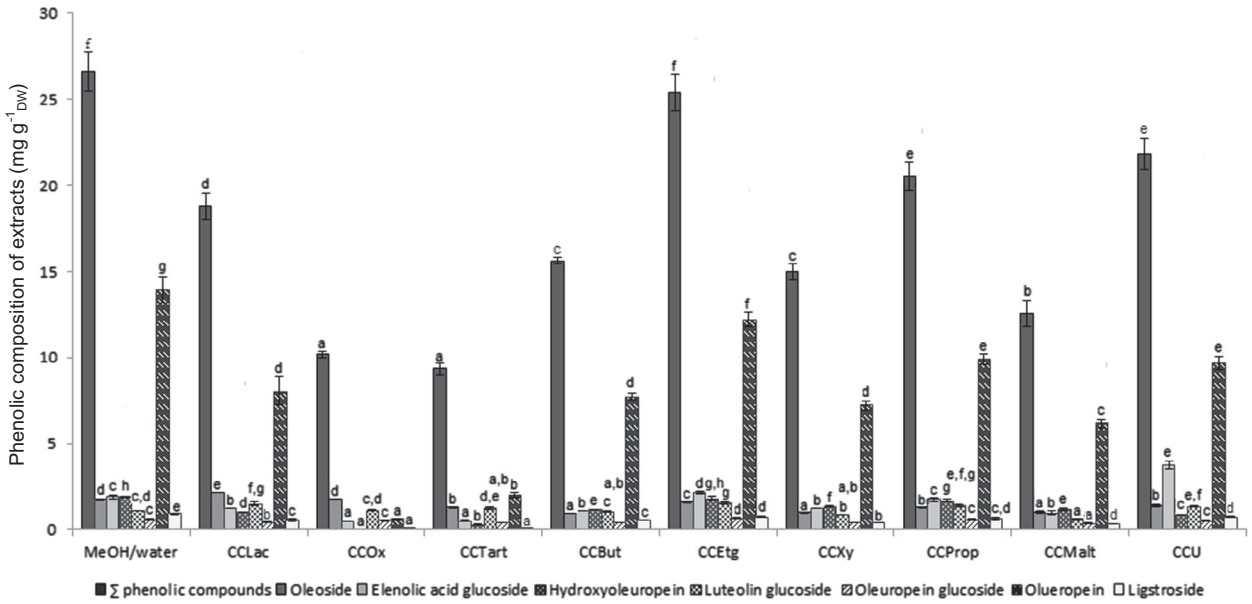

Fig. 2 Effect of different DESs on extraction efficiency of phenolic composition in comparison with a mixture of methanol/water (80:20). Extraction conditions were water content: 25%; extraction temperature: 65C, irradiation time: 20 min. Different superscripts for the same compound denoted significant differences among solvents tested according to the Student–Newman–Keuls method at $P < .05$.

(CCTart) and choline chloride-oxalic acid (CCOx). Comparing these two solvents, not statistical differences were found in the total yield of phenolic compounds. However, interestingly, the extraction of oleuropein seemed to be better for CCTart than for CCOx. This result highlighted the fact that the extraction of oleuropein seemed to be influenced by the pH value of solvents (pKa values are 1.25 and 2.89 for oxalic acid and tartaric acid, respectively). Better recoveries of oleuropein seemed to be achieved with solvents with not extreme pH values (García et al., 2016).

In general terms, polyalcohol-based DESs, showed high ability for extraction of phenolic compounds from olive leaf compared to other tested solvents. This statement is in good agreement with those reported by other authors who have pointed to polyalcohol-based DESs as excellent extraction solvents for phenolic acids and other phenolic compounds from several types of oils and plants, such as, *Lonicerae japonicae* and *Pyrola incarnata* (García et al., 2016; Khezeli, Daneshfar and Sahraei, 2016; Peng et al., 2016; Yao et al., 2015). This fact is likely to be explained by the lowest viscosity and the highest hydrogen bonding ability of these solvents. In our specific case, several polyalcohols such as 1,4-butanediol, ethylene glycol, xylitol and 1,2-propanediol were tested. Among them, choline chloride-ethylene glycol (CCEtg) was found to be the best alcohol-based DES for the extraction of phenolic compounds from olive leaf. Due to its polarity, ethylene glycol has significant polar interactions (dipole-type and hydrogen bonding interactions) with polar compounds such as phenolic compounds. Furthermore, the linear structure of ethylene glycol, seems to provide easier interactions between the targeted compounds and choline chloride than those achieved with branched structures, such as 1,2-propanediol (CCProp) or xylitol (CCXy). This could imply a major steric hindrance (Khezeli et al., 2016). Indeed, CCEtg was revealed as the best of the DES tested for the extraction of phenolic compounds from olive leaf. In comparison with conventional extraction by using methanol:water (80:20) as solvent, no significant differences were found in the extraction efficiency of the total phenolic compounds using CCEtg. The use of CCEtg exhibited significant slightly lower quantities of oleuropein in comparison with methanol:water. However, when CCEtg was employed as solvent extraction major recoveries of glycoside derivatives of some secoiridoids and flavonoids, such as, elenolic acid glucoside, luteolin glucoside and oleuropein glucoside were detected.

For the last few years, special attention has been paid to DESs based on sugars, since they are primary metabolites, classifying them as NADES. In this study, it is noteworthy the poor affinity of choline chloride-maltose (CCMalt) with phenolic compounds from olive leaf in comparison with the rest of DESs tested. However, it has been demonstrated the extraction capacity of DES based on sugars for other application such as, the isolation of anthocyanins from the grape skin and, the main phenolic metabolites from *Cartahmus tinctorius*: hydroxysafflor yellow A (HSYA), cartormin and carthamin (Dai et al., 2013b; Jeong et al., 2015). On the other hand, urea can also act as an hydrogen bond donor and form an eutectic mixture with choline chloride. According to the results obtained, choline chloride-Urea (CCU) was revealed as an excellent solvent for the extraction of phenolic compounds form olive leaf. CCU exhibited similar extraction capacity than CCProp for the total phenolic compounds and for almost all of the individual compounds under consideration, with the exception of the elenolic acid glucoside content which was significantly higher in extracts obtained by CCU.

