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Batch Stirred-Tank Green Extraction of *Salvia fruticosa* Mill. Polyphenols Using Newly Designed Citrate-Based Deep Eutectic Solvents and Ultrasonication Pretreatment

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Abstract: A series of citrate salts were tested as hydrogen bond acceptors to synthesize deep eutectic solvents (DES) based on lactic acid and glycerol, used as hydrogen bond donors. The DES produced were then screened to identify the highest performing system for the effective extraction of polyphenolic phytochemicals from the medicinal plant *Salvia fruticosa* Mill. (Greek sage). The most efficacious DES was the one composed of lactic acid and sodium citrate dibasic, at a molar ratio of 15:1 (LA-SCDB15). Furthermore, for the first time there has been evidence concerning DES pH and extraction efficiency. Using this solvent, a batch, stirred-tank extraction process was developed, by employing ultrasonication pretreatment and response surface methodology. The optimal settings determined were stirring speed 900 rpm, proportion of DES/water 77% (*w*/*v*), and ultrasonication pretreatment time 15 min. By adjusting these optimal settings, the predicted maximum total polyphenol yield was calculated to be 79.93 ± 1.92 mg gallic acid equivalents g^{-1} dry mass. The examination of temperature effects demonstrated that the batch, stirred-tank extraction stage was very energy-efficient, with a barrier of 7.64 kJ mol^{-1}. Comparison of the extraction of *Salvia fruticosa* polyphenols with other green processes previously developed, illustrated the high extraction capacity of LA-SCDB15. The major polyphenols identified in the extracts produced under optimized settings were chlorogenic acid, luteolin 7-*O*-glucuronide and rosmarinic acid.

Keywords: antioxidants; deep eutectic solvents; extraction kinetics; polyphenols; *Salvia fruticosa*; ultrasonication

1. Introduction

Medicinal and aromatic plants (MAPs) are routinely used as food and folk remedies for centuries worldwide, and to-date substantial scientific evidence has accumulated to support their reputed nutritional and pharmacological properties. The knowledge derived by long-term traditional uses of MAPs has now been acknowledged as a sound basis to support health claims for numerous botanicals [1], and there has been a climbing interest for products originating from MAPs with a spectrum of bio-functionalities. Consumer trends for natural commodities with health-promoting activities has raised awareness and high demand for botanical-based supplements, and ignited a large

development of novel products, thus enabling the launch of a variety of functional ingredients [2] and cosmetic additives [3].

Currently, there is a great interest for the development and implementation of cutting-edge sustainable extraction methods for polyphenols from medicinal plants. In this direction, numerous green and low-cost approaches have gained acceptance as being more efficient and precise than traditional ones [4,5]. In compliance with green chemistry principles, a crucial concern towards establishing eco-friendly extraction processes is the replacement of conventional petroleum-based volatile solvents with bio-based alternatives. In this line, the utilization of a benign, eco-friendly solvent is of prime importance to the sustainable profile of an extraction process. Such a solvent should be non-toxic, it should have satisfactory extraction efficiency, it should be inexpensive and readily available, and it should originate from recyclable materials, such as waste biomass [6,7].

Deep eutectic solvents (DES) are innovative liquids, composed of low-cost, non-toxic and recyclable materials, which can be naturally occurring compounds (e.g., organic acids and salts, polyols, sugars, etc.). DES are usually composed of a substance functioning as hydrogen bond donor (HBD) (e.g., glycerol, organic acids) and another one as hydrogen bond acceptor (HBA) (e.g., choline chloride, amino acids), and their synthesis is straightforward and benign. DES possess features such as absence of flammability water (im)miscibility and low vapor pressure, and these attributes make DES suitable solvents for a spectrum of sustainable applications, such as extraction, synthesis, etc. [8]. To-date, by virtue of their unique properties, the use of DES for natural product extraction has been rapidly expanding, and there has been a bewildering number of substances used for DES synthesis.

The family of Lamiaceae is widespread and embraces 220 genera and 4000 species occurring around the globe. The chemistry of Lamiaceae species is exceptionally wide and versatile, as it concerns chemical constituents such as terpenes (diterpenes and triterpenes) and polyphenols, two major and multitudinous groups of biologically active compounds. *Salvia* L. is the largest genus of the Lamiaceae, represented by over than 1000 species [9,10]. *Salvia fruticosa* (syn. *S. triloba*), is known as Greek or Cretan sage, and it is a Lamiaceae species occurring in several parts of the East Mediterranean. A number of biological properties have been ascribed to this medicinal plant, which are mainly attributed to its polyphenolic load and composition [11–13]. However, up to now the development of green extraction processes for the production of polyphenol-containing bioactive extracts from *S. fruticosa* is extremely limited. Given the current expanding interest by several cosmetics and food supplement industries in Greece for this particular botanical, the current study had as objective the establishment of a batch stirred-tank green extraction methodology, by blending ultrasonication pretreatment and a highly efficacious DES, selected out of a thorough screening.

2. Materials and Methods

2.1. Chemicals

Chromatography solvents were HPLC grade. L-lactic acid (80%) was obtained from Fisher Scientific (Loughborough, UK). Sodium carbonate, sodium citrate dibasic sesquihydrate (>99%), sodium citrate monobasic (99%), sodium acetate trihydrate, ascorbic acid, rosmarinic acid, luteolin 7-*O*-glucoside, chlorogenic acid and 2,2-diphenylpicrylhydrazyl (DPPH) were from Sigma-Aldrich (Darmstadt, Germany). Sodium citrate tribasic dihydrate (>99%), Folin–Ciacalteu reagent, glycerol (99%) and citric acid and were from Merck (Darmstadt, Germany). Methanol and ethanol were from Honeywell/Riedel-de Haen (Seelze, Germany). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) and iron chloride hexahydrate were from Honeywell/Fluka (Steinheim, Germany).

2.2. Plant Material

Details regarding plant material source and handling have been described elsewhere [14]. In short, dry and powdered *S. fruticosa*, with mean particle size of 1.28 mm, was used in all experiments.

