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Ultrasound-assisted extraction of phenolic compounds from blueberry leaves using natural deep eutectic solvents (NADES) for the valorization of agrifood wastes

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ABSTRACT

The food industry demands novel green solvents for the extraction of bioactive compounds, particularly from residues of the agrifood industry. Herein, an ultrasound-assisted method has been developed for the environmentally friendly extraction of phenolic compounds from blueberry leaves using natural deep eutectic solvents (NADES). After the screening of multiple NADES, the best extraction efficiencies in terms of total phenol content and antioxidant activity were provided by NADES composed of lactate, sodium acetate, and water (3:1:2), and of choline chloride and oxalic acid (1:1). Using a Box–Behnken experimental design, the optimal extraction conditions were achieved by sonicating for 45 min at 65 °C and using solvent:sample ratios of 15 and 75 (v/w) for the NADES based on lactic acid and choline, respectively. Compared with conventional organic solvents, the use of these NADES composed of lactic acid and choline provided superior performance for the recovery of phenolic compounds (1.6-fold and 2.2-fold greater efficacy, respectively) and antioxidant compounds (1.6-fold and 2.8-fold greater efficacy, respectively). The chromatographic characterization of the extracts obtained under these optimized conditions evidenced that the lactic-based NADES enabled the extraction of a wide range of hydroxycinnamic acids and flavonol derivatives, whereas the choline-based NADES was selective towards the extraction of anthocyanins. These results indicate that the proposed method could represent an excellent green alternative for the recovery of phenolic compounds from plant materials and agrifood wastes, with improved extraction efficacy and/or selectivity compared to that provided by traditional organic solvents.

1. Introduction

Within the framework of the sustainable and green chemistry concept, numerous efforts have been made to develop novel solvents as an alternative to toxic and polluting volatile organic compounds [1]. In this context, the use of deep eutectic solvents (DES) has dramatically emerged in recent years [2]. Traditionally, DES have been defined as eutectic mixtures of two or more hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) components which, at a certain molar ratio and temperature (i.e., eutectic point), undergo a phase change from solid to liquid at temperatures much lower than the melting point of their separate components [3]. More recently, Martins et al. proposed the following definition: "DES are mixtures of two or more pure compounds for which the eutectic point temperature is below that of an ideal

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liquid mixture, presenting significant negative deviations from ideality" [4]. Among these green solvents, natural deep eutectic solvents (NADES) refer to a subclass of DES that are composed of natural origin primary metabolites, such as amino acids, carboxylic acids, sugars, choline chloride, and urea [5]. Depending on the nature and molar ratios of their components, NADES can present diverse physicochemical properties (e.g., density, viscosity, melting point, polarity, ionic conductivity, surface tension, acidity or alkalinity), which in turn will determine their applicability [5]. Of note, NADES present excellent extractant properties in terms of negligible vapor pressure, which minimizes volatility problems during the extraction process; high biodegradability, which consequently results in low or no toxicity; high recyclability; minimal flammability; and easy preparation [6]. In contrast, the major drawbacks of NADES are their usual high density and viscosity, which can partially be solved by adding a third component (e.g., water, halides, carboxylic acids) to form ternary eutectic mixtures [2]. Furthermore, it should be noted that some NADES (e.g., oxalate-based NADES), despite their natural origin, may display potential cytotoxic effects. Concerning the preparation method, different methodologies have previously been reported for the synthesis of NADES, mainly based on stirring and heating, lyophilization, evaporation, and microwave-assisted synthesis [7]. As an alternative strategy, ultrasound-assisted synthesis has recently been proposed as a more sustainable, faster, and more efficient process [8,9].

Currently, one of the major applications of NADES is the extraction of bioactive compounds from different raw materials, particularly from residues of the agrifood industry [10]. Food wastes may contain a myriad of bioactive and functional components, including phenolic compounds, proteins, alkaloids, sugars, lipids, pigments, dietary fibers, essential oils, and many others. In particular, phenolic compounds are widely distributed in plants and fruits, and high contents of these phytochemicals have been reported in residues such as peels, stems, seeds, and leaves [11]. In recent years, numerous studies have shown that their consumption can cause beneficial effects on health, especially on cancer diseases, and cardiovascular and neurological systems. Therefore, their recovery from agrifood subproducts would be of great interest for the food, pharmaceutical, and cosmetic industries. This valorization of food wastes into high-value-added products allows their reinstatement into the supply chain, thus complying with the principles of the circular economy and approaching the concept of zero-waste generation [12]. In this respect, various authors have demonstrated the potential of NADES as solvents for the highly efficient extraction of bioactive compounds in different plant origin materials, such as olive leaves [13], pomegranate peels [14], strawberry and raspberry extrudates [15], and seaweeds [16], among other wastes. In this context, berries are a valuable feedstock because of their high content in sugars, organic acids (e.g., malic, succinic, citric, and quinic acids), vitamin C, minerals (e.g., manganese, calcium, iron, potassium, magnesium, phosphorus, selenium), carotenoids, and polyphenols (e.g., phenolics acids, anthocyanins, flavonols, flavan-3-ols) [17,18]. The cultivation of blueberry and other berry fruits has considerably grown over the last years, being one of the most important agricultural sectors in the province of Huelva (southwest Spain). Nowadays, approximately 2000 farms are dedicated to this crop, with a production surpassing 57,000 tons. This activity generates large amounts of agricultural wastes with potential usability in the framework of green chemistry and green engineering.

In this context, this work was aimed to develop a novel ultrasound-assisted method for the extraction of phenolic compounds from blueberry leaves using NADES. To this end, a preliminary screening of the extraction efficiency of multiple NADES was first conducted based on spectrophotometric determinations, and the extraction conditions were then optimized using an experimental design methodology. Under these optimal conditions, the potential of the NADES to recover the phenolic fraction from blueberry leave wastes was investigated by chromatographic analysis of a wide range of individual polyphenol compounds.

Table 1
List of the 18 NADES that were prepared and tested for the extraction of phenolic compounds from blueberry leave samples.

