# Analytical Methods

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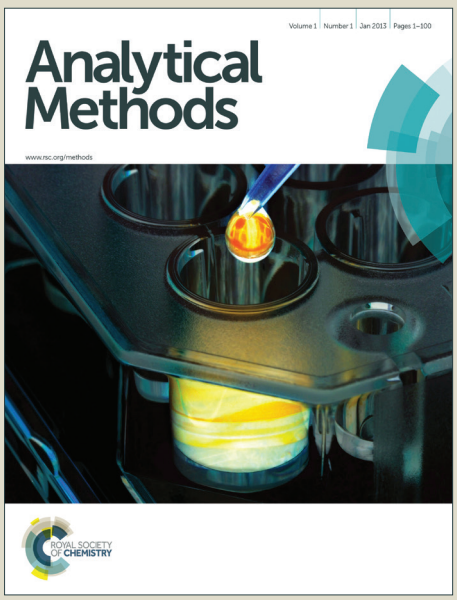





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PAPER

# Determination of phenolic acids in Prunella vulgaris L : a safe and green extraction method using alcohol-based deep eutectic solvent

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**Bohou Xia[abc], Dong Yan[abc], Yubing Bai[abc], Jiachi Xie[abc], Yi Cao[abc], Duanfang Liao[abc]* and Limei Lin[abc]***

The extraction of natural products with available green and safe solvents is rather limited. Recently, deep eutectic solvents (DESs) have been growing in interest as sustainable and safe solvents. However, few studies have applied DESs to the extraction and determination of phenolic acids. Therefore, in the present study, $DES_S$ (alcohol-based) was applied to extract rosmarinic acid and salviaflaside, which were the predominant phenolic acids in Prunella vulgaris. The extraction yield acted as the comprehensive evaluation indexes, and a Central Composite design (CCD) of response surface liquid/solid ratiomethodology (RSM) was employed to further optimize the alcohol-based DES extraction conditions. The results showed that the optimized extraction conditions included a water/DES ratio of 36 %（v/v）, liquid/solid ratio of 14 mL/g, extraction temperature of 86℃ and extraction time of 46 min for rosmarinic acid as well as a water/DES ratio of 30 %（v/v）, liquid/solid ratio of 12 mL/g, extraction temperature of 89℃ and extraction time of 32 min for salviaflaside in ChCl/ethylene glycol at 1/4 ratio. Under these conditions, the mean experimental value of the extraction yield( 3.658 and 1.049 mg/g for rosmarinic acid and salviaflaside) corresponded well with the predicted values. Moreover, these experimental values were higher and safer than those obtained from previously reported conventional extraction methods. This study suggests that DESs can be utilized as sustainable and safe extraction media for natural products.

## 1. Introduction

*Prunella vulgaris* L (Labiatae) is a perennial and herbaceous plant found throughout Europe, Asia and North America, as well as most temperate climates. In china, for its flowering season (from May to September), it is known as Xia Ku Cao.[1] The dried fruit spike of *Prunella vulgaris* is not only applied in traditional formulations as well as in modern herbal medications, but also used for making tea, as an agent for changing the course of a chronic disease.[2] According to the Chinese Pharmacopoeia (2010 version),[3] *Prunella vulgaris* is used for the treatment of a variety of diseases, such as conjunctival congestion, vertigo, headache and thyroid gland malfunction. Chemical constituent investigations have indicated that phenolic contents are the main bioactive components in *Prunella vulgaris*.[4] Rosmarinic acid**(Fig. 1a**), the only criterion for the quality control of *Prunella vulgaris* in the Chinese Pharmacopoeia (2010 version), as well as another phenolic substance named salviaflaside **(Fig. 1b)**, are attracting more and more interest due to their wide spectrum of bioactivities,[5] including antioxidant,[6] anti-inflammatory.[7] anti-cancer,[8] antiestrogenic,[9] neuroprotective,[10] immunomodulatory.[11] Due to these excellent roles, considering the high cost and toxicity of volatile organic solvents extraction, it is an attempt of practical significance to develop an efficient method for green extraction and separation of them from *Prunella vulgaris.*

Up to now, the number of available green solvents for extracting natural products is rather limited. In this sense, some of these new "Green Solvents" including water,[12] supercritical fluids [13] and ionic liquids(ILs) [14, 15]have received growing interest over the last two decades[16]. Water and supercritical fluids like $scCO_2$ are beneficial because they are non-toxic, relatively inert, easily removable when applied to extracting natural products. However they are hampered by the low yield of active principle and demand for expensive advanced apparatus respectively,[17] during widespread application in research and development. ILs currently receive much attention as a new class of solvents, due to their dissolution capacity of chemically diverse compounds, in microorganisms and plant.[18] Meanwhile, most of ILs have negligible vapour pressures at room temperature which, in

[a] College of Pharmacy, Hunan University of Chinese Medicine, Changsha 410208, P. R. China.
[b] Hunan Research Center of Engineering Technology for Rapid Test and Removal of Toxic and Harmful Substances in Chinese Medicine, Hunan University of Chinese Medicine, Changsha 410208, P. R. China.
Hunan Engineering Laboratory for Prevention and Control Technology of Toxic Substances in Chinese Medicine, Hunan University of Chinese Medicine, Changsha 410208, P. R. China.
* Corresponding author. Tel.: +86 0731 8458230; E-mail: lizasmile@163.com; E-mail: dfliao66@aliyun.com.

turn, facilitates their recovery and reusability in separation and purification processes.[19] In fact, some of the anions and cations commonly used in ILs have been recently shown to be are highly toxic and poorly biodegradable.[20] To overcome these limitations, a new ionic solvent, which is known as deep eutectic solvents (DESs), has emerged. DESs are novel, advanced class of solvents, which combines the interesting features characteristic of ILs and can be produced from cheap, non-toxic, completely biodegradable and biocompatible materials.[21, 22] Moreover, their preparation is very simple, requiring only heating and mechanical stirring to perform the homogeneous liquid in just one step. DESs are eutectic mixtures of solid halide salts(*eg*, choline chloride ) with hydrogen bond donors (HBD) such as amines, amides, alcohols or carboxylic acids that have much lower melting points than the starting materials.[23] Among them, choline chloride-based DESs have been found a number of interesting applications in electrochemistry,[24] functional materials,[25] organic synthesis,[26] catalytic conversion[27] and pretreatment process[28]. Recently, there is continuing interest in choline chloride-based DESs and their successful applications in separation and extraction of natural compounds. Flavonoids,[29, 30] aromatics,[31] saponins,[32] and chalcones[33] are successfully separated and extracted by choline chloride-based DESs from natural plants, and this method is proved to be more efficient than conventional method. However, there has little reports about the application of choline chloride-based DESs for separation and extraction of phenolic acids from natural plants.