The material was from the area of Chania (Crete, southern Greece) and it composed of the aerial parts of the plant.

2.3. Preparation of the DES

To protocol followed for DES synthesis was based on a previously reported one [15]. Precise mass of HBD and HBA were mixed at various molar proportions, and the mixtures were heated at 70 °C, under continuous stirring at 500 rpm, until the formation of perfectly transparent liquids. This process usually required 60 min, depending on HBD/HBA combination and molar ratio. All DES produced were stored in glass screw-cap vials, at ambient temperature, in the dark, and they were periodically inspected for appearance of crystals over 5 weeks.

2.4. Ultrasonication Pretreatment

Ultrasonication of samples was applied prior to batch stirred-tank extraction, using an ultrasonication bath (Sonorex Bandeline, Berlin, Germany). The ultrsonication was carried out at ambient temperature, with the following settings: frequency, 100 Hz; power, 120 W; acoustic energy density, 120 W L^{-1}.

2.5. Batch Stirred-Tank Extraction Process

For the screening process, all DES were tested as 70% (*w*/*v*) aqueous mixtures. Control solvents were 60% (*v*/*v*) ethanol, 60% (*v*/*v*) methanol and deionized water [14,16]. Extractions were accomplished in a 20-mL glass vial, using 15 mL of each solvent and 0.375 g of plant material, for 150 min. Continuous stirring at 500 rpm and regulation of temperature at 50 °C were provided by a stirring hot plate (VELP Scientifica, San Francisco, CA, USA). After the extraction, extracts were centrifuged at 10,000× *g* for 10 min, to obtain a clear supernatant used for all determinations.

2.6. Design of Experiment and Response Surface Methodology

Response surface methodology was implemented through a Box–Behnken design with three central points, to assess the effect of selected process variables on the total polyphenol yield (Y_{TP}, mg GAE g^{-1} dm). The variables considered were the stirring speed (S_S, rpm), the DES/water proportion (C_{DES}, % *w*/*w*) and the ultrasonication pretreatment time (t_{US}, min), which were assigned as X_1, X_2 and X_3, respectively. Codification of the variable levels (Table 1) was done as described in detail elsewhere [16]. Model fitting to the experimental data was evaluated by performing ANOVA and lack-of-fit analysis, and non-significant dependent terms were excluded from the model (mathematical equation).

Table 1. Actual and coded levels of the independent variables selected to set up the experimental design.

Independent Variables	Code Units	Coded Variable Level		
		−1	**0**	**1**
S_S (rpm)	X_1	300	600	900
C_{DES} (%, *w*/*w*)	X_2	55	70	85
t_{US} (min)	X_3	5	10	15

2.7. Extraction Kinetics

The kinetic model employed has been previously reported [17]. The model is described by the following equation:

$$Y_{TP(t)} = \frac{Y_{TP(s)}t}{t_{0.5} + t} \quad (1)$$

$Y_{TP(t)}$ and $Y_{TP(s)}$ correspond to the yield in total polyphenols at any time, t, and at saturation (equilibrium). The term $t_{0.5}$ corresponds to the time at which $Y_{TP(t)} = \frac{Y_{TP(s)}}{2}$. The initial extraction rate, h, and the second-order extraction rate, k, can be determined by the following equations:

$$h = \frac{Y_{TP(s)}}{t_{0.5}} \quad (2)$$

$$k = \frac{1}{Y_{TP(s)}\ t_{0.5}} \quad (3)$$

The influence of temperature on k was portrayed by non-linear regression between k and T. Effective description of this correlation could be given by an exponential model, as previously proposed [18]:

$$k = k_0 + ae^{-bT} \quad (4)$$

Term k corresponds to the second-order extraction rate and k_0 is a pre-exponential factor. Where a and b are fitting parameters. Estimation of the activation energy (E_a) of the process was calculated as follows [19]:

$$ln\left(\frac{k}{k_{ref}}\right) = \left(-\frac{E_a}{R}\right)\left(\frac{1}{T} - \frac{1}{T_{ref}}\right) \quad (5)$$

T_{ref} and T represent a reference temperature (K) and a temperature at which kinetics was traced, k_{ref} and k correspond to the second-order extraction rate constants, R is the universal gas constant (8.314 J K^{-1} mol^{-1}) and E_a the activation energy (J mol^{-1}).

2.8. Determinations

Total polyphenol concentration was determined with the Folin–Ciocalteu methodology and yield in total polyphenols was expressed as mg gallic acid equivalents (GAE) per g dry mass [20]. Total flavonoid determination was carried out with the aluminum chloride reagent and results were expressed as mg rutin equivalents (RtE) per g dry mass [21]. The antiradical activity (A_{AR}) was estimated with a stoichiometric methodology [16], using DPPH as the radical probe. The ferric-reducing power was measured with a modified FRAP assay and expressed as µmol ascorbic acid equivalents (AAE) per g dry mass [16].

2.9. Chromatographic Analyses

The equipment used was a FinniganMAT P4000 pump, coupled with a UV6000LP diode array detector (Thermo Scientific, Waltham, MA, USA), and a TSQ Quantum Access LC/MS/MS, with a surveyor pump (Thermo Scientific, Walltham, MA, USA), interfaced by XCalibur 2.1, TSQ 2.1 software. Chromatographic analyses were performed on a Superspher RP-18 column, 125 mm × 2 mm, 4 µm, at 40 °C, with a 10 µL injection loop. The eluents were (A) 2.5% acetic acid and (B) methanol. The flow rate was 0.3 mL min^{-1}, and the elution program used was: 0 min, 100% A; 22 min, 65% A; 32 min, 65% A; 60 min, 0% A; 65 min, 0% A. Mass spectra acquisition was performed with negative ionization, capillary temperature 300 °C, sheath gas pressure 30 mTorr, auxiliary gas pressure 15 mTorr, and collision pressure at 1.5 mTorr; quantification was done with external standards, using a rosmarinic acid (50–3000 µg L^{-1}, R^2 = 0.9985) and a luteolin 7-*O*-glucoside (5–1500 µg L^{-1}, R^2 = 0.9982) calibration curve. Standards were prepared in HPLC grade methanol and stored at −17 °C.