Therefore, taking into account the results obtained, the order of efficiency of DES tested for the extraction of phenolic compounds from olive leaf based on the sum of phenolic compounds detected was: CCEtg > CCU ≈ CCProp > CCLac > CCBut ≈ CCXy > CCMalt > CCOx > CCTart.

Table 3 Mean values and metrological parameters for the methodology used by means of MeOH (80:20) and different DES evaluated for the phenolic compounds determination from olive leaf.

Parameter	Value							
Analyte	TPI	Oleoside	Elenolic acid glucoside	Hydroxyoleuropein	Luteolin glucoside	Oleuropein glucoside	Oleuropein	Ligstroside
Calibration curve								
Standard compound	*Gallic acid*	*Oleuropein*	*Oleuropein*	*Oleuropein*	*Luteolin*	*Oleuropein*	*Oleuropein*	*Oleuropein*
N	7	9	9	9	10	9	9	9
b ± ts_a (95%)	0.0009 ± 0.00001	29.103 ± 9.199	29.103 ± 9.199	29.103 ± 9.199	84.898 ± 8.327	29.103 ± 9.199	29.103 ± 9.199	29.103 ± 9.199
a ± ts_b (95%)	0.0536 ± 0.012	2.743 ± 0.230	2.743 ± 0.230	2.743 ± 0.230	−3.022 ± 0.216	2.743 ± 0.230	2.743 ± 0.230	2.743 ± 0.230
r^2	0.9951	0.9999	0.9999	0.9999	1.000	0.9999	0.9999	0.9999
LOD		0.035	0.035	0.035	0.042	0.035	0.035	0.035
LOQ		0.28	0.28	0.28	0.12	0.28	0.28	0.28
Uncertainty (standard)								
Mass of sample-$u_{(sample)}$	$2.55{\cdot}10^{-6}$	$2.55{\cdot}10^{-6}$	$2.55{\cdot}10^{-6}$	$2.55{\cdot}10^{-6}$	$2.55{\cdot}10^{-6}$	$2.55{\cdot}10^{-6}$	$2.55{\cdot}10^{-6}$	$2.55{\cdot}10^{-6}$
Autopipett 5 mL-$u_{(vol)}$	$1.36{\cdot}10^{-6}$	$1.36{\cdot}10^{-6}$	$1.36{\cdot}10^{-6}$	$1.36{\cdot}10^{-6}$	$1.36{\cdot}10^{-6}$	$1.36{\cdot}10^{-6}$	$1.36{\cdot}10^{-6}$	$1.36{\cdot}10^{-6}$
Standard preparation-$u_{(std)}$	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013
Calibration curve-$u_{(cal)}$	0.729	0.729	0.729	0.729	0.729	0.729	0.729	0.729
Uncertainty (depended on the extraction solvent used)								
MeOH (control)								
Concentration (mg g^{-1})	23.57	1.80	1.93	1.95	1.12	0.68	14.08	0.95
Repeatability-RSD (%)	6.1	1.0	6.8	1.9	4.2	1.3	5.5	4.2
Repeatability-$u_{(rep)}$	1.29	0.02	0.09	0.03	$3.54{\cdot}10^{-3}$	0.01	0.54	0.03
Combined uncertainty	1.49	0.07	0.11	0.07	0.02	0.06	0.56	0.06
Expanded uncertainty (k = 2)	2.97	0.13	0.22	0.13	0.03	0.12	1.11	0.13
Result (mass ± $U_{(k=2)}$ mg g^{-1})	**23.57 ± 2.97**	**1.80 ± 0.13**	**1.93 ± 0.22**	**1.95 ± 0.13**	**1.12 ± 0.03**	**0.68 ± 0.12**	**14.08 ± 1.11**	**0.95 ± 0.13**
CCLac								
Concentration (mg g^{-1})	23.92	2.22	1.25	1.04	1.45	0.42	8.20	0.60
Repeatability-RSD (%)	5.1	1.4	1.3	1.2	7.4	3.9	10.5	14.2
Repeatability-$u_{(rep)}$	1.03	0.02	0.01	0.01	0.08	0.01	0.59	0.06
Combined uncertainty	1.26	0.07	0.06	0.06	0.08	0.06	0.60	0.09
Expanded uncertainty (k = 2)	2.52	0.13	0.12	0.12	0.16	0.12	1.20	0.17
Result (mass ± $U_{(k=2)}$ mg g^{-1})	**23.92 ± 2.52**	**2.22 ± 0.13**	**1.25 ± 0.12**	**1.04 ± 0.12**	**1.45 ± 0.16**	**0.42 ± 0.12**	**8.20 ± 1.20**	**0.60 ± 0.17**
CCOx								
Concentration (mg g^{-1})	26.61	1.83	1.03	0.03	1.11	0.46	0.65	**Below LOQ**
Repeatability-RSD (%)	4.7	1.8	3.4	13.3	4.0	6.8	4.3	
Repeatability-$u_{(rep)}$	0.88	0.02	0.01	$2.11{\cdot}10^{-4}$	0.03	0.03	0.02	
Combined uncertainty	1.14	0.07	0.06	**Below LOQ**	0.03	0.07	0.06	
Expanded uncertainty (k = 2)	2.28	0.13	0.12		0.07	0.13	0.13	
Result (mass ± $U_{(k=2)}$ mg g^{-1})	**26.61 ± 2.28**	**1.83 ± 0.13**	**1.03 ± 0.12**		**1.11 ± 0.07**	**0.46 ± 0.13**	**0.65 ± 0.13**	