The objective of this study was to optimize the DESs conditions for the extraction of phenolic acids (rosmarinic acid and salviaflaside) from *Prunella vulgaris*. Central composite design-response surface method (CCD-RSM) was employed to study the optimal extraction temperature, extraction time, liquid/solid ratio and DES/water ratios, which could maximize the yield of phenolic acids from *Prunella vulgaris*. In addition, the comparing extraction efficiency of yields of phenolic acids with conventional method was studied.

## 2. Experimental

### 2.1. Chemicals

1,3-butanediol (>99.5%)，1,2-propylene glycol (>99.0%), 1,4-butanediol (>99.%), 2,3-butanediol (>99.0%), Glycerol (>99.0%), and choline chloride(ChCl, >98.0%) were obtained from Sinopharm Chemical Reagent Co,Ltd (Shanghai, China). Ethylene glycol (>99.5%) was acquired from Guangdong Guanghua Sci-Tech Co. Ltd (Guangdong, China).

HPLC-grade acetonitrile (Tedia, USA) and acetic acid glacial (Tianjing Chemical Reagent Research Institute, China) were used for mobile phase preparation. Purified water was prepared with a Mili-Q water-purification system (Millipore, USA). Other solvents used for analyses were of analytical grade.

### 2.2. Materials

*Prunella vulgaris* was purchased from GaoQiao natural herbal special market (ChangSha,China).The *Prunella vulgaris* samples were identified by Dr. Zhi Wang, and the voucher specimens were deposited in the College of Pharmacy of Hunan University of Chinese Medicine (ChangSha, China).

Rosmarinic acid and salviaflaside were isolated from the whole plant of *Prunella vulgaris* and their structures were elucidated by different spectroscopic methods including $^{1}$H nuclear magnetic resonance spectrometry ($^{1}$H-NMR), $^{13}$C nuclear magnetic resonance spectroscopy ($^{13}$C-NMR), and mass spectrometry (MS), and were confirmed by comparing the data with those of previous studies. The compound purity was over 98% based on the HPLC area normalization method. The standard structures were illustrated in **Fig. 1**.

**Insert Fig. 1** The chemical structures of salviaflaside (a) and rosmarinic acid (b)

### 2.3. Apparatus and procedures

UPLC analysis was performed on a Waters ACQUITY UPLC H-Class system (Waters, USA) equipped with a binary solvent delivery pump, an auto sampler, and a Tunable UV detector and was controlled by the Empower 3 software. Separation was achieved at 30 °C on a Waters ACQUITY UPLC BEH C18 (1.7 μm, 2.1 mm × 50 mm). A binary gradient elution system composed of acetonitrile (phase A) and 0.5% acetic acid glacial in water (phase B), with the gradient elution as follows: 0 min to 4 min, 5% A; 4 min to 10 min, 5% to 12% A; 10 min to 20 min, 12% A. Detection was performed at a wavelength of 320 nm [34], with mobile flow rate at 0.4 mL min$^{-1}$. UPLC chromatograms of DESs extraction of *Prunella vulgaris* and two standards were shown in Fig.2.

### 2.4. Preparation of alcohol-based DESs

DESs mixtures were prepared accordingly to the method reported by Abbot *et al*.[21] Briefly, different alcohol-based HBDs and ChCl were heated to 80.0℃ with constant stirring until a homogeneous liquid formed. **Table 1** lists the abbreviations of the alcohol-based DESs synthesized and tested for extraction.

**Insert Table 1** List of alcohol-based DESs synthesized and tested for extraction.

### 2.5. Preparation of phenolic acid extraction from *Prunella vulgaris*.

The *Prunella vulgaris* samples were dried at room temperature, ground into fine powder. Subsequently, the powdered sample passed through suitable sieve for selection in order to achieve uniformity of particles.

### 2.6 Initial extraction procedure

The pulverized *Prunella vulgaris* (1.00g) was introduced into a flask, and 15 mL of different alcohol-based DESs (Salt/HBD ratio=1:2) solvents was added. Stirring while heating extraction



 

(temperature = 80°C), stirring extraction (temperature = 20 °C), and ultrasonic extraction (temperature = 20 °C, ultrasonic power = 79 W) were applied, respectively, when other variables were fixed at the extraction time of 30 min, and liquid/solid ratio of 15 mL/g. After extraction, the extracts were separated by centrifugation at 5000 rpm for 10 min. The collected solutions were diluted with 2 times methanol and filtered through a 0.45 μm nylon membrane prior to UPLC analysis. Each extraction was performed in triplicate.

### 2.7. Experimental design

Influence of process parameters was investigated using single-factor-test to determine the preliminary range of the extraction variables, and a three-level-four-factor CCD of RSM was used to determine the optimal combination of the variables for both the contents of rosmarinic acid and salviaflaside. On the basis of the single-factor experimental results, the four independent variables included water/DES ratios ($X_1$), liquid/solid ratio ($X_2$), extraction temperature ($X_3$) and extraction time ($X_4$), whereas the response variables included the extraction yields of both rosmarinic acid and salviaflaside (**Table 2**). Each variable was designated at three levels and coded as +1, 0 and 1 for high, intermediate and low values, respectively. And α= 2 was employed to evaluate the effects of operating variables on the yield of response variables. The response could be related to the selected variables by the following reduced cubic-order polynomial model:

$$y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j + \sum_{i=1}^{n-2} \sum_{j=i+1}^{n-1} \sum_{k=i+2}^{n} b_{ijk} x_i x_j x_k + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{iij} x_i^2 x_j \quad (1)$$

Where $y$ is the predicted response, $X_i$, $X_j$, $X_k$ refers to the coded levels of the design variables, $b_0$, $b_i$, and $b_{ii}$ are the regression coefficients for the intercept, linear, quadratic, respectively, and $b_{ij}$, $b_{ijk}$ and $b_{iij}$ are the interaction coefficients.

**Insert Table 2** Independent variables and their coded and actual values used for optimization.

### 2.8. Statistical analysis

All the experiments were carried out in triplicate, and the data presented are the mean values of these independent experiments. The experimental design and the regression analysis of the experimental data were performed using Design-Expert 8.0.6 (Stat-Ease Inc., Minneapolis, MN, USA). For multiple comparisons, analysis of variance (*ANOVA*) was applied, and $p$ values of <0.05 were considered statistically significant. The adequacy and significance of the model were tested from the $F$-value, determination coefficient ($R^2$) and a lack of fit, as determined from *ANOVA*. Student's $t$-test and Fischer's $F$-test was employed for evaluating the statistical significance of the regression coefficient and determining the cubic-order polynomial model equation, respectively. Its fitness was expressed by the regression coefficient $R^2$. $R^2$ values closer to 1, means the model is more accurate. The high adjusted and predicted coefficient of determination also illustrate whether the model adequately fits the data.