2.10. Statistics

Design of experiment, statistics associated with response surface methodology (ANOVA, lack-of-fit) and distribution analysis was performed with JMP™ Pro 13 (SAS, Cary, NC, USA). Linear regressions, non-linear regressions and kinetics model fitting were performed with SigmaPlot™

12.5 (Systat Software Inc., San Jose, CA, USA). Extraction experiments were carried out at least twice and all determinations in triplicate. Values given are averages ± standard deviation.

3. Results and Discussion

3.1. Screening of DES for Extraction Efficiency

The evidence emerged from a previous investigation suggested that citrate salts may form DES with increased extraction efficiency [15]. Thus, the generation of a series of DES was systematically approached, by selecting two widely used HBDs, glycerol (GL) and L-lactic acid (LA), and citrate salts as the HBAs (Figure 1). The salts tested were sodium citrate monobasic (SCMB), sodium citrate dibasic (SCDB) and sodium citrate tribasic (SCTB). However, as attempts to synthesize DES with either GL or LA and SCMB did not meet with success, even when HBD:HBA molar ratio ($R_{mol}^{D/A}$) was 15. Thus, SCMB was not further considered. With regard to SCDB, it formed stable DES (no crystallization) with GL and LA at $R_{mol}^{D/A} \geq 9$; therefore, a series of GL-SCDB and LA-SCDB DES were synthesized with $R_{mol}^{D/A}$ varying from 9 to 15. On the other hand, SCTB formed stable DES with GL only at a $R_{mol}^{D/A}$ of 15. By contrast, stable DES with LA and SCTB were formed at $R_{mol}^{D/A} \geq 7$. Thus, LA-SCTB DES were tested within a $R_{mol}^{D/A}$ range of 7 to 15.

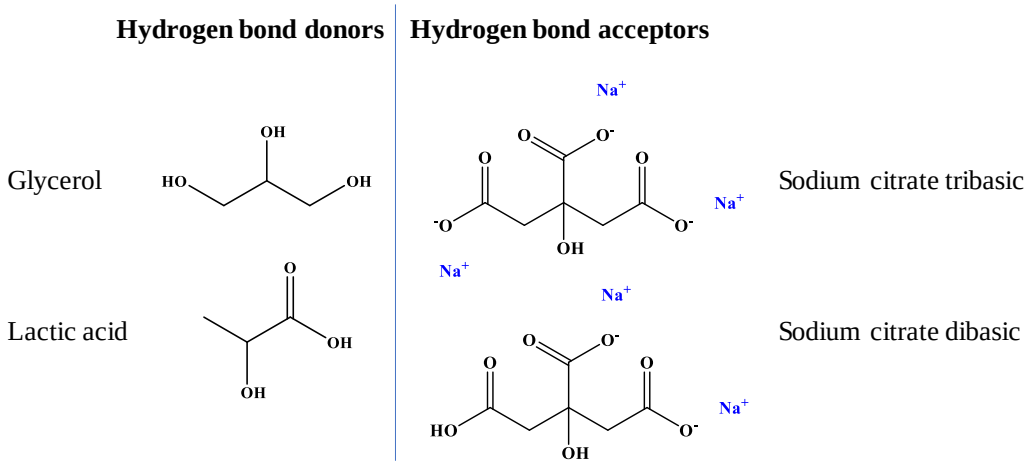


Figure 1. Hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) tested in the current investigation.

In total, 14 DES were tested covering a wide pH range, from 2.86 (LA-SCDB15) to 7.50 (GL-SATB15). The extraction efficiency of the DES synthesized was compared to other green solvents, including water and 60% (*v*/*v*) ethanol, but also 60% (*v*/*v*) methanol, which is a commonly used solvent for polyphenol extraction (Figure 2). The highest Y_{TP} was found for the extraction with 60% (*v*/*v*) methanol (67.86 ± 1.70 mg GAE g^{-1} dm), followed by 60% (*v*/*v*) ethanol (55.64 ± 1.39 mg GAE g^{-1} dm). Regarding the DES, the LA-SCDB with $R_{mol}^{D/A}$ = 15, termed as LA-SCDB15, gave a Y_{TP} of 52.31 ± 1.31 mg GAE g^{-1} dm and it was the most efficient one (php < 0.05), as opposed to GL-SATB15, which was the least efficient (31.49 ± 0.63 mg GAE g^{-1} dm). Because it was observed that these extreme Y_{TP} values coincided with the corresponding extreme pH values, concerns were raised as to what extent the pH of a DES could affect polyphenol extractability.

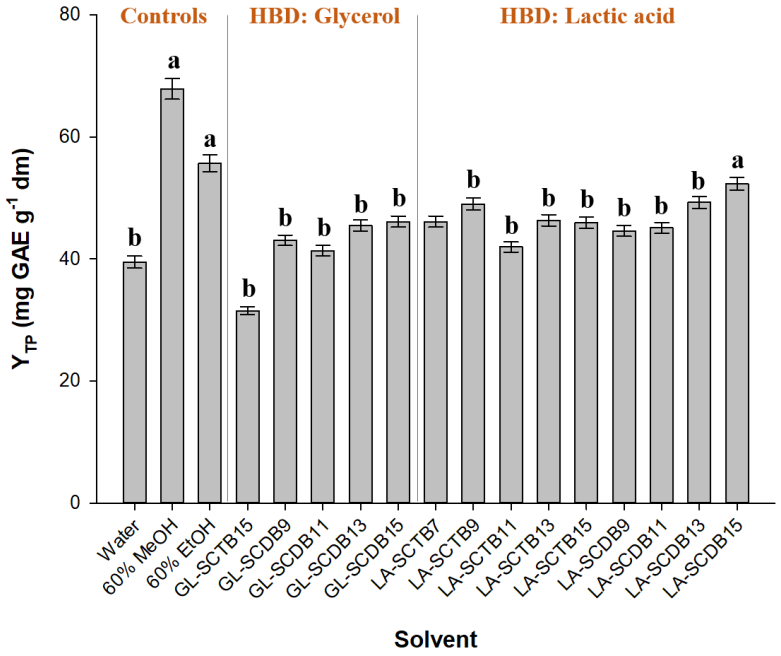


Figure 2. Graph showing the results of screening of the deep eutectic solvents (DES) tested. Extractions were accomplished at 50 °C, for 150 min, under continuous stirring at 500 rpm. All DES were tested as 70% (*w*/*v*) aqueous mixtures. Values designated with different letters are statistically different ($p < 0.05$).