CCTart								
Concentration (mg g^{-1})	24.10	1.35	0.52	0.30	1.26	0.28	1.92	**Below LOQ**
Repeatability-RSD (%)	6.3	2.3	5.5	3.3	4.0	1.4	8.3	
Repeatability-$u_{(rep)}$	0.52	0.02	0.02	0.01	0.04	$4.24 \cdot 10^{-3}$	0.12	
Combined uncertainty	1.29	0.07	0.07	0.06	0.04	0.06	0.15	
Expanded uncertainty ($k = 2$)	2.58	0.13	0.13	0.12	0.08	0.12	0.29	
Result (mass ± $U_{(k=2)}$ mg g^{-1})	**24.10 ± 2.58**	**1.35 ± 0.13**	**0.52 ± 0.13**	**0.3 ± 0.12**	**1.26 ± 0.08**	**0.28 ± 0.12**	**1.92 ± 0.29**	
CCBut								
Concentration (mg g^{-1})	15.68	0.97	1.11	1.20	1.06	0.39	7.85	0.57
Repeatability-RSD (%)	4.8	2.1	9.9	0.4	0.2	1.4	2.4	2.1
Repeatability-$u_{(rep)}$	0.52	0.02	0.01	$3.53 \cdot 10^{-3}$	$1.41 \cdot 10^{-3}$	$4.24 \cdot 10^{-3}$	0.13	0.01
Combined uncertainty	1.00	0.06	0.06	0.06	0.01	0.06	0.16	0.06
Expanded uncertainty ($k = 2$)	2.00	0.12	0.12	0.12	0.03	0.12	0.31	0.12
Result (mass ± $U_{(k=2)}$ mg g^{-1})	**15.68 ± 2.00**	**0.97 ± 0.12**	**1.11 ± 0.12**	**1.20 ± 0.12**	**1.06 ± 0.03**	**0.39 ± 0.12**	**7.85 ± 0.31**	**0.57 ± 0.12**
CCEtg								
Concentration (mg g^{-1})	25.00	1.60	2.15	1.82	1.54	0.60	12.06	0.76
Repeatability-RSD (%)	5.3	1.3	3.5	6.7	5.9	4.0	3.2	9.7
Repeatability-$u_{(rep)}$	0.92	0.01	0.05	0.08	0.06	0.02	0.28	0.05
Combined uncertainty	1.17	0.06	0.08	0.10	0.07	0.07	0.29	0.08
Expanded uncertainty ($k = 2$)	2.34	0.12	0.16	0.21	0.13	0.13	0.58	0.16
Result (mass ± $U_{(k=2)}$ mg g^{-1})	**25.00 ± 2.34**	**1.60 ± 0.12**	**2.15 ± 0.16**	**1.82 ± 0.21**	**1.54 ± 0.13**	**0.60 ± 0.13**	**12.06 ± 0.58**	**0.76 ± 0.16**
CCXy								
Concentration (mg g^{-1})	16.22	1.06	1.25	1.40	0.87	0.39	7.33	0.85
Repeatability-RSD (%)	4.2	2.9	0.1	1.5	1.3	1.1	3.7	2.5
Repeatability-$u_{(rep)}$	0.48	0.02	$5.44 \cdot 10^{-5}$	0.02	0.01	$3.39 \cdot 10^{-3}$	0.18	0.01
Combined uncertainty	0.97	0.07	0.06	0.07	0.02	0.06	0.20	0.06
Expanded uncertainty ($k = 2$)	1.93	0.13	0.12	0.13	0.03	0.12	0.40	0.12
Result (mass ± $U_{(k=2)}$ mg g^{-1})	**16.22 ± 1.93**	**1.06 ± 0.13**	**1.25 ± 0.12**	**1.40 ± 0.13**	**0.87 ± 0.03**	**0.39 ± 0.12**	**7.33 ± 0.40**	**0.85 ± 0.12**
CCProp								
Concentration (mg g^{-1})	21.06	1.36	1.78	1.71	1.40	0.65	9.88	0.65
Repeatability-RSD (%)	6.2	3.7	7.4	4.9	4.6	6.9	2.8	6.5
Repeatability-$u_{(rep)}$	0.93	0.03	0.09	0.06	0.05	0.03	0.20	0.03
Combined uncertainty	1.20	0.07	0.11	0.08	0.05	0.07	0.22	0.07
Expanded uncertainty ($k = 2$)	2.39	0.14	0.22	0.17	0.10	0.14	0.43	0.13
Result (mass ± $U_{(k=2)}$ mg g^{-1})	**21.06 ± 2.39**	**1.36 ± 0.14**	**1.78 ± 0.22**	**1.71 ± 0.17**	**1.40 ± 0.10**	**0.65 ± 0.14**	**9.88 ± 0.43**	**0.65 ± 0.13**
CCMalt								
Concentration (mg g^{-1})	12.10	1.04	0.98	1.17	0.60	0.30	6.22	0.36
Repeatability-RSD (%)	11.4	7.7	9.2	7.8	6.8	2.5	4.3	6.7
Repeatability-$u_{(rep)}$	0.96	0.05	0.06	0.07	0.03	6.72 ± 10^{-3}	0.18	0.02
Combined uncertainty	1.35	0.08	0.09	0.09	0.03	0.06	0.20	0.06
Expanded uncertainty ($k = 2$)	2.70	0.16	0.18	0.18	0.07	0.12	0.39	0.13
Result (mass ± $U_{(k=2)}$ mg g^{-1})	**12.10 ± 2.70**	**1.04 ± 0.16**	**0.98 ± 0.18**	**1.17 ± 0.18**	**0.60 ± 0.07**	**0.30 ± 0.12**	**6.22 ± 0.39**	**0.36 ± 0.13**