## 3. Results and discussion

### 3.1 Effect of the single factor test

#### 3.1.1. Effect of the extraction methods on the yields of rosmarinic acid and salviaflaside

Diffusion, solubility, viscosity, surface tension, polarity and HBD interaction are important factors for extracting target compounds in choline chloride-based deep eutectic solvent extraction.[21, 35] Different DESs and extraction methods affects these factors. A suitable extraction method is a substaintial factor that could prominently influence the extraction efficiency. The effect of different DESs and extraction methods on the yields of rosmarinic acid and salviaflaside is shown in Fig. 2. The yields of rosmarinic acid and salviaflaside by Stirring while heating extraction were remarkably higher than those extracted by room temperature stirring and ultrasonic extraction. This result indicating that temperature is the major factor on the yields of rosmarinic acid and salviaflaside due to its influence on the diffusion, solubility, viscosity, and so on.

**Insert Fig. 2** here

#### 3.1.2. Effect of the types of DESs on the yields of rosmarinic acid and salviaflaside

To further optimize the types of DESs and the ChCl/HBD ratio, the selection process was carried out with 6 types of DESs and 5 ranges of ChCl/HBD ratio(,from 1/2 to 1/6 (mol/mol) ) while other variables(*i.e*, extraction methods, extraction time, extraction temperature) were fixed at heating extraction, 30min and 80℃. As is shown in table 3, extracting by the Ethylene glycol-based *DESs*, glycerol-based *DESs*, and 1,2-propylene glycol-based *DESs* , among which Ethylene glycol-based DESs gained the highest yields, owned higher yields of rosmarinic acid and salviaflaside than the others. With the decreased amount of ChCl(1/2 to 1/4), yields of rosmarinic acid and salviaflaside increased simultaneously. When the ChCl/HBD ratio reached to 1/5 and 1/6, yields of rosmarinic acid and salviaflaside decreased apparently. Consequently, it is suggested that a ChCl/HBD ratio of 1/4 of ethylene glycol-based *DESs* was favorable for subsequent factor selection experiments.

**Insert Table 3** here

#### 3.1.3. Effect of the water/DES ratio on the yields of rosmarinic acid and salviaflaside

The properties of the DESs affected by the presence of water.[36, 37] Water is a polar molecule. Commonly used as extracting solvent in natural product chemistry, water is a low-

cost, non-polluting, renewable source. Moreover, it decreases the viscosity and modulates the polarity, which makes the DESs optimal to extract phenolic compounds and increases the dissolution rate of phenolic compounds. The effect of DES/water ratio on the yields of rosmarinic acid and salviaflaside are shown in **Fig. 3a**. Extraction was carried out at different ratio of water to DESs (0-100 vol %). The following conditions were kept as follows: extraction methods, heating extraction; extraction time, 30min; extraction temperature, 80 ℃. The result suggested that the miscibility with 40% water offers the highest yields while the lowest yields of rosmarinic acid and salviaflaside was observed in 100% water. Therefore, although several advantages of water take, a suitable ratio of water in DESs is important in extracting phenolic compounds.

**3.1.4. Effect of the extraction temperature on the yields of rosmarinic acid and salviaflaside**

Generally, the increased temperature of extraction medium can increase the diffusivity of the solvent into cells and enhances the desorption and solubility of compounds from the cells, which results in the dissolution of components.[38-39] To investigate the effect of temperature *o*n the yields of rosmarinic acid and salviaflaside, the extraction process was carried out at different temperatures of 40, 50, 60, 70, 80, 90 and 100 °C while other variables (*i.e*, extraction methods, extraction time, extraction solvent) were fixed at heating extraction, 30min and ethylene glycol-based DESs(1/4), respectively. As shown in **Fig. 3b**, the yields of both rosmarinic acid and salviaflaside continued to increase as the extraction temperature raised from 40 to 80°C, where a maximum yields were achieved. Although the extraction yields were also high from 80 to 100°C (the extraction reached an equilibrium of desorption and solubility from 80 to 100°C), this high temperature range can increase the cost of the extraction process.

**3.1.5. Effect of the extraction time on the yields of rosmarinic acid and salviaflaside**

The effects of different times on rosmarinic acid and salviaflaside productions are shown in **Fig. 3c**. Extraction process was carried out for different times (20, 30, 40, 50, 60 min) while other extraction variables were fixed as follows: heating extraction, ethylene glycol-based DESs (1/4) and extraction temperature of 80 °C, respectively. As the extraction time increased from 20 min to 40 min, the extraction yields of both rosmarinic acid and salviaflaside remarkably increased, reaching a maximum value at about 40 min. With the prolonged extraction time to 60 min, the yields of both had insignificant changes. This result showed that the extraction time of 40 min was sufficient to obtain the targeted phenolic acid.

**Insert Fig. 3** here

**3.2. Optimization of the extraction conditions by RSM**

**3.2.1 Statistical analysis and the model fitting**

The CCD of RSM in the experimental design involves four independent variables, three levels and six replicates at the center point, which was carried out to measure the inherent variability and process stability. The experimental conditions and the fit statistics of the extraction yields of 30 runs are performed in triplicate and the obtained results are depicted in **Table 4**.

**Insert Table 4** here
**Insert Table 5** here
**Insert Table 6** here

The effects of the four variables on the yields of rosmarinic acid and salviaflaside were highly significant except extraction time ($X_4$) (**Table 5** and **Table 6**). The predicted models of the extraction yield were obtained from the following reduced cubic-order polynomial equation:

$$Rosmarinic\ acid = -2.63523 - 0.79298X_1 + 1.23914X_2 + 0.20192X_3 + 0.054057X_4 + 0.015552X_1X_2 + 4.13641 \times 10^{-3}X_1X_3 - 4.06780 \times 10^{-4}X_1X_4 - 0.010913X_2X_3 + 4.66000 \times 10^{-3}X_2X_4 - 4.86777 \times 10^{-4}X_3X_4 + 0.014454X_1^2 - 0.065847X_2^2 - 1.31642 \times 10^{-3}X_3^2 - 1.24680 \times 10^{-3}X_4^2 + 2.74400 \times 10^{-4}X_1X_2X_3 - 1.44682 \times 10^{-4}X_1X_2X_4 + 2.03312 \times 10^{5}X_1X_3X_4 + 2.47393 \times 10^{-5}X_2X_3X_4 - 7.47529 \times 10^{-4}X_1^2X_2 - 9.93400 \times 10^{-5}X_1^2X_3 + 1.49563 \times 10^{-5}X_1^2X_4 + 1.02429 \times 10^{-3}X_1X_2^2 \quad (2)$$

$$salviaflaside = -2.63523 - 0.79298X_1 + 1.23914X_2 + 0.20192X_3 + 0.054057X_4 + 0.015552X_1X_2 + 4.13641 \times 10^{-3}X_1X_3 - 4.06780 \times 10^{-4}X_1X_4 - 0.010913X_2X_3 + 4.66000 \times 10^{-3}X_2X_4 - 4.86777 \times 10^{-4}X_3X_4 + 0.014454X_1^2 - 0.065847X_2^2 - 1.31642 \times 10^{-3}X_3^2 - 1.24680 \times 10^{-3}X_4^2 + 2.74400 \times 10^{-4}X_1X_2X_3 - 1.44682 \times 10^{-4}X_1X_2X_4 + 2.03312 \times 10^{5}X_1X_3X_4 + 2.47393 \times 10^{-5}X_2X_3X_4 - 7.47529 \times 10^{-4}X_1^2X_2 - 9.93400 \times 10^{-5}X_1^2X_3 + 1.49563 \times 10^{-5}X_1^2X_4 + 1.02429 \times 10^{-3}X_1X_2^2 \quad (3)$$