NADES code	Components			Molar ratio
	HBA component	HBD component	Ternary component	
Ca:Glu:W	Citric acid	d-Glucose	Water	1:2:7.5
La:Glu:W	l-Lactic acid	d-Glucose	Water	6:1:6
La:Gly:W		Glycine	Water	3:1:3
La:AcNa:W		Sodium acetate	Water	3:1:2
ChCl:U	Choline chloride	Urea		1:2
ChCl:U:W		Urea	Water	1:2:1
ChCl:Glyc		Glycerol		1:2
ChCl:Glyc:W		Glycerol	Water	1:2:1
ChCl:Eg		Ethylene glycol		1:2
ChCl:Eg:W		Ethylene glycol	Water	1:2:1
ChCl:La		l-Lactic acid		1:2
ChCl:Ox		Oxalic acid (dihydrate)		1:1
ChCl:But		1,4-Butanediol		1:6
Bet:Glyc:W	Betaine	Glycerol	Water	1:2:1
Bet:Eg:W		Ethylene glycol	Water	1:2:1
Pro:La	l-Proline	l-Lactic acid		1:1
Pro:Ox		Oxalic acid (dihydrate)		1:1
Pro:Glyc		Glycerol		2:5

2. Material and methods

2.1. Chemicals and samples

Reagents of analytical purity and HPLC-grade solvents were obtained from various commercial sources, as detailed in Table S1. Ultrapure water was obtained from a 109 Milli/Q water purification system (Millipore, Bedford, MA, USA). Blueberry leaves were identified and provided by specialized personnel from Driscoll's company (Huelva, Spain). Leaves were washed with deionized water and left to dry at room temperature for 7 days. Then, dried blueberry leaves (DBL) were ground using a kitchen grinder and stored at room temperature until use.

2.2. Ultrasound-assisted synthesis and spectroscopic characterization of NADES

The NADES were obtained by combining different HBA (e.g., quaternary amines, organic acids, amino acids) and HBD (e.g., sugars, alcohols, urea) components. All NADES were prepared on a weight basis according to the molar ratios depicted in Table S2. Initially, 73 mixtures were tested, but only 18 combinations yielded eutectic mixtures, as evaluated by NMR determinations, and were finally used for extraction purposes (Table 1). All the NADES were prepared by sonication and heating at 50 °C using an ultrasonic bath (Power Sonic 405, 350 W, 40 kHz) until the formation of a homogeneous liquid. Nuclear magnetic resonance (NMR) spectroscopy measurements of selected NADES and their components were recorded at 500 MHz on a Bruker Avance 500 spectrometer (Billerica, MA, USA), operating at 25 °C and using deuterated dimethyl sulfoxide (DMSO-d_6) as the solvent.

2.3. Ultrasound-assisted extraction of phenolic compounds from blueberry leaves

The potential of the NADES for extracting phenolic compounds from blueberry leaves was evaluated using an ultrasound-assisted procedure. This extraction performance was compared with that provided by a conventional organic solvent consisting of methanol:water (80:20, v:v), previously proposed by other authors as a suitable solvent for the

extraction of phenolic compounds from leaf materials [19,20]. For a preliminary screening of the extraction efficacy of the NADES, 0.2 g of DBL was mixed with 1.5 mL of the extractant in an Eppendorf tube (solvent-sample ratio: 7.5). Then, samples were vigorously vortexed until homogenization, and sonicated for 45 min in a water bath at 65 °C. Subsequently, the mixtures were centrifuged at 13400 rpm for 15 min and the supernatants were filtered using glass wool. The extracts were stored at 4 °C until analysis.

2.4. Spectrophotometric determinations

The total phenol content was determined by means of the Folin-Ciocalteu spectrophotometric method with some modifications [21]. Briefly, 20 μL of the diluted samples (1:50, v/v) were mixed with 1.58 mL of distilled water, 100 μL of the Folin's reagent, and 300 μL of 20% sodium carbonate (w/v). The mixture was vortexed and heated at 40 °C for 90 min. Finally, the absorbances were measured at 725 nm. The results were expressed as milligrams of gallic acid equivalents per grams of dried blueberry leaf (mg GAE/g DBL), using a gallic acid calibration curve within the concentration range 0–750 mg/L. On the other hand, the antioxidant activity of the extracts was measured using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) spectrophotometric method [22]. For this purpose, 100 μL of diluted samples (1:50, v/v) were mixed with 3.9 mL of a 0.1 mM DPPH solution. After 30 min, the absorbance was measured at 515 nm. The results were expressed as milligrams of Trolox equivalents per grams of dried blueberry leaf (mg TEAC/g DBL), using a Trolox calibration curve within the concentration range 0.0–1.6 mM. Finally, the total anthocyanin content was determined by using a modification of the pH differential method [23]. Two aliquots of 100 μL of the diluted samples (1:2, v/v) were mixed with 2.5 mL of pH 4.5 buffer (0.2 M Na_2HPO_4, 0.1 M citric acid) and 2.5 mL of pH 1.0 buffer (2% HCl), respectively. The mixtures were vortexed and the absorbances were measured at 510 nm and 700 nm. The results were expressed as milligrams of cyanidin-3-glucoside equivalents per 100 g of dried blueberry leaf (mg C3GE/100 g DBL). All these spectrophotometric tests were performed in triplicate, and the results were expressed as the mean ± standard deviation.

2.5. Chromatographic determination of individual phenolic compounds

The sample extracts were five-fold diluted with ultrapure water, centrifuged at 10000 rpm for 3 min, and filtered through 0.45 μm PVDF filters. Then, phenolic compounds were determined using an Agilent 1260 Infinity HPLC system coupled to a photodiode array detector (DAD), using a previously optimized and validated methodology [24,25]. Briefly, the separation of phenolic compounds was performed on a Kinetex EVO C18 column (2.6 μm, 100 mm × 2.1 mm), thermostated at 40 °C. The injection volume was 5 μL and the flow rate was set at 0.5 mL/min. For the analysis of phenolic acids and flavonoids, water with 0.1% formic acid and acetonitrile were used as mobiles phases A and B, respectively. The elution gradient was programmed as follows: 0–3 min, 0% B; 3–16 min, 0–12% B; 16–16.5 min, 12–16% B; 16.5–21min, 16% B; 21–25 min, 16–20% B; 25–30 min, 20% B; 30–31 min, 20–100% B; 31–34 min, 100% B; 34–39 min, 0% B. For anthocyanins, the mobile phase A consisted of water:formic acid (95:5, v/v) and mobile phase B of acetonitrile:formic acid (95:5, v/v), using the following gradient of elution: 0–10 min, 0–15% B; 10–14 min, 15–100% B; 14–18 min, 100% B; 18–23 min, 0% B. For quantification, the following wavelengths were employed: 260 nm for ellagic acid, 280 nm for phenolic acids and flavan-3-ols, 320 nm for cinnamic acids, 360 nm for flavonols, and 520 nm for anthocyanins.