(*continued on next page*)

Table 3 (*continued*)

Parameter	Value							
Analyte	TPI	Oleoside	Elenolic acid glucoside	Hydroxyoleuropein	Luteolin glucoside	Oleuropein glucoside	Oleuropein	Ligstroside
CCU								
Concentration (mg g^{-1})	23.75	1.45	4.03	0.83	1.34	0.51	9.79	0.72
Repeatability-RSD (%)	4.4	3.5	6.4	1.4	2.8	4.7	4.0	5.7
Repeatability-$u_{(rep)}$	0.74	0.03	0.18	0.01	0.03	0.02	0.28	0.03
Combined uncertainty	1.04	0.07	0.18	0.06	0.03	0.06	0.29	0.06
Expanded uncertainty ($k = 2$)	2.07	0.14	0.35	0.12	0.06	0.13	0.58	0.13
Result (mass ± $U_{(k=2)}$) mg g^{-1})	**23.75 ± 2.07**	**1.45 ± 0.14**	**4.03 ± 0.35**	**0.83 ± 0.12**	**1.34 ± 0.06**	**0.51 ± 0.13**	**9.79 ± 0.58**	**0.72 ± 0.13**

Thus, no significant differences were found between the total phenolic compounds extracted with CCEtg and the conventional solvent (methanol:water).

3.2. Optimization of DES extraction conditions by using an experimental design

Choline chloride-ethylene glycol (CCEtg) based DES was selected as the most promising for the extraction of phenolic compounds from the olive leaf. As mentioned above, in order to optimize the most important MAE extraction parameters, an experimental design was applied. The response surface methodology (RSM) is a useful way for studying the effect of several factors influencing the system, by varying them simultaneously when carrying out a limited number of experiments. To the best of our knowledge, the effect of microwaves on the extraction of olive leaf phenolic compounds has not been yet studied. In the present work, the focus was on the temperature, the solvent composition and the microwave irradiation time, which are recognized as the most important parameters affecting the MAE efficiency (Pellati et al., 2013). Microwave power and pressure are parameters directly dependent on the selected temperature, and were not chosen for method optimization.

According to the recently published results, it was found out that the use of these solvents at higher temperatures has some limitations related to the thermal-stability (Haz et al., 2016; Craveiro et al., 2016). Therefore, in order to avoid the degradation of the DES, the extraction temperature ranged from 40 °C to 80 °C. On the other hand, the percentage of water in DES is also an important factor to take into account, due to the fact that the addition of water is essential to reduce viscosity and improve handling. However, excessive addition of water could cause the breaking of hydrogen bonds and consequently destroy the eutectic nature of the DES solvents (Paiva et al., 2014). For that reasons, the water content evaluated ranged from 0 to 70%. The irradiation time is another factor that could have certain influence on the extraction yield. The optimization of this parameter is required to avoid lengthening the isolation process but without compromising the extraction efficiency. Consequently, the range of irradiation time varied from 10 to 40 min in the study.

These three independent variables were coded at three levels (−1, 0, +1), and a total of 15 experiments with three replications in the central point were performed in random order to avoid systematical mistakes. The sum of the total individual phenolic compounds characterized by HPLC-DAD-ESI-TOF-MS, the TPI and the extraction yield of oleuropein, as the most represented phenolic compounds in olive leaf, were selected as the main depended variables. Table 4 shows the coded and uncoded levels of the independent variables provided by the BBD experimental design together with the experimental values of the main responses, expressed in mg g^{-1}, and the predicted values provided by the statistical model. All the predicted values were in a level of confidence interval of 95%.

The experimental results obtained were further mathematically processed, in order to build a quadratic model, by applying second-order polynomial equations without data transformation. Table 5 summarizes all important parameters related to the analysis of variance (ANOVA). ANOVA tests showed that the models adequately represented the

Table 4 Box-Behnken design (BBD) experimental design with the independent variables and experimental data for the total phenol index, oleuropein and sum of individual phenolic compounds detected. The predicted values provided by the statistical model for the responses are also included.