The statistical significance of the regression equation for the response surface reduced cubic model, as checked from the *F*-test, *T*-test and ANOVA are presented in **Table 5** and **Table 6**. The results of the high model *F*-value (*F*=237.9807 for rosmarinic acid and *F*=214.8000 for salviaflaside) and low *p*-value ($p$ <0.0001 for both) showed that the models were highly significant. The models showed good fitness and less variation around the mean explained by the model ($R^2$=0.9987 for rosmarinic acid and $R^2$=0.9985 for salviaflaside). The adjusted $R^2$ ($R^2_{adj}$ = 0.9945 for rosmarinic acid, $R^2_{adj}$ = 0.9939 for salviaflaside) was reasonable for the generated model, thus indicating that only 0.1% of the variation of response was due to the unpredicted variables. In addition, the models showed a reasonable prediction power ($R^2_{pred}$ = 0.9885 for rosmarinic acid, $R^2_{pred}$ = 0.9288 for salviaflaside). The difference between $R^2_{adj}$ and $R^2_{pred}$ was less than 0.2, which was within the acceptable limit.[40] The adequate precision (62.46 for rosmarinic acid, 52.64 for salviaflaside), which was calculated by dividing the difference between the maximum and minimum predicted responses, showed good signal to noise ratio (a ratio greater than 4 is desirable). The non-significant ($p$ > 0.05 for both) lack of fit for the final reduced model also



 

revealed that the model adequately is fitted to the significant independent variable effects.

In this case, $X_1$, $X_2$, $X_3$, $X_1X_2$, $X_1X_4$, $X_2X_3$, $X_2X_4$, $X_3X_4$, $X_1^2$,$X_2^2$,$X_3^2$,$X_4^2$, $X_1X_2X_3$, $X_1X_2X_4$, $X_1^2X_2$, $X_1^2X_3$, $X_1X_2^2$ were significant model terms for rosmarinic acid, and $X_1$, $X_2$, $X_3$, $X_4$, $X_1X_2$, $X_1X_3$, $X_1X_4$, $X_2X_4$, $X_3X_4$, $X_1^2$,$X_2^2$,$X_3^2$,$X_4^2$, $X_1X_2X_3$, $X_1X_3X_4$, $X_2X_3X_4$, $X_1^2X_2$, $X_1X_2^2$ were significant model terms for salviaflaside ($p$<0.05). The other terms were considered non-significant ($p$ > 0.05).

### 3.2.2 Analysis of response surfaces

The three-dimensional (3D) response surface and the contour plots were used to provide a means of visualizing the relationship between the responses and experimental levels of each variable and illustrate the interaction effects between the variables. In this study, the results of the extraction yields of rosmarinic acid and salviaflaside were highly affected by water/DES ratio ($X_1$), liquid/solid ratio ($X_2$) and extraction temperature ($X_3$), as shown in **Fig. 4 and Fig.5**.

**Fig. 4a and 5a**, which give the extraction yields of responses variables as a function of water/DES ratio ($X_1$), liquid/solid ratio ($X_2$) at a fixed extraction temperature (80℃) and extraction temperature (40min), indicated that the extraction yield increased rapidly with an increase in the liquid/solid ratio from 8 to 14 mL/g and decreased slowly with an increase from 14 to 15 mL/g. The extraction yields of responses variables decreased with the increase in the water/DES ratio from 35% to 60%. It can be seen that the maximum extraction yield of both responses variables were attained when water/DES ratio ($X_1$) and liquid/solid ratio ($X_2$) were around 35% and 14mL/g, respectively. A similar trend was seen in **Fig. 4b and 5b** as well as **Fig. 4c and 5c**.

**Fig. 4d and 5d**, show the 3D response surface plot and the contour plot at varying liquid/solid ratio ($X_2$) and extraction temperature ($X_3$) settings, at a fixed water/DES ratio(40%, v/v) and extraction temperature (40min),. It indicated that the maximum extraction yields of responses variables can be achieved when the extraction temperature and liquid/solid ratio were at the threshold level of around 85℃ and 13 mL/g, respectively. A similar trend was seen in **Fig. 4e and 5e** as well as **Fig. 4f and 5f**.

**Insert Fig. 4** here

**Insert Fig. 5** here

It can be concluded that optimal extraction conditions of responses variables were following ranges of the examined variables: $X_1$=36.18, $X_2$=14.14, $X_3$=86.67, $X_4$=46.69 for rosmarinic acid and $X_1$=30.25, $X_2$=12.26, $X_3$=89.15 and $X_4$=32.44 for salviaflaside . Under these conditions, the maximum yields of rosmarinic acid and salviaflaside were predicted as 3.6613 and 1.0530, respectively. Taking convenience into account, the optimum experimental parameters were modified as follows: $X_1$=36, $X_2$=14, $X_3$=86, $X_4$=46 for rosmarinic acid and $X_1$=30, $X_2$=12, $X_3$=89 and $X_4$=32 for salviaflaside . To compare the predicted results with experimental values, rechecking was performed using modified optimal conditions. The result showed that experimental value (3.658 and 1.049 mg/g of rosmarinic acid and salviaflaside, respectively) and predicted results were not significant ($p$ > 0.05).

### 3.2.3 Analytical performance and comparison of extraction methods and solvents

The linearity for each reference, together with the precision and LOD values are shown in **Table 7**. Both of the phenolic acids showed good linearity ($r^2$ > 0.9991) in a relatively wide concentration range. The precision was determined over five extractions of rosmarinic acid and salviaflaside under optimized operation parameters with DES and the RSD was 2.84–1.47%. The limits of determination (LODs) of the two phenolic acids under the present chromatographic conditions were determined at a signal-to-noise (S/N) ratio of 3, ranging from 0.08-0.14 μg/mL. Therefore, the method is precise enough, which highlights its potential applications in the determination of other medicinal products.

**Insert Table 7** here

Furthermore, alcohol-based DES extraction was compared with previously reported extraction methods and solvents, and as seen in **Table 8**, the alcohol-based DES extraction obtained higher mean values of phenolic acids yield than other previously reported extraction methods and solvents. A similar result was seen in flavonoids extraction with alcohol-based deep eutectic solvent method.[30]

**Insert Table 8** here

## 4. Conclusion

A highly efficient, green extraction method using alcohol-based DES was described for phenolic acids extractions from *Prunella vulgaris*. In this study, ChCl/ethylene glycol at 1/4 ratio was proved to be the best alcohol-based DES for the extraction of rosmarinic acid salviaflaside, after a systematic screening and comparing of various types of alcohol-based DESs and the ChCl/HBD ratio. Subsequent optimization of the operational parameters using CCD-RSM further improves the extraction efficiency. The optimal conditions were validated, under which the current extraction method was definitely a green and safe method with higher efficiency than conventional extraction methods. This study suggests that DESs can be utilized as sustainable and efficient extraction media for natural products.