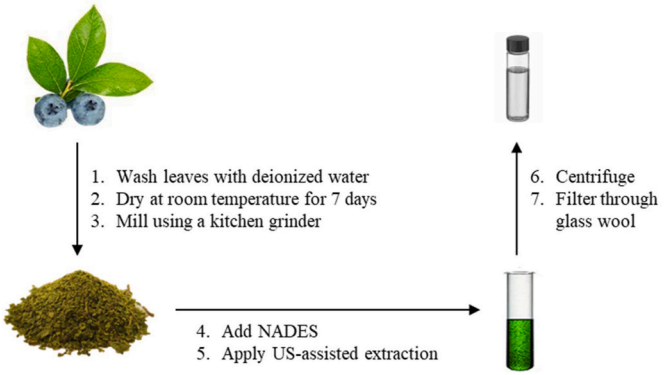

Fig. 1. Flow diagram of the extraction process.

2.6. Statistical analysis

Statistical analysis of the data was performed using the STATISTICA 7.0 software (StatSoft, Tulsa, OK, USA). One-way analysis of variance (ANOVA) and principal component analysis (PCA) was applied to the chemical data (i.e., spectrophotometric and chromatographic determinations) to compare the extraction efficacy of the different solvents under study.

A Box-Behnken experimental design, combined with response surface methodology (RSM), was employed to optimize the ultrasound-assisted extraction conditions, namely temperature (T), extraction time (ET), and solvent:sample ratio (v/w) (SSR). For this purpose, a three-level, three-factor Box-Behnken factorial design was applied using the following experimental levels: 25, 45 and 65 °C for T; 30, 45 and 60 min for ET; 15, 30 and 75 for SSR. In total, 15 trials (including three replicates at the center points) were conducted in random order to avoid any systematic error. The spectrophotometric determinations (i.e., total phenol content, antioxidant activity, and total anthocyanin content) were used as dependent responses, and the experimental data were fitted to a second-order polynomial model. Regression analyses were carried out according to Equation (1):

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

where X_i and X_j ($I = 1, 3$; $j = 1, 3$; $I \neq j$) represent the factors which influence the responses Y (i.e., spectrophotometric determinations). B_0, β_i, β_{ii}, and β_{ij} are the regression coefficients in the intercept, linear, quadratic, and the interaction terms of the model, respectively.

In total, four different models were tested (i.e., multiple regression, factorial regression, polynomial regression, and response surface regression). Their regression adequacy was assessed by computing the coefficient of determination (R^2 and R^2_{adj}), and the lack of fit was tested by computing the p-value. An analysis of variance (ANOVA) was conducted for each response variable at a significance level of $p = 0.05$. Furthermore, the F test was applied to determine the significance of each regression coefficient. To investigate the effects of the independent variables on the response variables, three-dimensional surface and contour graphs were built from the regression models. In addition, the desirability function method was used to predict optimal conditions for all dependent variables. The extraction conditions and optimization design were selected based on previous experience [26,27] and literature [19,28,29].

3. Results and discussion

3.1. Preliminary screening of different NADES for the extraction of bioactive compounds from blueberry leaves based on spectrophotometric determinations

The composition of NADES determines their physicochemical properties and, therefore, influences their extraction efficiency. For this reason, the performance of several NADES commonly reported in the

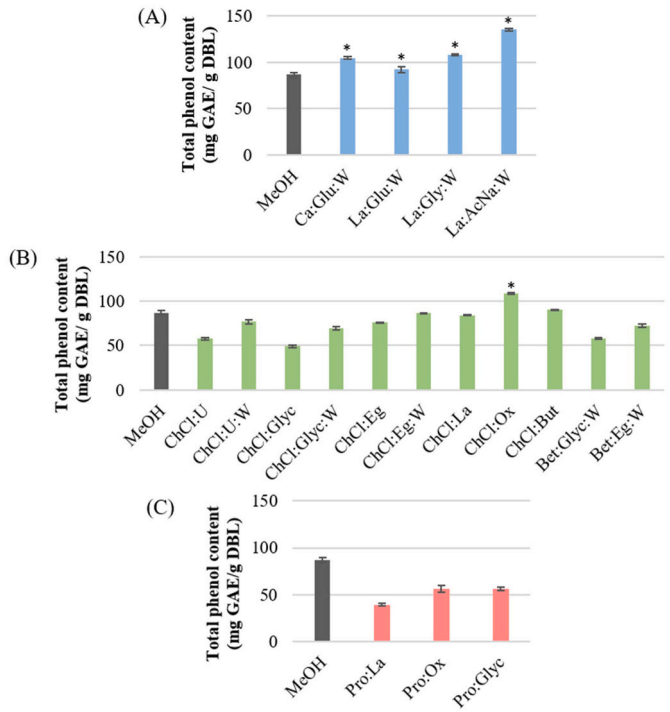

Fig. 2. Column plots with standard deviation bars representing the total phenol content (expressed as mg GAE/g DBL) measured in extracts prepared with 80% methanol and with NADES consisting of organic acids (A), quaternary amines (B), and amino acids (C) as the HBA component. * Denotes those NADES with statistically significant improved extraction efficacy compared to that provided by 80% methanol.