To obtain evidence for such an effect, the pH values of all DES tested were plotted against Y_{TP} (Figure 3). The linear regression gave $R^2 = 0.67$ ($p = 0.0003$), revealing a trend that should not be overlooked, which evidenced higher extraction efficiency for DES with lower pH. Although previous studies on polyphenol extraction with DES stressed emphatically the importance of $R^{D/A}_{mol}$ on the extraction yield [16,21–24], a correlation of yield with pH is heretofore unreported.

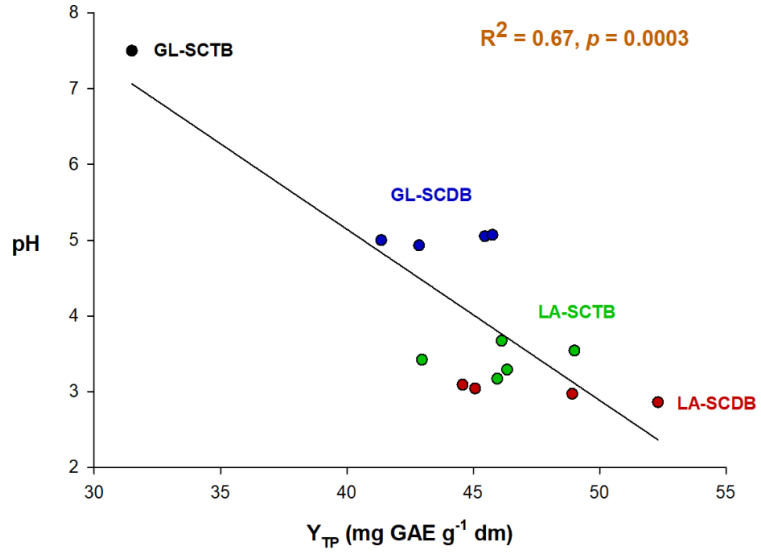


Figure 3. Linear regression between the pH of the DES tested and Y_{TP}. All DES were tested as 70% (*w*/*v*) aqueous mixtures.

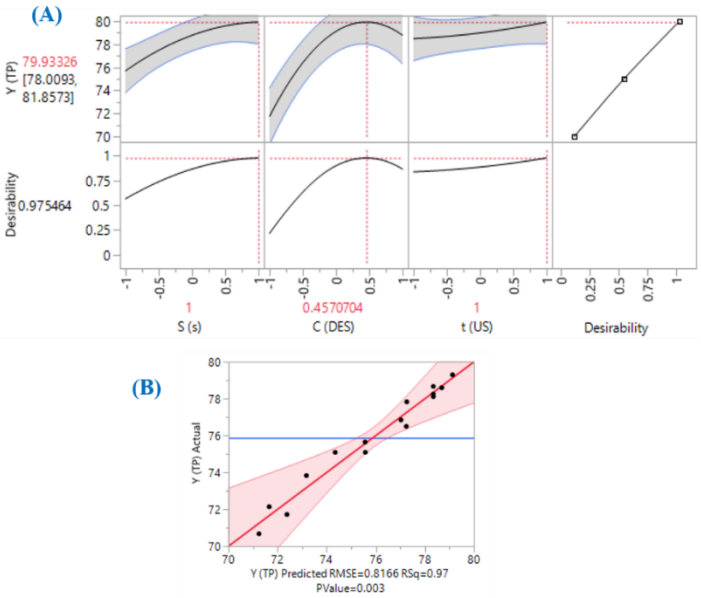
As also reported for classical solvents, pH is critical for DES extraction efficiency. Earlier investigations with conventional volatile solvents addressed the role of pH on polyphenol extractability, demonstrating that higher total polyphenol yield from olive leaves could be achieved at pH 2 [25]. Results from following studies on onion solid wastes were in the same line, indicating pH 2 as optimal

to maximize polyphenol extraction [26]. Furthermore, examinations on grape stem [27] and grape seed [28] polyphenol extraction showed that in most cases higher yields in total polyphenols, total flavanols and proanthocyanidins were favored at pH < 3.5. For other plant tissues, such as *Solanum melongena*, effective extraction was accomplished with 70% ethanol adjusted at pH 3 [29]. Such a phenomenon was ascribed to the protective effect of low pH on polyphenol against polyphenol oxidation because polyphenol oxidizability is higher at neutral or alkaline environment, due to phenolic hydroxyl dissociation. Likewise, it could be argued that acidic DES might act protectively with regard to polyphenol oxidation, and this would be likely to contribute to achieving higher Y_{TP}.

3.2. Extraction Process Optimization

Since LA-SCDB15 provided significantly higher Y_{TP} compared to all other DES tested, this solvent was chosen to further optimize the extraction process. To this end, three process variables that can critically affect polyphenol extraction [16,21,24], namely the S_S, the C_{DES} and the t_{US}, were included in the experimental design. The design deployed aimed at assessing the effect of the process variables and identifying any synergistic functions between them. The evaluation of model fitting and validity was based on the ANOVA and lack-of-fit test (Figure 4), taking into account the proximity of measured and predicted values (Table 2). The mathematical model derived after omitting non-significant terms, was as follows:

$$Y_{TP} = 78.34 + 1.31X_1 + 1.70X_2 + 1.09X_2X_3 - 3.86X_2^2 \ (R^2 = 0.97, p = 0.003) \quad (6)$$