Run	T, °C	Variables		Responses mg g_{DW}^{-1}								
				Experimental values			Predicted values			Confidence Interval, 95%		
		I.T[ϑ], min	% water	TPI[*,#]	Oleuropein[#]	∑ phenolic compounds[γ]	TPI[*]	Oleuropein	∑ phenolic compounds	TPI[*]	Oleuropein	∑ phenolic compounds
1	80 (1)	25 (0)	0 (−1)	22.28 ± 2.63	10.25 ± 0.23	20.64 ± 1.36	21.96	10.18	20.60	20.99–22.92	9.71–10.64	19.24–21.97
2	40 (−1)	25 (0)	70 (1)	26.10 ± 2.72	10.35 ± 0.42	20.31 ± 0.99	26.43	10.42	20.34	25.46–27.39	9.95–10.89	18.98–21.70
3	60 (0)	40 (1)	70 (1)	27.61 ± 2.53	10.15 ± 0.57	22.62 ± 1.20	27.66	10.27	22.73	26.69–21.37	9.80–10.73	28.62–24.10
4	80 (1)	40 (1)	35 (0)	28.55 ± 1.12	11.56 ± 0.32	26.37 ± 0.63	28.55	11.44	25.83	27.58–29.52	10.98–11.91	24.47–27.19
5	60 (0)	40 (1)	0 (−1)	18.67 ± 1.36	8.11 ± 0.32	16.60 ± 0.23	18.99	8.30	17.17	18.03–19.96	7.84–8.77	15.81–18.54
6	40 (−1)	40 (1)	35 (0)	27.21 ± 2.52	11.00 ± 0.34	22.99 ± 0.39	26.84	10.82	22.83	25.87–27.80	10.35–11.29	21.47–24.20
7	80 (1)	25 (0)	70 (1)	31.62 ± 1.65	11.40 ± 0.30	26.57 ± 0.85	31.57	11.40	26.98	30.61–32.54	10.94–11.87	25.62–28.35
8	40 (−1)	25 (0)	0 (−1)	20.64 ± 1.42	8.50 ± 0.35	17.29 ± 0.36	20.69	8.50	17.40	19.72–21.65	8.03–8.96	16.04–18.77
9	60 (0)	10 (−1)	0 (−1)	20.18 ± 1.09	9.21 ± 0.22	18.56 ± 0.10	20.13	9.10	18.44	19.17–21.10	8.63–9.56	17.08–19.80
10	80 (1)	10 (−1)	35 (0)	29.83 ± 4.95	12.37 ± 0.19	27.97 ± 0.86	30.20	12.56	28.12	29.24–31.17	12.09–13.03	26.76–29.49
11	60 (0)	10 (−1)	70 (1)	27.16 ± 4.25	10.48 ± 0.17	22.77 ± 0.13	26.83	10.29	22.20	25.87–27.80	9.82–10.76	20.83–23.56
12	40 (−1)	10 (−1)	35 (0)	25.50 ± 2.51	10.40 ± 0.35	20.74 ± 0.45	25.50	10.52	21.27	24.53–26.46	10.05–10.99	19.91–22.64
13	60 (0)	25 (0)	35 (0)	30.63 ± 2.23	11.44 ± 0.76	24.44 ± 0.14	30.63	11.38	24.06	29.71–31.00	11.07–11.69	23.15–24.97
14	60 (0)	25 (0)	35 (0)	30.45 ± 1.96	11.37 ± 0.38	23.92 ± 0.22	30.35	11.38	24.06	29.71–31.00	11.07–11.69	23.15–24.97
15	60 (0)	25 (0)	35 (0)	29.98 ± 2.32	11.33 ± 0.63	23.82 ± 0.54	30.35	11.38	24.06	29.71–31.00	11.07–11.69	23.15–24.97

I.T[ϑ].: Irradiation time expressed in minutes

[*] TPI: total phenol index expressed as mg of gallic acid equivalents g^{-1} of olive leaf dry weight (n = 6).

[#] Selected responses are expressed as mean value ± expanded uncertainties (k = 2) at 95% confidence level (for TPI n = 6, for oleuropein n = 2).

[γ] Sum of individual phenolic compounds detected in HPLC-DAD-ESI-TOF-MS expressed as mg g^{-1} of olive leaf dry weight ± standard deviation (n = 2).

Table 5 Analysis of variance for response surface polynomial model of TPI, sum of phenolic compounds and oleuropein content.

Polynomal term	Responses								
	TPI			Oleuropein			∑ phenolic compounds		
	Sum of squares	*p* value	Coefficient	Sum of squares	*p* value	Coefficient	Sum of squares	*p* value	Coefficient
Model	241.95	< .0001**		19.38	.0002**		150.80	.0003**	
Intercept			30.35			11.38			24.06
Linear term									
A (Temperature)	20.59	.0001**	1.60	3.55	.0003**	0.67	48.46	< .0001**	2.46
B (Extraction time)	0.05	.6294	−0.079	0.33	.0404*	−0.20	0.27	.4366	−0.18
C (Water)	117.93	< .0001**	3.84	4.98	.0001**	0.79	43.40	.0001**	2.33
Quadratic term									
AA	0.63	.1273	−0.41	0.32	.0428*	0.30	2.52	.0486*	0.83
BB	17.36	.0002**	−2.17	0.43	.0267*	−0.34	0.50	.2989	−0.37
CC	84.36	< .0001**	−4.78	8.88	< .0001**	−1.55	46.58	.0001**	−3.55
Interactions									
AB	2.24	.0183*	−0.75	0.50	.0203*	−0.35	3.71	.0255*	−0.96
AC	3.75	.0066**	0.97	0.12	.1582	−0.17	2.96	.0375*	0.86
BC	0.96	.0734	0.49	0.15	.1267	0.19	0.81	.2008	0.45
Lack of fit	0.72	.3369		0.21	.072		0.55	.1739	
R^2	0.9961			0.9887			0.9877		
Adjusted R^2	0.9899			0.9684			0.9656		
Predicted R^2	0.9508			0.8240			0.8238		
Adeq. Precision	35.519			24.785			21.903		

** Significant at $p < .01$.
* Significant at $p < .05$.

experimental data at a 95% and 99% confidence level. The correlation coefficients (R^2) of the main three variables of response were calculated to indicate the difference between experimental and predictive values. R^2 values were 0.996, 0.988 and 0.989 for TPI, sum of the phenolic compounds detected and oleuropein, respectively. All correlation coefficients indicated that the responses were in really good agreement with the predicted extraction yields. Table 5 also shows the coefficient of the second order polynomial equations to represent the extraction yield adequately for each response.

For TPI, temperature (A), percentage of water (C), the quadratic term of irradiation time (BB), the quadratic term of percentage of water (CC), and the interaction between temperature and irradiation time (AB) and temperature and percentage of water (AC) were significant (*p* value < .05). This means that these terms had a significant impact on the extraction yield of TPI. Consequently, the model should be reduced as follows to represent the extraction yield of TPI adequately:

$$Y_1 = 30.35 + 1.60A + 3.84C - 2.17BB - 4.78CC - 0.75AB + 0.97BC$$

The model for TPI was significant at a 99% confidence level (*p* value < .01) and the "Lack of Fit *F-value*" was insignificant which confirmed that the model could perfectly fit the responses variables with a good prediction. The maximal value of TPI (32.67 mg g^{-1}) was obtained with 79.79 °C of temperature, 19.86 min of irradiation time and 50.19% of water.