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## Captions

**Fig. 1** The chemical structures of salviaflaside (a) and rosmarinic acid (b)

**Fig.2** UPLC chromatograms of DESs extraction of *Prunella vulgaris* (A) and two standards (B) detected at 320 nm.

**Fig. 3** Effects of different DESs and extraction methods on the yields of rosmarinic acid (a) and salviaflaside(b).

**Fig. 4** Effects of different (a) Water ratios in DES-water mixture, (b) Extraction temperatures, (c) Extraction time and on the yields of rosmarinic acid and salviaflaside.

**Fig. 5** (a-f) Response surface (3D) showing the effect of of water/DES ratio ($X_1$), liquid/solid ratio ($X_2$), extraction temperature ($X_3$) and extraction time ($X_4$) on the yield of rosmarinic acid.

**Fig. 6** (a-f) Response surface (3D) showing the effect of of water/DES ratio ($X_1$), liquid/solid ratio ($X_2$), extraction temperature ($X_3$) and extraction time ($X_4$) on the yield of salviaflaside.

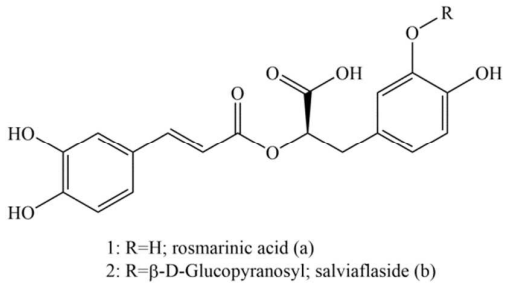
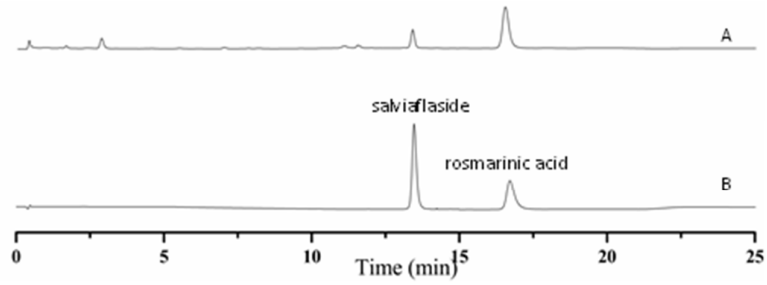



**Fig. 1** The chemical structures of rosmarinic acid (a) and salviaflaside (b)

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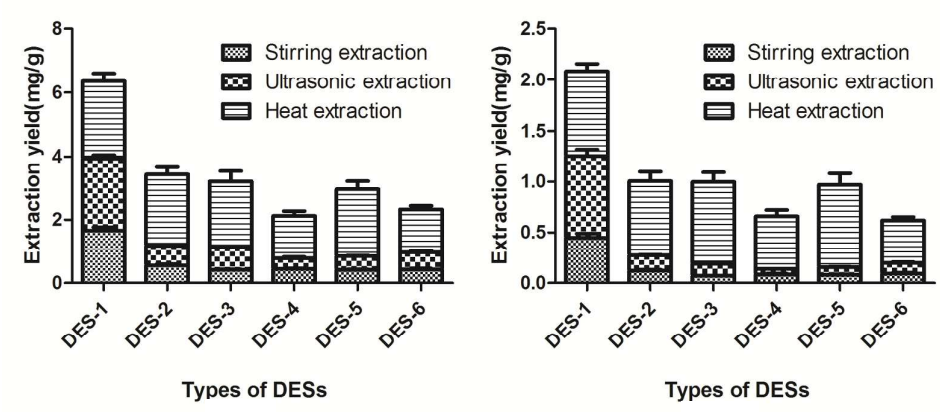



**Fig. 3** Effects of different DESs and extraction methods on the yields of rosmarinic acid (a) and salviaflaside(b).



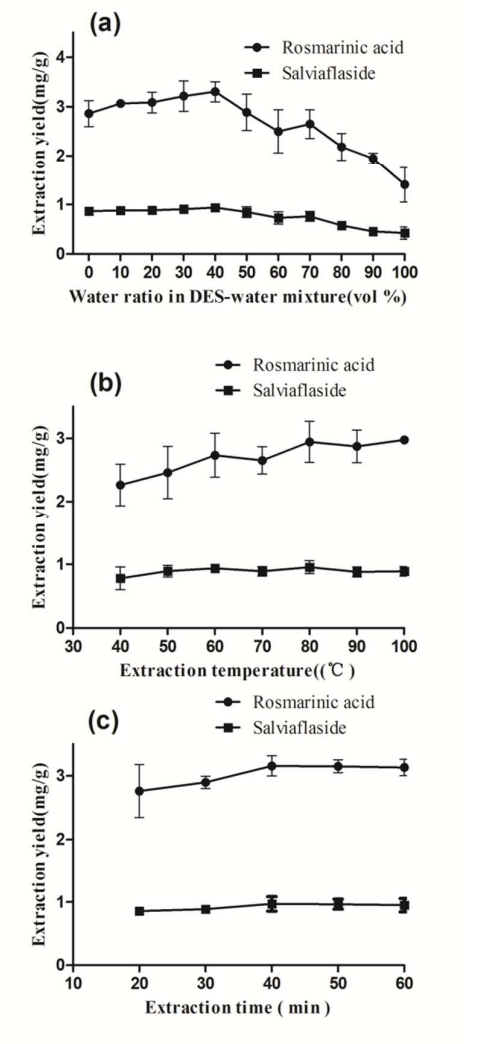



**Fig. 4** Effects of different (a) Water ratios in DES-water mixture, (b) Extraction temperatures, (c) Extraction time and on the yields of rosmarinic acid and salviaflaside.