Lack Of Fit

Source	DF	Sum of Squares	Mean Square	F Ratio
Lack Of Fit	3	3.1640250	1.05468	12.4177
Pure Error	2	0.1698667	0.08493	Prob > F
Total Error	5	3.3338917		0.0754
				Max RSq

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	78.343333	0.471444	166.18	<.0001*
S (s)	1.30625	0.288699	4.52	0.0063*
C (DES)	1.70375	0.288699	5.90	0.0020*
t (US)	-0.255	0.288699	-0.88	0.4175
S (s)*C (DES)	0.7375	0.408282	1.81	0.1307
S (s)*t (US)	0.475	0.408282	1.16	0.2972
C (DES)*t (US)	1.09	0.408282	2.67	0.0444*
S (s)*S (s)	-0.967917	0.424954	-2.28	0.0717
C (DES)*C (DES)	-3.862917	0.424954	-9.09	0.0003*
t (US)*t (US)	0.2245833	0.424954	0.53	0.6198

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
S (s)	1	1	13.650312	20.4720	0.0063*
C (DES)	1	1	23.222112	34.8273	0.0020*
t (US)	1	1	0.520200	0.7802	0.4175
S (s)*C (DES)	1	1	2.175625	3.2629	0.1307
S (s)*t (US)	1	1	0.902500	1.3535	0.2972
C (DES)*t (US)	1	1	4.752400	7.1274	0.0444*
S (s)*S (s)	1	1	3.459185	5.1879	0.0717
C (DES)*C (DES)	1	1	55.097078	82.6318	0.0003*
t (US)*t (US)	1	1	0.186231	0.2793	0.6198

Figure 4. Statistics associated with model fitting, performed by implementing response surface methodology. (**A**), Desirability function; and (**B**), actual-by-predicted plot. Inset tables (lack-of-fit, parameter estimates and effect test) illustrate the effect of independent (process) variables on the response. Asterisk (*) on values in the "parameter estimates" and "test effects" inset tables signify statistically significant values (at least at a 95% significance level).

Table 2. Analytical presentation of the design of experiment (design points), including predicted and measured values of the response.

Design Point	Independent Variables			Response	
	X_1 (S_S, rpm)	X_2 (C_{DES}, % *w*/*v*)	X_3 (t_{US}, min)	Y_{TP} (mg GAE g^{-1} dm)	
				Measured	Predicted
1	−1 (300)	−1 (55)	0 (10)	70.67	71.24
2	−1 (300)	1 (85)	0 (10)	73.83	73.17
3	1 (900)	−1 (55)	0 (10)	71.72	72.38
4	1 (900)	1 (85)	0 (10)	77.83	77.26
5	0 (600)	−1 (55)	−1 (5)	75.09	74.35
6	0 (600)	−1 (55)	1 (15)	72.14	71.66
7	0 (600)	1 (85)	−1 (5)	75.09	75.57
8	0 (600)	1 (85)	1 (15)	76.50	77.24
9	−1 (300)	0 (70)	−1 (5)	76.85	77.02
10	1 (900)	0 (70)	−1 (5)	78.60	78.69
11	−1 (300)	0 (70)	1 (15)	75.65	75.56
12	1 (900)	0 (70)	1 (15)	79.30	79.13
13	0 (600)	0 (70)	0 (10)	78.67	78.34
14	0 (600)	0 (70)	0 (10)	78.25	78.34
15	0 (600)	0 (70)	0 (10)	78.11	78.34

The square correlation coefficient (R^2) was a good indicator of the total variability around the mean provided by the Equation (6). Assuming a confidence interval of 95% and considering the R^2 the *p* value for lack-of-fit (Figure 4), it could be supported that the mathematical model displayed very satisfactory adjustment to the experimental data. The 3D graphs that represent a visualization of the model, can portray at-a-glance the effect of the process variables on the response (Y_{TP}) (Figure 5). The desirability function (Figure 4) provided the theoretical optimized values for each of the variables considered, which were S_S = 900 rpm, C_{DES} = 77% (*w*/*v*), and t_{US} = 15 min. By adjusting these optimal settings, the predicted maximum response was calculated to be 79.93 ± 1.92 mg GAE g^{-1} dm. To ascertain the validity of the model, three individual extracts were performed using the optimized values and the outcome was 78.39 ± 2.96 mg GAE g^{-1} dm, illustrating the accuracy of response prediction.

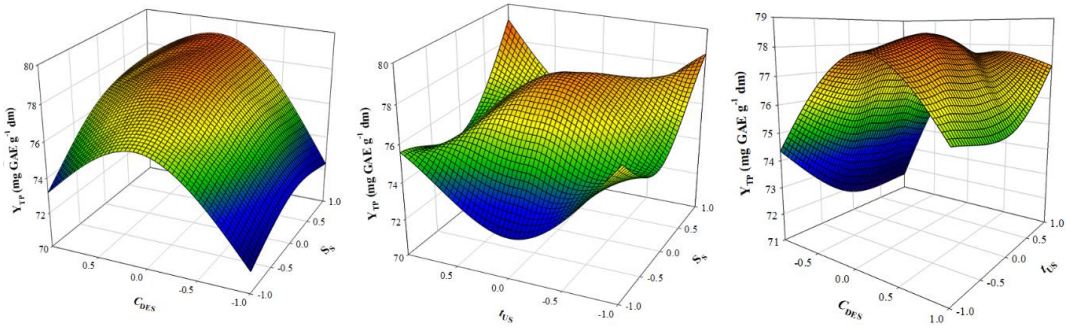


Figure 5. The effect of independent variables on the response (Y_{TP}), illustrated as three-dimensional plots.

The ANOVA results indicated that X_3 (t_{US}) was not statistically significant, as opposed to its cross term with X_2 (C_{DES}) (Figure 4). This finding pointed to a combined effect of these two variables and evidenced that the efficiency of the ultrasonication pretreatment might be dependent on the proportion of DES/water. On the other hand, X_1 (S_S) had a clear positive effect on Y_{TP}, which showed that increased speed of agitation favored polyphenol extraction yield. Recent studies on the effect of S_S on Y_{TP} gave contradictory results, suggesting that the influence exerted by S_S might not follow a specific pattern.