With regards to the sum of individual phenolic compounds detected in extracts, the model was also significant as opposed to "Lack of Fit F-value". For this response, the linear and quadratic term of irradiation time (B, and BB) as well as the interaction between irradiation time and percentage of water (BC) did not show influence on the extraction process. Therefore, the model was reduced as follows:

$$Y_2 = 24.06 + 2.46A + 2.33C + 0.83AA - 3.55CC - 0.96AB + 0.86AC$$

The highest value of the sum of individual phenolic compounds was found to be 28.52 mg g^{-1} which was obtained at a temperature of 79.98 °C, 15.28 min of irradiation time and 48.63% of water.

The model was also significant for the most representative compound of olive leaf, oleuropein. The interaction between temperature and percentage of water of DES (AC) as well as the interaction between irradiation time and percentage of water (BC) was insignificant. Consequently, the reduced equation was:

$$Y_3 = 11.38 + 0.67A + 0.79C + 0.30AA + 1.55CC - 0.35AB$$

The maximal value of oleuropein (10.58 mg g^{-1}) was obtained with at a temperature of 79.64 °C, for 16.69 min of irradiation time and with a percentage of water of 43.34%.

Quadratic models based on second order polynomial equations were also built for the rest of the individual phenolic compounds detected in olive leaf. Significant models were found for olesoide/secologanoside (R^2 = 0.985), caffeoylglucoside (R^2 = 0.991), elenolic acid glucoside (R^2 = 0.992), phenethyl primeveroside (R^2 = 0.986), apigenin rutinoside (R^2 = 0.987) and 2″-metoxyoleuropein (R^2 = 0.979). Analysis of variance for response surface polynomial moles are compiled in the supplementary material (Table S2).

To investigate the interactive effects of operational parameters on the extraction yields, the three-dimensional profiles of multiple non-linear regression models were depicted, as shown in Fig. 3. The response surface plots are the graphical output of the interaction between two independent variables, while the third is fixed in the central point. The response surface (Fig. 3A–C) showed the interaction percentage of water, temperature and irradiation time on the extraction yield of TPI. It was possible to observe that the TPI yield was enhanced by the increase in the water content. However, when a certain percentage of water is reached, the extraction yield of TPI decreased. This fact can be explained by a decrease in the viscosity of the DES which improved the transfer of mass. However major quantities of water seemed to have a detrimental effect on extraction yield, likely to be, the breakage of the eutectic mixture (Dai et al., 2013a). An increase in the temperature showed a positive effect on the extraction efficiency as result of its influence on diffusion, viscosity, surface tension and solubility of target compounds. High temperature can increase the diffusion coefficient of the solute from the solvent, reduce the viscosity and the surface tension and improve solute's solubility (Chen et al., 2015). The effect of irradiation time on the TPI yield was less noticeable than for the other parameters. Similar trends were observed for both the sum of individual phenolic compounds (Fig. 3D–F) and the oleuropein (Fig. 3G–I). In order to maximize extraction yields of all the variables taken into account, the operational