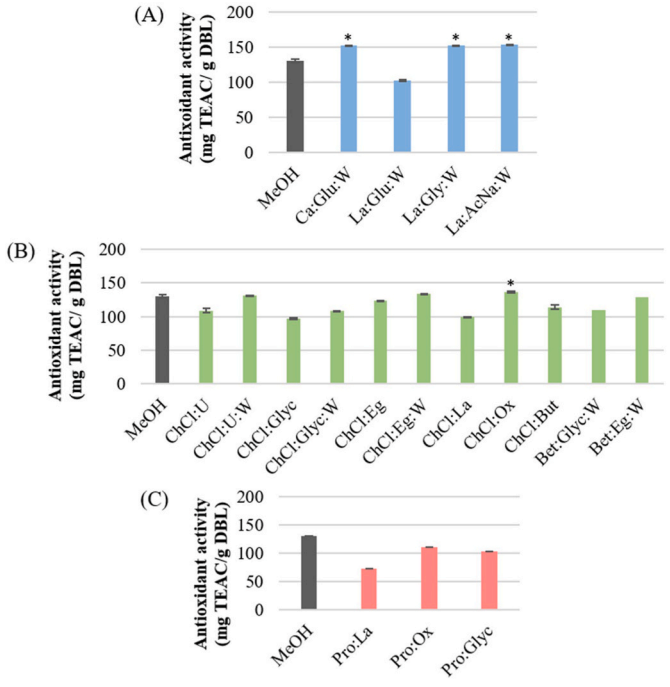

Fig. 3. Column plots with standard deviation bars representing the antioxidant activity (expressed as mg TEAC/g DBL) measured in extracts prepared with 80% methanol and with NADES consisting of organic acids (A), quaternary amines (B), and amino acids (C) as the HBA component. * Denotes those NADES with statistically significant improved extraction efficacy compared to that provided by 80% methanol.

Table 2
Extraction efficacy by using selected NADES and reference organic solvent.

		Methanol (80%)	La:AcNa:W (3:1:2)	ChCl:Ox (1:1)
Total phenol content (GAE/g DBL)	**Preliminary screening**	86.9 ± 2.4	135 ± 1	109 ± 1
Antioxidant activity (mg TEAC/g DBL)		130.1 ± 2.1	153 ± 0	137 ± 1
Total anthocyanin content (mg C3GE/100 g DBL)		–	–	50.2 ± 0.7
Total phenol content (GAE/g DBL)	**After Box-Behnken optimization**	86.9 ± 2.4	142.5 ± 2.6	195.5 ± 1.1
Antioxidant activity (mg TEAC/g DBL)		130.1 ± 2.1	220.0 ± 1.7	368.3 ± 9.8
Total anthocyanin content (mg C3GE/100 g DBL)		–	–	217.9 ± 4.3

literature, which were prepared by ultrasound-assisted synthesis, was compared in the present study for extracting bioactive compounds from blueberry leaves. In total, 73 combinations were tested based on three groups of HBA components (quaternary amines such as choline chloride and betaine; amino acids such as glycine and proline; and carboxylic acids such as citric acid, malic acid, and lactic acid) and four types of HBDs (carboxylic acids, alcohols, amides, and sugars) (Table S2). From all these tests, only 18 combinations yielded satisfactory eutectic mixtures (Table 1), and ^{1}H NMR experiments confirmed that no reactions occurred during the synthesis process (i.e., the same NMR signals were observed in the eutectic liquid and in the original components, see Figs. S1–S6) [30,31].

To assess their extraction efficiency, these 18 NADES were tested for the ultrasound-assisted extraction of phenolic compounds from blueberry leaves (Fig. 1). For this preliminary screening, the extracts were subjected to various spectrophotometric determinations with the aim of obtaining a general overview about their bioactive content in terms of total phenol content (Fig. 2) and antioxidant activity (Fig. 3). ANOVA was applied to compare their extractant capabilities with that provided by a conventional organic solvent (i.e., methanol:water, 80:20).

The NADES based on organic acids as the HBA component provided the higher extraction efficacy for both parameters (Figs. 2A and 3A). As summarized in Table 2, the best results were obtained by using the ternary NADES composed of lactic acid, sodium acetate, and water (La:AcNa:W), which yielded a total phenol content of 135 ± 1 mg GAE/g DBL and an antioxidant activity of 153 ± 0 mg TEAC/g DBL, thereby surpassing the extraction yield obtained with the organic solvent by a 55% and 15%, respectively. These findings are in line with previous studies reporting the potential of this specific NADES to extract phenolic compounds from medicinal plants (total phenol content: 19–108 mg GAE/g plant material, antioxidant activity: 574 mg TEAC/g plant material) [32]. Choline-based NADES also proved to be useful for the recovery of bioactive compounds from blueberry leaves (Figs. 2B and 3B). In particular, the extracts that were prepared with the NADES composed of choline chloride and oxalic acid (ChCl:Ox) presented a total phenol content of 109 ± 1 mg GAE/g DBL (i.e., 25% improvement with respect to methanol 80%) and an antioxidant activity of 137 ± 1 mg TEAC/g DBL (i.e., 5% improvement with respect to methanol 80%) (Table 2). Similarly, Barbieri et al. also described the use of this NADES for the satisfactory ultrasound-assisted extraction of phenolic compounds from rosemary plant [33]. Interestingly, the extracts prepared with ChCl:Ox presented a distinctive reddish color, which could be due to the anthocyanin compounds that are present at high concentrations in blueberry, and which was not observed for any other of the NADES under study. Thus, the total anthocyanin content of these extracts was analyzed by means of the pH differential method, and results corroborated the ability of this NADES to extract this flavonoid subclass (total anthocyanin

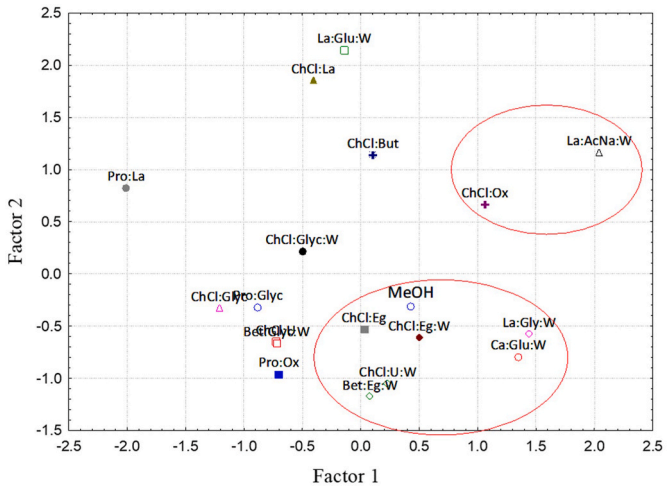

Fig. 4. PCA scores plot showing the distribution of the blueberry leave extracts prepared with NADES and the conventional organic solvent (MeOH 80%) in the plane defined by the two first principal components.