In certain cases, such as polyphenol extraction from hop [16], saffron processing wastes [22] and onion solid wastes [30], relatively high S_S (>650 rpm) were demonstrated to provide Y_{TP} maximization.

To the contrary, the requirement in S_S for optimum extraction of olive leaf polyphenol was shown to be either low (300 rpm) [31] or moderate (500 rpm) [21]. In another study on the extraction of polyphenols from *M. oleifera* leaves, the optimum S_S was determined to be 800 rpm, but when ultrasonication was integrated as pretreatment, the optimum S_S was 200 rpm [23]. In general, S_S is considered to play important role in solid–liquid extraction, and its careful adjustment may end up in significantly higher yields [32,33]. It has been supported that a sufficient level of S_S results in turbulence in the extraction tank, which is appropriate to boost mass transfer rate, and increases in S_S have been correlated to higher polyphenol diffusivity [33].

C_{DES} had also a significant positive effect on Y_{TP}, and the optimum value estimated was 77% (*w*/*v*). This level lies between 75 and 80% (*w*/*v*) found for polyphenol extraction with DES from *M. oleifera* leaves [23,34], and 78 and 80% (*w*/*v*) from olive leaves [21,35]. Other investigations reported 80% (*w*/*w*) for tartary buckwheat hull [36], 80% (*w*/*w*) for sea buckthorn leaves [37], 74% (*w*/*w*) for *Cymbidium kanran* [38] and 76.2% (*w*/*w*) for grape skin [39]. In all these optimization studies, the appropriate adjustment of water amount was shown to be critical for the extraction efficiency, because the DES/water proportion regulates features such as viscosity and polarity [40], which profoundly affect solute solubility, hence extraction performance. Such hypothesis has been well exemplified by a recent examination, which demonstrated that the higher the lipophilicity of the HBA in a DES, the higher the water amount required to achieve polyphenol extraction maximization from *O. dictamnus* [41].

3.3. Extraction Kinetics—Temperature Effects

Previous studies on the extraction of polyphenols from *S. fruticosa* using methyl β-cyclodextrin (m-β-CD) showed that extracts with increased polyphenol concentration and improved antioxidant characteristics could be obtained at 80 °C [14]. However, a following investigation with a 60% (*w*/*v*) hydroglycerolic mixture demonstrated that Y_{TP} displayed a gradual decrease when extraction temperature varied from 50 to 80 °C [42], although differences were non-significant ($p < 0.05$). Therefore, to obtain a reliable picture of the effect of temperature, extraction kinetics was traced within the range of 40 to 80 °C (Figure 6), under optimized conditions, that is, S_S = 900 rpm, C_{DES} = 77% (*w*/*v*), and t_{US} = 15 min.

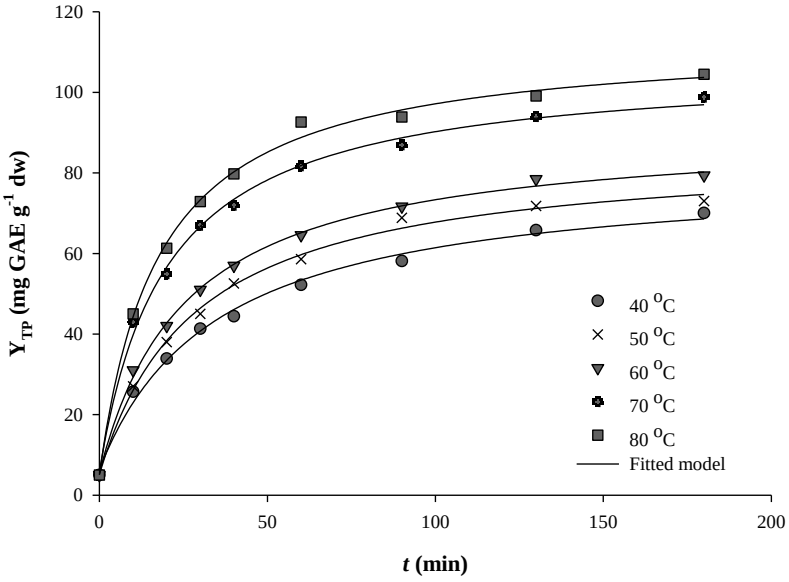


Figure 6. Kinetics of polyphenol extraction from *S. fruticosa*, traced under optimized conditions (S_S = 900 rpm, C_{DES} = 77% (*w*/*v*), and t_{US} = 15 min).

Y_{TP} exhibited an increasing trend, and at 80 °C the $Y_{TP(s)}$ determined was 113.39 mg GAE g^{-1} dm (Table 3). Likewise, the initial extraction rate, h, increased from 2.314 mg g^{-1} min^{-1} at 40 °C to 6.439 mg g^{-1} min^{-1} at 80 °C, and $t_{0.5}$ showed a declining tendency over this range, which manifested acceleration of the extraction. In a similar manner, the second-order extraction rate, k, increased from 0.356×10^{-3} g mg^{-1} min^{-1} at 40 °C to 0.501×10^{-3} g mg^{-1} min^{-1} at 80 °C, and k values correlated well with T ($R^2 = 0.96$, $p = 0.0413$), using the exponential model described by the Equation (4) (Figure 7). Comparison with extraction using hydroglycerolic solvent [42] showed that the fitting parameter b (Equation (4)), which is a measure of the sensitivity of k with regard to T changes, was 0.0136 for the extraction with LA-SCDB15 and 0.0765 for the extraction with hydroglycerolic solvent. This finding suggested that the extraction with hydroglycerolic solvent was more energy-demanding.

Table 3. Illustration of the data derived by implementing kinetics to assess the effect of T on the extraction of *S. fruticosa* polyphenols, under optimized conditions.