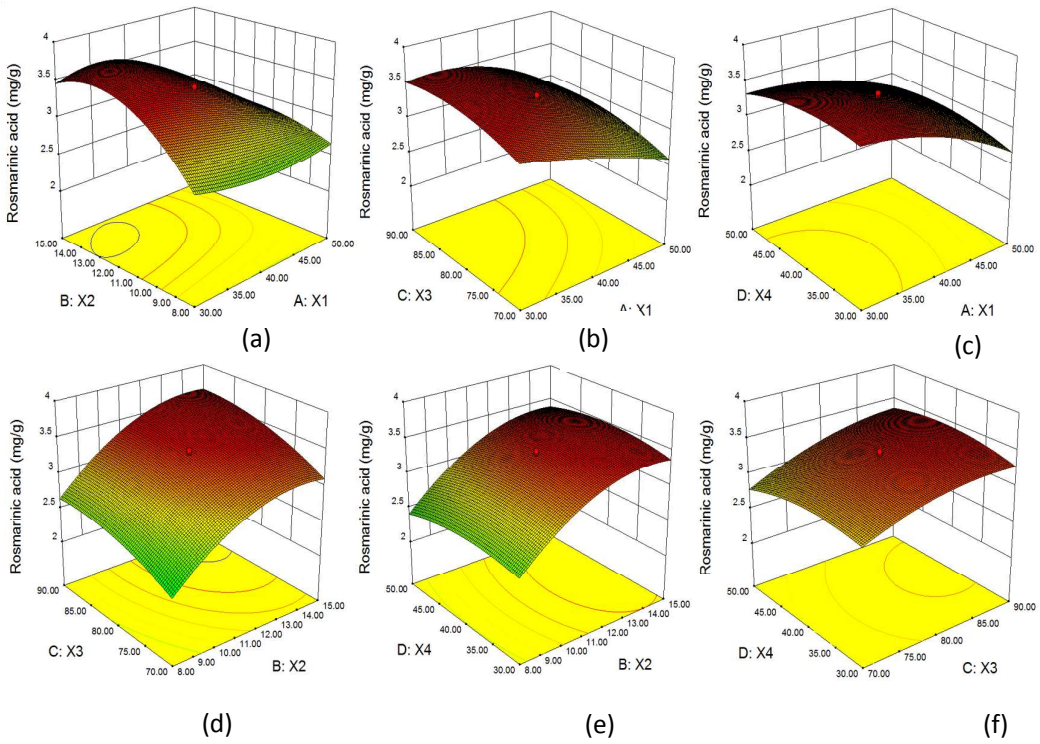



**Fig. 5** (a-f) Response surface (3D) showing the effect of of water/DES ratio ($X_1$), solid–liquid ratio ($X_2$), extraction temperature ($X_3$) and extraction time ($X_4$) on the yield of rosmarinic acid.

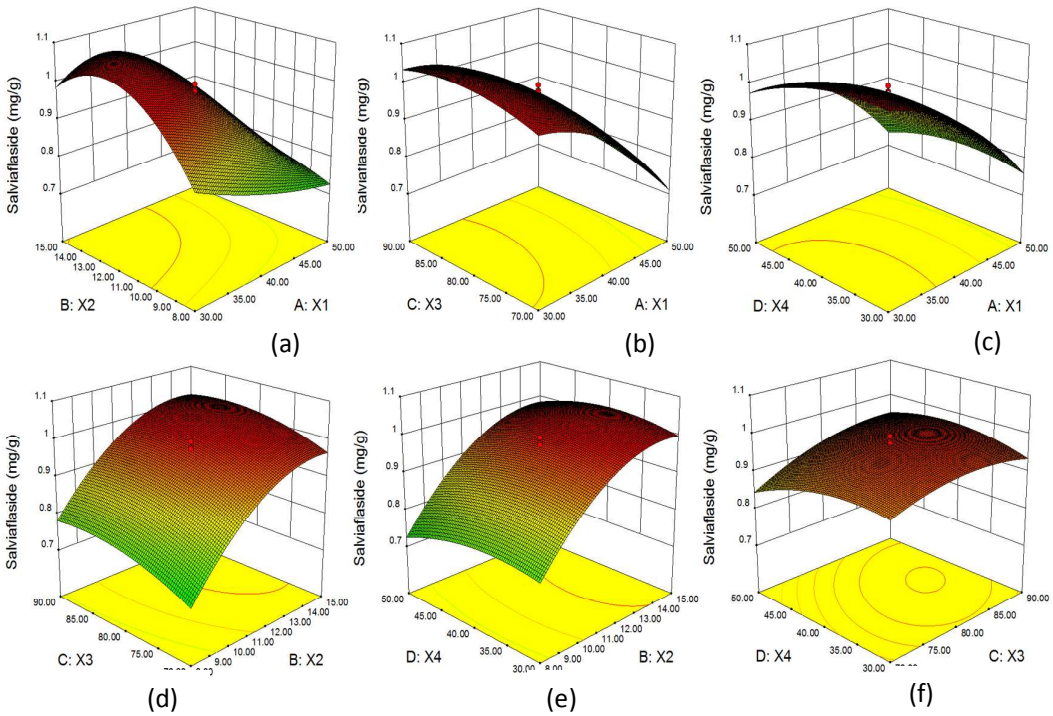



**Fig. 6** (a-f) Response surface (3D) showing the effect of of water/DES ratio ($X_1$), solid–liquid ratio ($X_2$), extraction temperature ($X_3$) and extraction time ($X_4$) on the yield of salviaflaside.

**Table 1** List of alcohol-based DESs synthesized and tested for extraction.

| Abbreviation | Salt | HBD | Salt/HBD ratio |
|---|---|---|---|
| **DES-1** | ChCl (choline chloride） | Ethylene glycol | 1:2 |
| **DES-2** | | glycerol | |
| **DES-3** | | 1,2-propylene glycol | |
| **DES-4** | | 1 4-butanediol | |
| **DES-5** | | 1 3-butanediol | |
| **DES-6** | | 2 3-butanediol | |

**Table 2** Independent variables and their coded and actual values used for optimization.

| Independent variable | Symbol | Coded level | | | | |
|---|---|---|---|---|---|---|
| | | -α(-2) | -1 | 0 | +1 | +α(+2) |
| **Water/DES ratios(%, v/v)** | $X_1$ | 20 | 30 | 40 | 50 | 60 |
| **Liquid/solid ratio(mL/g)** | $X_2$ | 4.5 | 8 | 11.5 | 15 | 18.5 |
| **extraction temperature(℃)** | $X_3$ | 60 | 70 | 80 | 90 | 100 |
| **extraction time（min）** | $X_4$ | 20 | 30 | 40 | 50 | 60 |

**Table 3** Yields of tested phenolics under the DESs with different ChCl/HBD ratios ( liquid/solid ratio= 15 mL/g, temperature = 80.0℃, time = 30.0 min).

| Tested phenolics | ChCl/ HBD ratios | Types of HBD and extraction yield (mg/g) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Ethylene glycol | 1,3-butan-ediol | 1,4-butan-ediol | glycerol | 2,3-butanediol | 1,2-propylene glycol |
| Rosmarinic acid | 1:2 | 2.47±0.34 | 1.98±0.35 | 1.34±0.52 | 2.12±0.48 | 1.53±0.17 | 1.90±0.40 |
| | 1:3 | 2.54±0.36 | 1.53±0.18 | 1.45±0.18 | 1.98±0.32 | 1.96±0.20 | 2.20±0.27 |
| | 1:4 | 2.87±0.39 | 1.40±0.22 | 1.82±0.31 | 2.33±0.25 | 1.62±0.11 | 2.12±0.21 |
| | 1:5 | 2.74±0.38 | 1.40±0.25 | 1.74±0.51 | 2.17±0.10 | 1.67±0.20 | 2.44±0.23 |
| | 1:6 | 2.68±0.38 | 1.71±0.29 | 1.36±0.10 | 1.99±0.25 | 1.14±0.15 | 2.47±0.19 |
| Salviaflaside | 1:2 | 0.82±0.11 | 0.69±0.09 | 0.48±0.26 | 0.76±0.15 | 0.65±0.08 | 0.71±0.14 |
| | 1:3 | 0.84±0.13 | 0.56±0.07 | 0.56±0.08 | 0.78±0.12 | 0.74±0.10 | 0.82±0.10 |
| | 1:4 | 0.92±0.09 | 0.48±0.11 | 0.72±0.15 | 0.75±0.08 | 0.66±0.06 | 0.77±0.08 |
| | 1:5 | 0.85±0.12 | 0.51±0.10 | 0.65±0.26 | 0.77±0.04 | 0.66±0.10 | 0.80±0.07 |
| | 1:6 | 0.83±0.13 | 0.64±0.14 | 0.53±0.06 | 0.73±0.11 | 0.43±0.06 | 0.81±0.10 |