content: 50.2 ± 0.7 mg C3GE/100 g DBL, Table 2). Noteworthy, the addition of water as a third component during the synthesis of the choline-based NADES positively influenced the extraction of phenolic compounds, as can be observed for ChCl:U, ChCl:Glyc, and ChCl:Eg and their aqueous analogs (ChCl:U:W, ChCl:Glyc:W, and ChCl:Eg:W) (Figs. 2B and 3B). This could be allocated to the decreased viscosity that presented these water-containing NADES, which would maximize their extractant power [34,35]. In this respect, it should be noted that the oxalic acid reagent that was employed for the synthesis of the ChCl:Ox NADES was in dihydrate form, which could at least in part explain its satisfactory extraction performance. Conversely, NADES composed of amino acids provided the lowest extraction yields, below those obtained with the organic solvent that was used as a reference (Figs. 2C and 3C), in agreement with previous studies [36]. In line with the above-mentioned rationale, this poor extraction efficacy could be due to the visible high viscosity of these NADES.

To better visualize the differences in the extractability of phenolic compounds from blueberry leaves depending on the NADES employed, principal component analysis (PCA) was applied to the spectrophotometric data. A two-PC model was able to explain 90% of the total data variance, the first PC being positively correlated with the total phenolic content and antioxidant activity (loadings equal to 0.95 in both cases). In the corresponding scores plot (Fig. 4), three clear clusters of samples could be observed. The first cluster was composed of La:AcNa:W and ChCl:Ox extracts, which presented positive loadings for PC1, thereby indicating their higher extraction efficiencies. Also in the right side of the scores plot, but with negative loadings for PC2, we found a series of NADES with similar extraction performance to that provided by the reference organic solvent (ChCl:Eg:W, ChCl:Eg, ChCl:U, La:Gly:W, Ca:Glu:W, and Bet:Eg:W). The rest of the NADES were clustered at the left side of the plot because of their poor extractant potential.

Taking all this into consideration, the NADES La:AcNa:W and ChCl:Ox were selected as the most efficient solvents for the extraction of phenolic compounds from blueberry leaves, and were subjected to further optimization.

3.2. *Optimization of the ultrasound-assisted extraction conditions using a Box-Behnken experimental design*

A Box-Behnken (BBD) experimental design, based on 15 runs and three replications in the central points, was applied to optimize the working variables that are most likely to influence the performance of the ultrasound-assisted extraction process using the NADES La:AcNa:W and ChCl:Ox, namely the extraction temperature, extraction time, and solvent:sample ratio (v/w). For this purpose, the response variables under study were the spectrophotometric determinations discussed above, i.e., total phenol content, antioxidant activity, and total anthocyanin content (this latter variable only for ChCl:Ox-based extractions). Response surface regression provided the best fitting of experimental data, as summarized in Table 3. For the NADES based on lactic acid, the total phenol content was found to be affected by the extraction temperature (linear effect), whereas both the temperature (linear and quadratic effects) and the solvent:sample ratio (linear effect) had a significant impact on the antioxidant activity. Similarly, when using the NADES ChCl:Ox, the extraction temperature was also a crucial factor for the extraction of bioactive compounds form blueberry leaves in terms of total phenol content (linear effect), antioxidant activity (linear effect), and total anthocyanin content (linear, interaction, and quadratic effects). Moreover, a linear effect of the extraction time and the solvent:sample ratio was found for the total phenol content and the antioxidant activity, respectively. For total anthocyanin content, all factors had a significant impact except for the linear effect of the time and the quadratic effect of the solvent:sample ratio.

By applying the response surface methodology (RSM), satisfactory models were built for total phenol content and antioxidant activity,

Table 3
Regression coefficients (with p-values in brackets) and analysis of the variance of the models that were obtained by applying the response surface methodology.

	La:AcNa:W		ChCl:Ox		
Responses	Total phenol content	Antioxidant activity	Total phenol content	Antioxidant activity	Total anthocyanin content
Intercept					
B_0	−105.550(0.00001)	−61.6027(0.001)	−38.9537 (0.004)	25.10272 (0.000001)	−1.36120 (0.0002)
Linear terms					
B_1	6.427 (0.187)	5.8900 (0.108)	1.2958 (0.094)	4.35136 (0.131)	0.11725 (0.597)
B_2	−0.881 (0.014)	−3.4888 (0.007)	−0.8946 (0.048)	−4.66022 (0.023)	−0.04844 (0.0004)
B_3	0.487 (0.117)	2.3293 (0.045)	1.9357 (0.496)	2.30717 (0.0002)	−0.01935 (0.001)
Interaction terms					
B_{12}	−0.013 (0.697)	0.0132 (0.582)	0.0242 (0.589)	0.02298 (0.623)	−0.00017 (0.074)
B_{13}	−0.003 (0.875)	−0.0243 (0.197)	−0.0066 (0.809)	−0.00232 (0.936)	0.00003 (0.487)
B_{23}	−0.017 (0.312)	−0.0214 (0.155)	−0.0142 (0.512)	0.00143 (0.947)	0.00054 (0.002)
Quadratic terms					
B_{11}	−0.055 (0.282)	−0.0505 (0.214)	−0.0042 (0.944)	−0.04557 (0.0.488)	−0.00123 (0.003)
B_{22}	0.040 (0.177)	0.0719 (0.045)	0.0255 (0.481)	0.05731 (0.155)	0.00064 (0.003)
B_{33}	0.010 (0.566)	−0.0103 (0.409)	−0.0087 (0.687)	0.00943 (0.678)	0.00003 (0.284)
Validation of model					
R^2	0.864	0.975	0.871	0.964	0.898
R^2_{adj}	0.618	0.931	0.939	0.899	0.715
p-value (model)	0.003	0.0001	0.05	0.004	0.04
p-value (lack of fit)	0.664	0.448	0.632	0.722	0.336