T (°C)	Kinetic Parameters				
	k ($\times 10^{-3}$) (g mg^{-1} min^{-1})	H (mg g^{-1} min^{-1})	$Y_{TP(s)}$ (mg GAE g^{-1})	$t_{0.5}$ (min)	E_a (kJ mol^{-1})
40	0.356	2.314	80.64 [a]	34.85	
50	0.407	2.994	85.73 [a]	28.63	
60	0.424	3.508	90.95 [a]	25.93	7.64
70	0.471	5.388	107.01 [a]	19.86	
80	0.501	6.439	113.39 [b]	17.61	

Values with different letters within the same column are statistically different ($p < 0.05$).

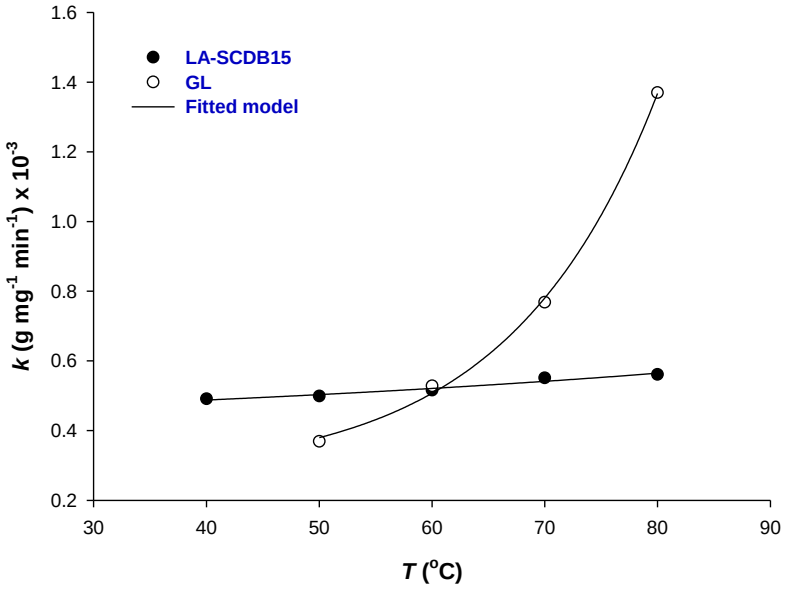


Figure 7. Non-linear regression between second-order extraction rate values, k, and T. Data concerning the extraction with 60% glycerol/water (GL) were obtained from Grigorakis et al., 2020.

To corroborate this hypothesis, the activation energy (E_a) of the process was estimated using the Equation (5). The barrier level of 7.64 kJ mol^{-1} found was significantly lower than 47.67 kJ mol^{-1} determined for the extraction with 60% (w/v) glycerol [42], thus affirming the higher efficiency of the extraction with LA-SCDB15. At this point, it should be stressed that in both cases stirred-tank extraction took place after ultrasonication pretreatment. This pretreatment stage resulted in washing out the most readily extracted compounds, a phenomenon also observed in other cases [16,21] and therefore the E_a determined corresponded to the extraction of the remaining solute, whose dissolution and entrainment into the liquid phase is governed by internal diffusion. The fact that the stirred-tank stage was far less energy-demanding using LA-SCDB15 than 60% (w/v) glycerol, evidenced that this

solvent might provide higher polyphenol solubility or that it might penetrate easier into the solid particles, or both.

3.4. Polyphenolic Profile and Antioxidant Activity—Comparative Assessment

To further bring out the efficiency of LA-SCDB15, the characteristics of an extract obtained under optimized conditions were compared to those from two preexisting green extraction methods, one performed with methyl β-cyclodextrin (m-β-CD) [14] and one with 60% (*w*/*v*) glycerol/water mixture (GL) [42], but also 60% (*v*/*v*) aqueous ethanol and 60% (*v*/*v*) aqueous methanol (Table 4).

Table 4. Comparative assessment of *S. fruticosa* extracts produced with LA-SCDB15 and other green solvents. Values given represent means ± standard deviation.

Extract	Y_{TP} (mg GAE g^{-1} dm)	A_{AR} (μmol DPPH g^{-1} dm)	P_R (μmol AAE g^{-1} dm)
Water	63.72 ± 0.96	613.07 ± 12.26 [a]	529.14 ± 7.94 [a]
60% MeOH	84.71 ± 1.27	828.54 ± 8.29 [b]	703.98 ± 10.56 [b]
60% EtOH	87.66 ± 1.31	820.45 ± 16.41 [b]	684.20 ± 10.26 [b]
m-β-CD	85.54 ± 1.28	820.93 ± 16.42 [b]	590.66 ± 14.77 [b]
GL	87.26 ± 1.31	817.58 ± 8.18 [b]	709.12 ± 17.73 [b]
LA-SCDB15	98.05 ± 1.47 [a]	751.74 ± 7.52 [b]	521.85 ± 7.83 [a]

Values with different letters within the same column are statistically different ($p < 0.05$).

The LA-SCDB15 extract was found to have significantly higher Y_{TP}, which demonstrated its high extraction capacity. Furthermore, the extract displayed A_{AR} comparable to the other extracts, except for water extract, where the A_{AR} was significantly weaker. On the other hand, both LA-SCDB15 and water extracts exhibited significantly lower P_R.

Three major *S. fruticosa* polyphenols occurring in LA-SCDB15 extracts were considered for quantification (Figure 8), and the results were compared to GL and m-β-CD. As can be seen in Table 5, extraction with LA-SCDB15 afforded by 31.8% higher yield in chlorogenic acid compared to m-β-CD, but by 8.3% less so compared to GL. On the other hand, the yield attained with LA-SCDB15 for luteolin 7-*O*-glucuronide was by only 2.7% higher than that attained with m-β-CD, but by 23% higher than that achieved with GL. Likewise, extraction with LA-SCDB15 performed by 38 and 37.6% higher than that with m-β-CD and GL, respectively, with regard to rosmarinic acid recovery. Overall, the extraction with LA-SCDB15 was by 27.6 and 32.9% more efficient than the corresponding carried out with m-β-CD and GL.

Table 5. Extraction yield in principal polyphenolic phytochemicals of *S. fruticose*, using LA-SCDB15, methyl β-cyclodextrin (m-β-CD) and 60% (*w*/*v*) glycerol/water (GL). Values reported are means ± standard deviation.