**Table 4** The CCD matrix and the experimental data for the responses(n=3)

| Run | Water/DES ratio (%, v/v) | Liquid/solid ratio (mL/g) | Extraction temperature (℃) | Extraction time（min） | Extraction yield (mg/g) | |
|---|---|---|---|---|---|---|
| | | | | | Rosmarinic acid | Salviaflaside |
| **1** | -1 | 1 | 1 | 1 | 3.21 | 0.96 |
| **2** | -1 | 1 | -1 | -1 | 3.19 | 0.95 |
| **3** | 1 | -1 | 1 | 1 | 2.57 | 0.73 |
| **4** | 0 | 0 | 0 | 0 | 3.39 | 0.99 |
| **5** | 0 | 0 | 0 | 0 | 3.37 | 0.96 |
| **6** | -1 | -1 | -1 | 1 | 2.11 | 0.71 |
| **7** | -1 | -1 | 1 | -1 | 2.78 | 0.87 |
| **8** | -1 | -1 | -1 | -1 | 2.50 | 0.81 |
| **9** | -2 | 0 | 0 | 0 | 3.39 | 0.99 |
| **10** | 0 | 2 | 0 | 0 | 3.05 | 0.93 |
| **11** | 0 | 0 | 2 | 0 | 3.28 | 0.92 |
| **12** | 0 | 0 | 0 | 0 | 3.29 | 0.96 |
| **13** | 0 | 0 | -2 | 0 | 2.36 | 0.79 |
| **14** | 0 | 0 | 0 | 0 | 3.32 | 0.98 |
| **15** | 0 | -2 | 0 | 0 | 1.20 | 0.44 |
| **16** | 0 | 0 | 0 | 0 | 3.41 | 0.97 |
| **17** | 0 | 0 | 0 | 2 | 2.80 | 0.82 |
| **18** | 1 | 1 | 1 | -1 | 2.62 | 0.74 |
| **19** | 1 | -1 | -1 | -1 | 2.37 | 0.70 |
| **20** | 1 | -1 | 1 | -1 | 2.26 | 0.66 |
| **21** | 1 | 1 | -1 | -1 | 2.25 | 0.70 |
| **22** | 2 | 0 | 0 | 0 | 2.10 | 0.47 |
| **23** | -1 | 1 | 1 | 1 | 3.30 | 0.89 |
| **24** | 1 | 1 | 1 | 1 | 2.91 | 0.80 |
| **25** | 1 | -1 | -1 | 1 | 2.41 | 0.56 |
| **26** | 0 | 0 | 0 | 0 | 3.32 | 0.97 |
| **27** | -1 | -1 | 1 | 1 | 2.53 | 0.79 |
| **28** | 0 | 0 | 0 | -2 | 2.89 | 0.88 |
| **29** | 1 | 1 | -1 | 1 | 2.16 | 0.64 |
| **30** | -1 | 1 | -1 | 1 | 3.10 | 0.89 |

**Table 5** ANOVA for response surface reduced cubic model and for the effect of water/DES ratio ($X_1$), liquid/solid ratio ($X_2$), extraction temperature ($X_3$) and extraction time ($X_4$) on the yield of rosmarinic acid

| Source | Sum of Squares | df | Mean Square | F Value | *p*-value | significant |
|---|---|---|---|---|---|---|
| **Model** | 8.4308 | 22 | 0.3832 | 237.9807 | < 0.0001 | *** |
| **$X_1$- water/DES ratio** | 0.8383 | 1 | 0.8383 | 520.5758 | < 0.0001 | *** |
| **$X_2$- liquid/solid ratio** | 1.7113 | 1 | 1.7113 | 1062.6950 | < 0.0001 | *** |
| **$X_3$- extraction temperature** | 0.4218 | 1 | 0.4218 | 261.9474 | < 0.0001 | *** |
| **$X_4$- extraction time** | 0.0036 | 1 | 0.0036 | 2.2202 | 0.1798 | |
| **$X_1X_2$** | 0.4016 | 1 | 0.4016 | 249.4239 | < 0.0001 | *** |
| **$X_1X_3$** | 0.0040 | 1 | 0.0040 | 2.4824 | 0.1591 | |
| **$X_1X_4$** | 0.0906 | 1 | 0.0906 | 56.2450 | 0.0001 | ** |
| **$X_2X_3$** | 0.0217 | 1 | 0.0217 | 13.4787 | 0.0080 | ** |
| **$X_2X_4$** | 0.0142 | 1 | 0.0142 | 8.8325 | 0.0208 | * |
| **$X_3X_4$** | 0.0597 | 1 | 0.0597 | 37.0904 | 0.0005 | ** |
| **$X_1^2$** | 0.6099 | 1 | 0.6099 | 378.7801 | < 0.0001 | *** |
| **$X_2^2$** | 2.5470 | 1 | 2.5470 | 1581.6695 | < 0.0001 | *** |
| **$X_3^2$** | 0.4753 | 1 | 0.4753 | 295.1788 | < 0.0001 | *** |
| **$X_4^2$** | 0.4264 | 1 | 0.4264 | 264.7859 | < 0.0001 | *** |
| **$X_1X_2X_3$** | 0.1476 | 1 | 0.1476 | 91.6473 | < 0.0001 | *** |
| **$X_1X_2X_4$** | 0.0410 | 1 | 0.0410 | 25.4789 | 0.0015 | ** |
| **$X_1X_3X_4$** | 0.0066 | 1 | 0.0066 | 4.1072 | 0.0823 | |
| **$X_2X_3X_4$** | 0.0012 | 1 | 0.0012 | 0.7449 | 0.4167 | |
| **$X_1^2X_2$** | 0.3651 | 1 | 0.3651 | 226.7178 | < 0.0001 | *** |
| **$X_1^2X_3$** | 0.0526 | 1 | 0.0526 | 32.6845 | 0.0007 | ** |
| **$X_1^2X_4$** | 0.0012 | 1 | 0.0012 | 0.7409 | 0.4179 | |
| **$X_1X_2^2$** | 0.0840 | 1 | 0.0840 | 52.1445 | 0.0002 | ** |
| **Residual** | 0.0113 | 7 | 0.0016 | | | |
| **Lack of Fit** | 0.0006 | 2 | 0.0003 | 0.1341 | 0.8776 | not significant |
| **Pure Error** | 0.0107 | 5 | 0.0021 | | | |
| **Cor Total** | 8.4421 | 29 | | | | |
| **R-Squared** | 0.9987 | | | | | |
| **Adj R-Squared** | 0.9945 | | | | | |
| **Pred R-Squared** | 0.9884 | | | | | |
| **Adeq Precision** | 62.46 | | | | | |