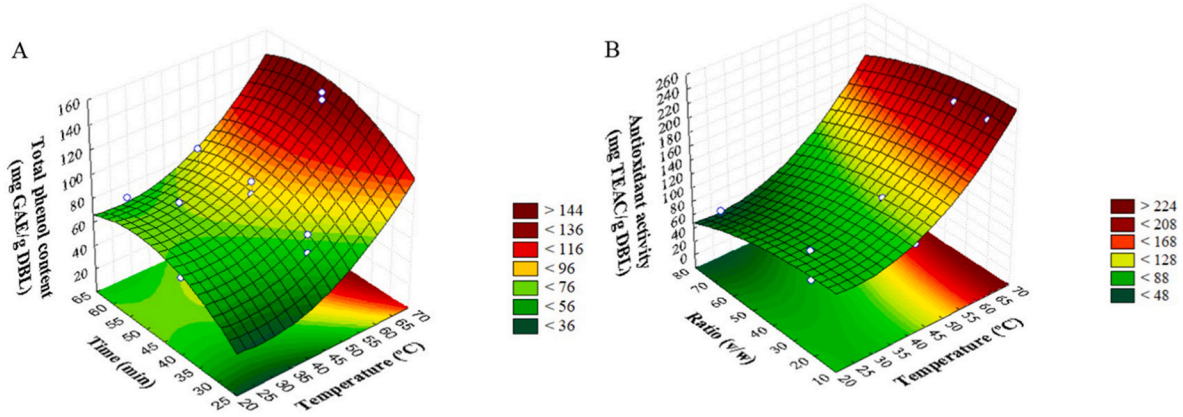

Fig. 5. Response surface plots showing the effect of experimental factors under study (i.e., extraction temperature, extraction time, solvent:sample ratio) on the total phenol content (A) and antioxidant activity (B) when using the NADES La:AcNa:W.

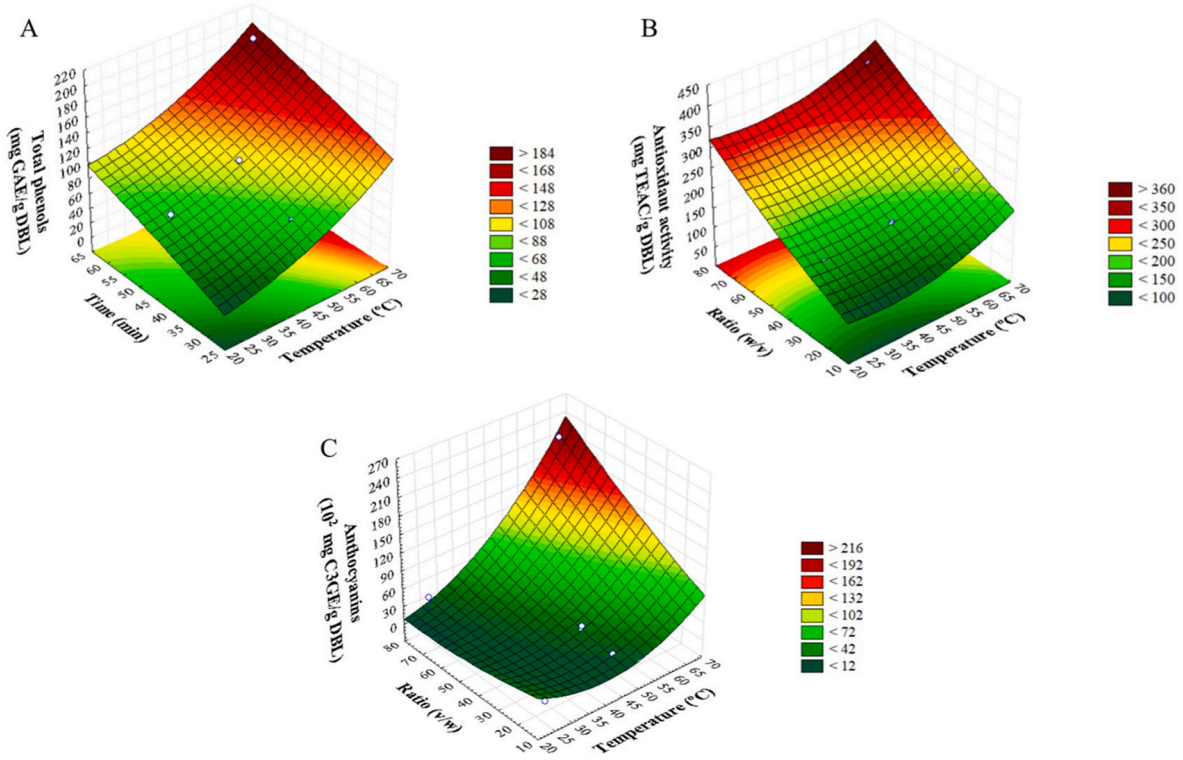

Fig. 6. Response surface plots showing the effect of experimental factors under study (i.e., extraction temperature, extraction time, solvent:sample ratio) on the total phenol content (A), antioxidant activity (B), and total anthocyanin content (C) when using the NADES ChCl:Ox.

showing valid regression fitness ($p < 0.05$ for the regression F-test) and non-significant lack of fit ($p > 0.05$) for both NADES under study (Table 3). Furthermore, the coefficients of determination were above 0.8 for both response variables and adjusted coefficients were close to the raw ones, thus evidencing the suitable statistical performance of the models.

Finally, these regression models were employed to obtain three-dimensional representations of the response surfaces with the aim of finding the optimal values for the three extraction variables investigated here (Figs. 5–6). For both NADES, the maximum response for the total phenol content, antioxidant activity, and total anthocyanin content (this latter only for ChCl:Ox) was found by applying high extraction temperatures and long extraction times, in line with previous studies reporting the positive influence of these factors in the extraction process of phenolic compounds from plant materials [37]. On the other hand, the optimum solvent:sample ratio was found to be different depending on the NADES, probably due to the lower viscosity of ChCl:Ox [38–40]. Accordingly, the application of a desirability function approach (Fig. 7) evidenced that the optimal extraction conditions are achieved by sonicating for 45 min in a water bath thermostated at 65 °C, and using solvent:sample ratios of 15 and 75 (v/w) for La:AcNa:W and ChCl:Ox, respectively. Although surface plots did not show that maximum extraction efficacy was reached, we decided not applying more extreme working conditions (e.g., higher temperatures, longer times) with the aim of not compromising the cost and environmental friendliness of the extraction method, as well as to avoid the degradation of labile phenolic compounds.