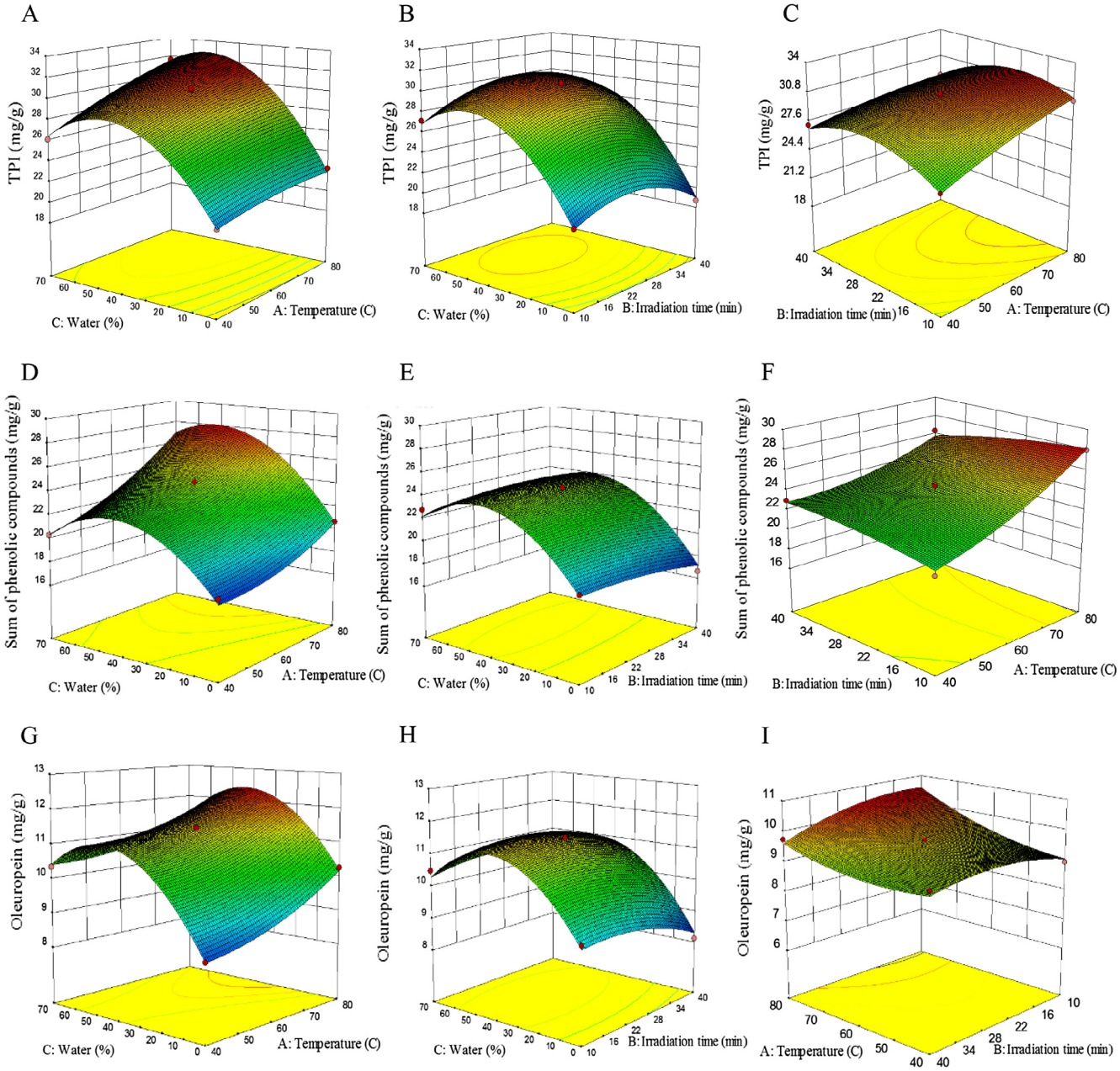

Fig. 3 Tridimensional response plots for interactions between three extraction variables on the total phenolic index (A–C), sum of the individual phenolic compounds (D–F) and oleuropein (G–I).

Table 6 Evaluation of the environmental impact of different techniques using organic solvents for analysis of phenolic compounds from olive leaf in comparison with the methodology proposed by means of deep eutectic solvents.

Ultrasound assissted extractor (UAE)*		Microwave extraction (MAE)**		Pressurized liquid extraction (PLE)***		MAE-Deep eutectic solvent extraction	
	Penalty points		*Penalty points*		*Penalty points*		*Penalty points*
Reagents:		*Reagents:*		*Reagents:*		*Reagents:*	
Methanol: 30 mL	12	Methanol: 10 mL	12	Ethanol: 20 mL	12	Choline chloride and ethylene glycol: 1.5 mL	1
Vapor emissions	3	Vapor emissions	3	Vapor emissions	3		
Waste	8	Waste	6	Waste	7		
Instrument:		*Instrument:*		*Instrument:*		*Instrument:*	
UAE	2	MAE	2	PLE	2	MAE	2
Rotary evaporator	2	LC-MS	2	Rotary evaporator	2	LC-MS	2
LC-MS	2			LC-MS	2		
Total penalti points	*29*	*Total penalti points*	*25*	*Total penalti points*	*28*	*Total penalti points*	*5*
Analytical Eco-Scale	*71*	*Analytical Eco-Scale*	*75*	*Analytical Eco-Scale*	*72*	*Analytical Eco-Scale*	*95*

* Methodology proposed byTalhaoui et al. (2014).
** Methodology proposed by Taamalli et al. (2012a, 2012b).
*** Methodology proposed by Herrero et al. (2011) and Quirantes-Pine et al. (2013).

parameters were optimized using the model equation provided by the statistical program. Thus, the optimum conditions applied were a temperature of 79.6 °C, 43.3% of water and an irradiation time of 16.7 min. Under these conditions, the maximum yield values of phenolic compounds from olive leaf could be reached using DES based on CCEtg.

3.3. Evaluation of green aspects for the methodology proposed

Concerned about rising environmental impact and waste generated by the chemical industry, new strategies and analytical procedures have been proposed or revised with the aim to enhance a sustainable development (Plotka-Wasylka et al., 2017; Tobiszewski et al., 2010; Tobiszewski 2016; Tobiszewski and Namieśnik, 2012; Tobiszewski and Namieśnik, 2017; Tobiszewski et al., 2018). Indeed, green analytical chemistry (GAC) emerged form green chemistry with the challenge of improving environmental friendliness of analytical methods. Recently 12 principles of green analytical chemistry have been proposed by Galuszka et al. (2013): (*i*) direct analytical techniques should be applied to avoid sample treatment; (*ii*) minimal sample size and minimal number of samples are goals; (*iii*) in situ measurements should be performed; (*iv*) integration of analytical processes and operations saves energy and reduces the use of reagents; (*v*) automated and miniaturized methods should be selected; (*vi*) derivatization should be avoided; (*vii*) generation of a large volume of analytical waste should be avoided and proper management of analytical waste should be provided; (*viii*) multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time; (*ix*) the use of energy should be minimized; (*x*) reagents obtained from renewable source should be preferred; (*xi*) toxic reagents should be eliminated or replaced; (*xii*) the safety of the operator should be increased.

Consequently, developing new green solvents is one of the key subjects in GAC in order to achieve a more eco-friendly media, shorter extraction times, simplicity, low cost and good extraction properties (Tobiszewski and Namieśnik, 2017; Tobiszewski, 2016; Tobiszewski et al., 2018; Plotka-Wasylka et al., 2017). In this sense, DESs seem to be promising green extraction solvents (Plotka-Wasylka et al., 2017). The methodology proposed by means of DESs for the extraction of phenolic compounds from olive leaf can be considered as greener alternative in comparison with the methodologies described until now for the same purpose such as ultrasound assisted extraction (Talhaoui et al., 2014), microwave extraction (Taamalli et al., 2012b) and pressurized liquid extraction (Herrero et al., 2011; Quirantes-Pine et al., 2013) using organic solvents like methanol or ethanol.

Each analytical methodology is characterized by its own specific requirements and limitations. Thus, it is important to evaluate and to improve the greenness of an analytical method or technique and to focus on its least green aspect. To evaluate the greenness of any analytical methodology according to their agreement with the principles of GAC various tools have been proposed in the last years. Among them, the use of Eco-scale seems to be helpful for finding and improving the weakest link in the method. It is based on assigning penalty points to parameters of an analytical process that are not in agreement with the ideal green analysis which has a score of 100 points in the eco-scale (Galuszka et al., 2012). The penalty points are given for each reagent type and amount, energy required, waste generated. The approach to scoring reagents is very intuitive. A penalty point is given for every hazard pictogram that corresponds to a chemical. Additionally, if the chemical is described with the word "danger", the number of hazard pictograms is multiplied by two, while the score does not change when the chemical is described with the word "warning". The number of penalty points related to the chemical hazards are

Table 7 Advantages and drawbacks of diverse protocols for extraction of phenolic compounds from olive leaf.