**Table 6** ANOVA for response surface reduced cubic model and for the effect of water/DES ratio ($X_1$), liquid/solid ratio ($X_2$), extraction temperature ($X_3$) and extraction time ($X_4$) on the yield of salviaflaside.

| Source | Sum of Squares | df | Mean Square | F Value | *p*-value | significant |
|---|---|---|---|---|---|---|
| **Model** | 0.7000 | 22 | 0.0320 | 214.8000 | < 0.0001 | *** |
| **$X_1$- water/DES ratio** | 0.1400 | 1 | 0.1400 | 937.1300 | < 0.0001 | *** |
| **$X_2$- liquid/solid ratio** | 0.1200 | 1 | 0.1200 | 822.3200 | < 0.0001 | *** |
| **$X_3$- extraction temperature** | 0.0078 | 1 | 0.0078 | 52.6300 | 0.0002 | ** |
| **$X_4$- extraction time** | 0.0016 | 1 | 0.0016 | 10.5600 | 0.0141 | * |
| **$X_1X_2$** | 0.0046 | 1 | 0.0046 | 30.9900 | 0.0008 | ** |
| **$X_1X_3$** | 0.0016 | 1 | 0.0016 | 10.9900 | 0.0128 | * |
| **$X_1X_4$** | 0.0034 | 1 | 0.0034 | 23.1100 | 0.0019 | ** |
| **$X_2X_3$** | 0.0002 | 1 | 0.0002 | 1.6800 | 0.2356 | |
| **$X_2X_4$** | 0.0009 | 1 | 0.0009 | 5.7900 | 0.0470 | * |
| **$X_3X_4$** | 0.0073 | 1 | 0.0073 | 49.2600 | 0.0002 | ** |
| **$X_1^2$** | 0.1000 | 1 | 0.1000 | 693.3600 | < 0.0001 | *** |
| **$X_2^2$** | 0.1500 | 1 | 0.1500 | 1010.5300 | < 0.0001 | *** |
| **$X_3^2$** | 0.0260 | 1 | 0.0260 | 174.1300 | < 0.0001 | *** |
| **$X_4^2$** | 0.0270 | 1 | 0.0270 | 183.1900 | < 0.0001 | *** |
| **$X_1X_2X_3$** | 0.0028 | 1 | 0.0028 | 18.7600 | 0.0034 | ** |
| **$X_1X_2X_4$** | 0.0000 | 1 | 0.0000 | 0.0005 | 0.9826 | |
| **$X_1X_3X_4$** | 0.0075 | 1 | 0.0075 | 50.5700 | 0.0002 | ** |
| **$X_2X_3X_4$** | 0.0012 | 1 | 0.0012 | 8.0300 | 0.0253 | * |
| **$X_1^2X_2$** | 0.0320 | 1 | 0.0320 | 214.2100 | < 0.0001 | *** |
| **$X_1^2X_3$** | 0.0000 | 1 | 0.0000 | 0.0300 | 0.8663 | |
| **$X_1^2X_4$** | 0.0005 | 1 | 0.0005 | 3.5500 | 0.1014 | |
| **$X_1X_2^2$** | 0.0130 | 1 | 0.0130 | 84.9300 | < 0.0001 | *** |
| **Residual** | 0.0010 | 7 | 0.0001 | | | |
| **Lack of Fit** | 0.0003 | 2 | 0.0002 | 1.2200 | 0.3705 | not significant |
| **Pure Error** | 0.0007 | 5 | 0.0001 | | | |
| **Cor Total** | 0.7000 | 29 | | | | |
| **R-Squared** | 0.9985 | | | | | |
| **Adj R-Squared** | 0.9939 | | | | | |
| **Pred R-Squared** | 0.9288 | | | | | |
| **Adeq Precision** | 52.64 | | | | | |

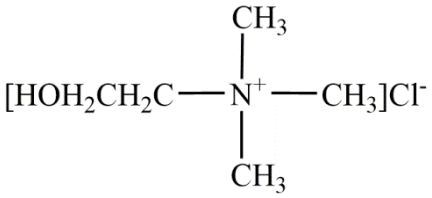
**Table 7** Linear range, LODs, and average recovery of the method.

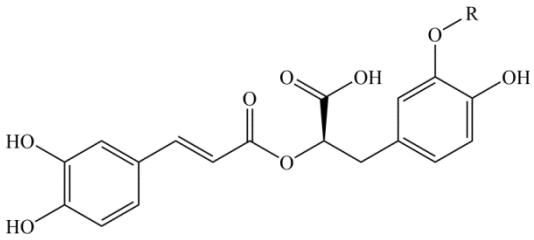
| Analyte | Linear range (μg/mL) | $r^2$ | RSD(%) | Average recovery (%) | RSD(%) | LODs(μg/mL) |
|---|---|---|---|---|---|---|
| **rosmarinic acid** | 1.97-246 | 0.9989 | 2.84 | 102.52 | 1.92 | 0.08 |
| **salviaflaside** | 4.11-257 | 0.9992 | 1.47 | 98.36 | 1.15 | 0.14 |

**Table 8** Extraction of phenolic acids from *Prunella vulgaris* L by different extraction methods and solvents.

| Solvent | Extraction method | Extracted amount (mg/g) | | Ref |
|---|---|---|---|---|
| | | Rosmarinic acid | Salviaflaside | |
| **75% methanol** | Ultrasonic extraction (79W) for 30.0 min | 3.137±0.028 | 0.938±0.018 | 41-42 |
| **50% ethanol** | maceration for 16h | 2.839±0.219 | 0.855±0.015 | 43 |
| **36% vol water in ChCl/ethylene glycol(1/4)** | Heating at 86℃ for 46.0min | 3.658±0.104 | 1.020±0.039 | |
| **30% vol water in ChCl/ethylene glycol(1/4)** | Heating at 89℃ for 32.0min | 3.568±0.116 | 1.049±0.015 | |

# Graphical Abstract



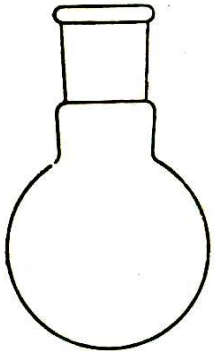




1: R=H; rosmarinic acid (a)
2: R=β-D-Glucopyranosyl; salviaflaside (b)

**Sustainable and Safe Process**

**High Yield**

**High Selectivity**



**Alcohols**

**DES**

**Extraction**