Using these optimized conditions, the NADES provided improved extraction recoveries compared with that obtained with the reference organic solvent in terms of total phenol content (80% methanol, 86.9 ±

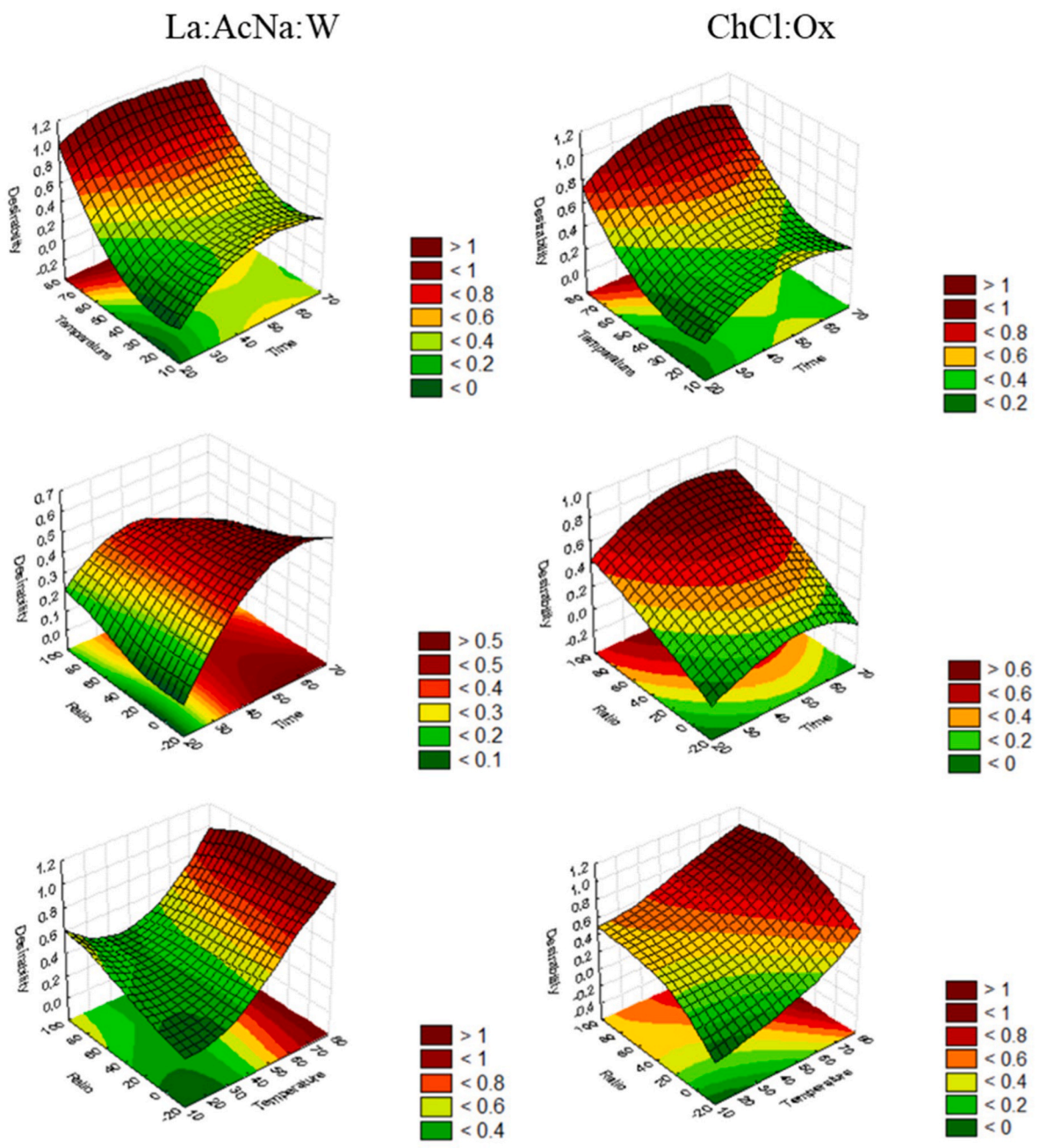

Fig. 7. Surface plots from desirability analysis for the NADES La:AcNa:W (left) and ChCl:Ox (right).

2.4 mg GAE/g DBL; La:AcNa:W, 142.5 ± 2.6 mg GAE/g DBL; ChCl:Ox, 195.5 ± 1.1 mg GAE/g DBL), antioxidant activity (80% methanol, 130.1 ± 2.1 mg TEAC/g DBL; La:AcNa:W, 220.0 ± 1.7 mg TEAC/g DBL; ChCl:Ox, 368.3 ± 9.8 1 mg TEAC/g DBL), and total anthocyanin content (80% methanol, negligible; ChCl:Ox, 217.9 ± 4.3 mg C3GE/100 g DBL) (Table 2). In general, the results presented here are in line to those reported by other authors who employed organic solvents for extracting phenolic compounds from blueberry leaves. For instance, Ştefănescu et al. applied ultrasound-assisted extraction during 30 min at 20 °C using a 40% ethanol solution, and found that the total phenolic content varied depending on the harvesting time of the blueberry leaves, in the range 105–152 mg/g blueberry leaves [28]. In another study, microwave-assisted extraction using 15 or 30% ethanol and 1.5 M citric acid as the solvent yielded sample extracts with total phenol contents in the range 92–127 mg/g leaves [41]. Altogether, our results highlight the potential of NADES as a green alternative to extract phenolic compounds from blueberry leaves.

Finally, to confirm the reliability of the extraction method, nine independent extractions were performed in a homogeneous sample of grounded DBL using the optimized conditions. To this end, the extracts were prepared in three non-consecutive days by triplicate. The repeatability was computed as the relative standard deviation of the ANOVA within-condition variance (S_W^2), whereas the intermediate precision (S_R^2) was estimated from the between-condition variance (S_B^2) and the within-condition variance (S_W^2) using the formula: $S_R^2 = S_W^2 + S_B^2$. The results evidenced satisfactory repeatability (<3.5%) and intermediate precision (<5%) for the determination of the total phenol content and the antioxidant activity in extracts prepared with both NADES. Only intermediate precision was slightly higher for the total anthocyanin content in ChCl:Ox-based extracts (8.8%), probably because of their low content.

Table 4
Individual phenolic compounds identified and quantified in blueberry leave extracts. The results are expressed as mean ± standard deviation of triplicate measurements (mg/kg). ND, non-detected.