Compound	Yield (mg g^{-1} dm) ± sd		
	m-β-CD	**GL**	**LA-SCDB15**
Chlorogenic acid	0.15 ± 0.02 [a]	0.24 ± 0.05 [b]	0.22 ± 0.00 [b]
Luteolin 7-*O*-glucuronide	6.96 ± 1.12 [a]	5.51 ± 1.57 [b]	7.15 ± 0.37 [a]
Rosmarinic acid	10.57 ± 1.37 [a]	10.63 ± 0.98 [a]	17.04 ± 0.15 [b]
Sum	17.68 [a]	16.38 [a]	24.41 [b]

Values with different letters within the same row are statistically different ($p < 0.05$).

The outcome presented in Tables 4 and 5 pointed out the higher efficiency of LA-SCDB15 and it was in line with earlier examinations, which demonstrated that polyphenol extraction with DES was more effective than those performed with common conventional solvents, such as aqueous methanol or ethanol [16,21,23,24]. At this point it should be stressed that the content of *S. fruticosa* in certain major polyphenolic phytochemicals depends to a large extent by the time of collection. For example, it has been illustrated that the content of rosmarinic acid, which is the main *S. fruticosa* polyphenol, may vary from 5.57 to as high as 45.06 mg g^{-1} dm, and that of chlorogenic acid from 0.46 to 1.82 mg g^{-1} dm [12].

Seasonal ranges between 4.73 and 6.29, and 0.042 and 0.15 mg g^{-1} dm, for rosmarinic and chlorogenic acid, respectively, have also been determined [43]. However, other authors reported seasonal variation of rosmarinic acid to be between 0.20–1.70 mg g^{-1} dm [44]. Levels of rosmarinic acid reported in Greek *S. fruticosa* specimens were 14.83 mg g^{-1} dm [45] and 27.8–76.6 mg g^{-1} dm [46].

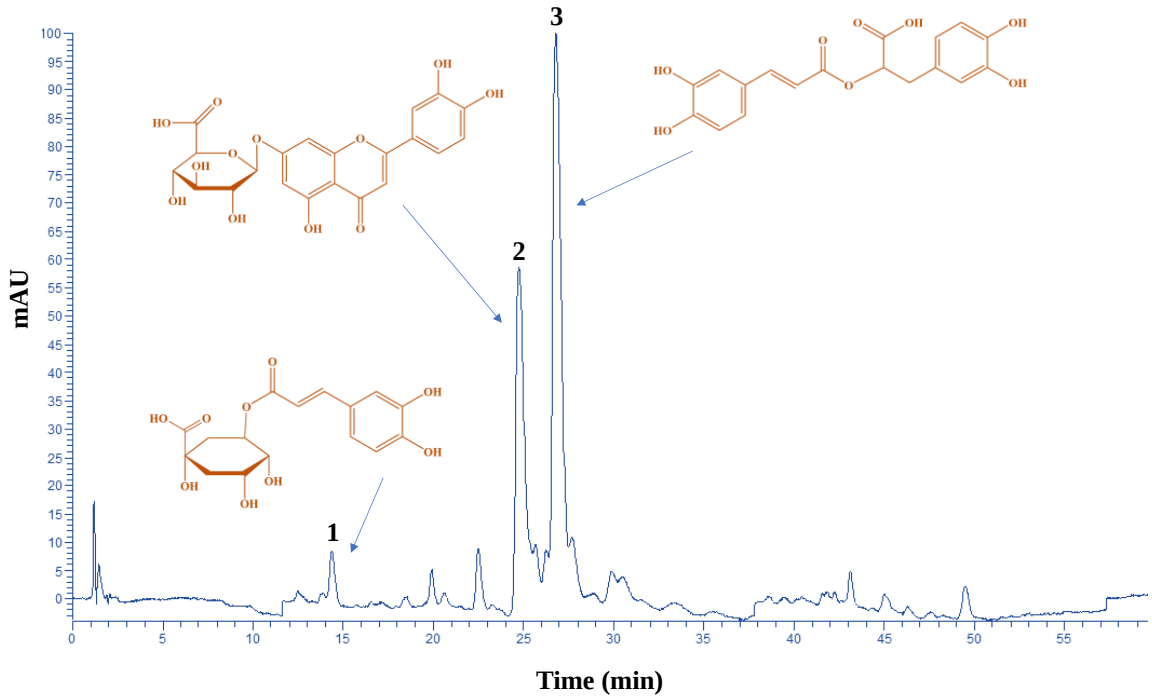


Figure 8. Chromatographic analysis of polyphenols in a *S. fruticosa* extract, produced under optimized conditions (S_S = 900 rpm, C_{DES} = 77% (*w*/*v*), and t_{US} = 15 min). The chromatogram was obtained at 330 nm. Peak assignment: 1, chlorogenic acid; 2, luteolin 7-*O*-glucuronide; 3, rosmarinic acid.

4. Conclusions

In the study presented herein, there has been a systematic approach to identify the most effective DES for the extraction of *S. fruticosa* polyphenols, by screening several citrate salts combined with two common HBDs, lactic acid and glycerol. The highest performing system was a DES composed of lactic acid and sodium citrate dibasic, at a molar ratio of 15:1, and for the first time, there has been evidence that the extraction performance of DES might depend on their pH. Optimization of the extraction and examination of the effect of temperature showed that blending ultrasonication pretreatment with optimized stirred-tank extraction may be a highly efficient green method to produce polyphenol-enriched extracts from *S. fruticosa*. This was also demonstrated by comparison with other pre-existing green extraction methodologies. The major polyphenolic phytochemicals identified in the extracts produced under optimized conditions were chlorogenic acid, luteolin 7-*O*-glucuronide and rosmarinic acid. The method developed is proposed as a green and efficacious methodology to recover bioactive polyphenols from the medicinal plant *S. fruticosa*. Testing of this solvent on several other matrices and comparison with other natural DES may reveal its full potential. Such a work is currently under progress.

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