Techniques	Advantages	Drawbacks	References
Ultrasound assisted extraction (UAE)	Inexpensive technique Easy and simple procedure	Uses organic solvents: MeOH Consumes large quantities of solvents (30 mL) Requires concentration step Produces large quantities of waste (30 mL) Poor extraction power Time consuming: > 60 min Vapor emissions	Talhaoui et al. (2014)
Microwave extraction (MAE)	Easy procedure Higher extraction yields Automated technique Good precision Multiple samples at the same run Short extraction time: 6 min	Uses organic solvents: EtOH or MeOH Consumes less quantities of solvents: 10 mL Requires concentration step Produce moderate quantities of waste (10 mL) Vapor emissions	Taamalli et al. (2012a, 2012b)
Pressurized liquid extraction (PLE)	Easy procedure Higher extraction yields Automated technique Good precision Use high temperature and pressure Multiple samples at the same run	Use organic solvents: EtOH Consumes large quantities of solvents: 20 mL Requires concentration step Produce high quantities of waste (20 mL) Vapor emissions Time consuming: > 20 min	Herrero et al. (2011) and Quirantes-Pine et al. (2013)
Deep Eutectic Solvents (DES-MAE)	Easy procedure Higher extraction yields Automated technique Good extraction power Low quantities of solvent: 1.5 mL Multiple samples at the same run Short extraction time: 17 min Non-toxic solvents Environmental friendly, benign Low cost of raw materials Minimal amount of reagent Reduce waste generation Negligible vapor pressure Low flammability	High viscosity Tedious manipulation	

multiplied by the amount of the chemical. If the amount of a reagent or solvent is less than 10 mL score is multiplied by 1, in case of an amount of 10–100 mL and above 100 mL the multiplication factors are 2 and 3, respectively. The emission of vapours to the air during the analytical process is additionally punished by 3 penalty points. The generation of waste in the amount less than 1 mL results in 1 penalty point; waste generated in a 1–10 mL results in assigning 3 penalty points, whereas greater amounts of generated waste give 5 penalty points. If the waste is not treated in any way, 3 additional points are given (Galuszka et al., 2012; Tobiszewski, 2016). For its convenience and simplicity, the use of eco-scale score is easy to read and compare different analytical methodologies. However, the main drawback of this tool is that the assessment procedure considers hazards in semi-quantitative way.

Based on this, Table 6 shows the eco-scale score of different techniques for the analysis of phenolic compounds from olive leaf using organic solvents reported in bibliography in comparison with the methodology proposed by means of deep eutectic solvents. The highest analytical eco-scale score was exhibited by the methodology proposed in this study using DESs. The replacement of organic solvents (methanol and ethanol) by non-toxic ones (choline chloride and ethylene glycol) meet with the principle *xi* of GAC. Other strong point of the DES methodology is the minimization of reagents. In our case, only 1.5 mL of DES was required, meanwhile higher volumes were used with other techniques such as microwave assisted extraction which was carried out with 30 mL of methanol (Table 6). On the other hand, the analytical methodologies differed of waste produced, resulting those methods which required large quantities of reagents such as UAE in higher waste generation. The minimal use of reagents and the reduction of waste production meet with the principle *iv* and *vii* of GAC, respectively. Another important issue is ensuring the proper treatment of analytical waste due to the toxicity of organic solvents. Furthermore, the negligible vapor pressure, low flammability and low toxicity of DESs contribute to increase the safety of the operator (principle *xii*). Therefore, the replacement of organic solvents by the use of choline chloride-ethylene glycol seems to be a greenness alternative for the extraction of phenolic compounds from olive leaf. The advantages and drawbacks of the diverse analytical methodology used to address this aim are summarized in Table 7. A comparison of extraction results obtained from the methodology proposed with those reported in the literature was not plausible due to the different varieties of olive leaf analyzed from different origins and with different ways of expressing results (Supplementary Table S3).

4. Conclusions

The results presented in this work show the possibility of recovering valuable compounds from olive leaf, a by-product from olive farming, through the use of sustainable green solvents with a base of choline chloride deep eutectic solvents (DESs) following microwave assisted extraction techniques (MAE). After the screening process, solvents such as choline chloride-oxalic acid (1:1), choline chloride-tartaric acid (2:1) and choline chloride-maltose (3:1) were discarded due to their poor power of extraction. However, choline chloride-ethylene glycol (1:2) was confirmed as an excellent solvent for the extraction of phenolic compounds from olive leaf. The results obtained were similar to those obtained by the use of conventional solvents. A response surface methodology and a Box-Behnken Design were successfully employed to set the optimized parameters for extraction of bioactive compounds form olive leaf, such as temperature, percentage of water and irradiation time.

In comparison with other analytical methodologies previously used, the highest analytical eco-scale score exhibited by the methodology proposed was due to the replacement of organic solvents by non-toxic ones such as choline-chloride and ethylene glycol. Furthermore, the use of this eco-friendly media meets with several principles of green analytical chemistry such as minimal amount of reagents, lower waste generation and safer solvents due to their low flammability and negligible vapor pressure.

In summary, the use of deep eutectic solvents in combination with microwave assisted extraction as a greenness extraction method seems to be a promising tool for the extraction of bioactive compounds from olive leaf. Their lower environmental and economic impact make them fit for a wide range of applications in e.g. food, cosmetic, agrochemical and pharmaceutical industry as new green technology.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.arabjc.2018.01.003.

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