Compound	RT (min)	λ (nm)	MeOH	La:AcNa:W	ChCl:Ox
Gallic acid	1.16	280	8.7 ± 0.02	3.5 ± 0.01	27.9 ± 0.9
3,4-Dihydroxybenzoic acid	2.02	260	15.1 ± 0.04	115.1 ± 0.4	341.9 ± 5.3
3,4-Dihydroxybenzaldehyde	3.00	280	22.5 ± 0.06	ND	ND
Hydroxycinnamic acid derivative (1)	5.30	320	39.9 ± 0.3	ND	ND
Caffeic acid	7.59	320	56.9 ± 0.2	185.5 ± 0.6	2466.3 ± 42.8
Hydroxycinnamic acid derivative (2)	7.74	320	58.1 ± 0.2	ND	ND
Catechin	8.06	280	60.5 ± 0.2	ND	ND
Hydroxycinnamic acid derivative (3)	8.33	320	62.5 ± 0.2	ND	ND
Chlorogenic acid	10.40	320	37,270 ± 132.5	25,816 ± 10.3	14,947 ± 29.5
Hydroxycinnamic acid derivative (4)	12.25	320	228.4 ± 1.6	152.8 ± 0.7	ND
Ferulic acid	13.05	320	245.6 ± 1.5	44.6 ± 0.4	189.3 ± 21.8
Flavonol derivative (1)	17.15	360	368.1 ± 0.4	ND	ND
Flavonol derivative (2)	17.44	360	70.1 ± 1.0	ND	ND
Quercetin 3-O-galactoside	19.05	360	2071.0 ± 11.9	769.1 ± 3.5	ND
Quercetin 3-O-rutinoside	19.32	360	3736.9 ± 21.4	1897.9 ± 7.5	ND
Flavonol derivative (3)	19.69	360	257.7 ± 2.3	ND	ND
Flavonol derivative (4)	19.91	360	387.9 ± 2.6	1369.3 ± 5.6	ND
Flavonol derivative (5)	20.14	360	704.1 ± 7.1	477.7 ± 1.5	ND
Quercetin 3-O-rhamnoside	20.52	360	598.0 ± 3.3	314.7 ± 1.9	ND
Flavonol derivative (6)	20.89	360	13.6 ± 0.4	27.1 ± 0.5	ND
Flavonol derivative (7)	21.01	360	46.6 ± 0.3	95.1 ± 1.1	ND
Quercetin	23.71	360	10.3 ± 2.5	5.8 ± 0.01	654.1 ± 25.2
Petunidin 3-O-glucoside	8.29	520	ND	ND	69.5 ± 1.6
Peonidin 3-O-glucoside	8.77	520	ND	ND	58.5 ± 1.1
Malvidin 3-O-glucoside	9.53	520	5.1 ± 0.1	ND	935.6 ± 11.7

3.3. Characterization of the phenolic profile of blueberry leave extracts

Leveraging a previously developed chromatographic method for high-throughput and comprehensive phenolic profiling [24,25], a wide range of polyphenol compounds were successfully detected and quantified in blueberry leaves (Table 4). Interestingly, the number of extracted compounds was significantly different between the two NADES under study and the reference organic solvent ($p < 0.0001$). In general, the phenolic profiles that were obtained by analyzing methanolic and La:AcNa:W extracts were quite similar regarding the total number of detected polyphenols and related metabolites, although recoveries were slightly higher when using the organic solvent. The major compounds that were detected in these extracts comprised several phenolic acids (mostly chlorogenic acid and other hydroxycinnamic acids) and flavonol derivatives (i.e., quercetin and kaempferol glycosides and aglycones), in line with previous studies conducted on blueberry leaves [42] and other berry fruits [43,44]. On the other hand, the content of these phenolic compounds was in general lower in the extracts derived from the use of choline-based NADES, but as a counterpart the extraction efficacy for anthocyanins was significantly improved, clearly surpassing that provided by the organic solvent. In this respect, Wang et al. reported for the first time the presence of various anthocyanin derivatives in blueberry leaves [17]. In our study, the major anthocyanin species that were detected resulted to be glucoside derivatives of petunidin, peonidin, and malvidin (Table 4).

4. Conclusions

To sum up, an ultrasound-assisted method has been optimized for the first-time extraction of phenolic compounds from blueberry leaves using NADES. The best extraction performances were obtained by using two different NADES, La:AcNa:W and ChCl:Ox, and by applying the following working conditions: extraction temperature, 65 °C; extraction time, 45 min; solvent:sample ratio, 15 (La:AcNa:W) and 75 (ChCl:Ox). This extraction conditions enabled the satisfactory recovery of phenolic compounds from blueberry leaves, as evaluated through spectrophotometric determinations (i.e., total phenol content, antioxidant activity) and chromatographic analysis of a broad spectrum of individual phenolic compounds. Compared with conventional organic solvents, the use of these NADES composed of lactic acid and choline provided superior performance for the recovery of phenolic compounds (1.6-fold and 2.2-fold greater efficacy, respectively) and antioxidant compounds (1.6-fold and 2.8-fold greater efficacy, respectively). In particular, it should be noted that the NADES based on lactic acid enabled the extraction of hydroxycinnamic acids and flavonols in a similar extent to that provided by conventional organic solvents, whereas the choline-based NADES proved to be selective for the extraction of anthocyanin compounds. Therefore, these results highlight the potential of NADES as a sustainable and environmentally friendly approach to recover bioactive compounds from food subproducts.

Author contributions

Conceptualization, A.S. and Á.F.-R.; methodology, M.S.-M. and R.G.-D.; formal analysis, M.S.-M. and R.G.-D.; investigation, M.S.-M., J.C.-C.; R.G.-D., E.C.-T., A.S., J.U. and Á.F.-R.; resources, A.S. and Á.F.-R.; data curation, M.S.-M. and Á.F.-R.; writing—original draft preparation, M.S.-M., R.G.-D. and Á.F.-R.; writing—review and editing, M.S.-M., J.C.-C.; R.G.-D., E.C.-T., A.S., J.U. and Á.F.-R.; supervision, A.S. and Á.F.-R.; project administration, A.S. and Á.F.-R.; funding acquisition, A.S. and Á.F.-R. All authors have read and agreed to the published version of the manuscript.

Data sharing

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2023.106882.

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