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# Enhanced extraction of bioactive natural products using tailor-made deep eutectic solvents: Application to flavonoid extraction from *Flos sophorae*

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Green and efficient extraction of natural products from biomass is considered an important field in the pharmaceutical and biochemical industries. Recently, deep eutectic solvents (DESs) have been growing in interest as sustainable and safe solvents. In this study, we aimed to provide a practical example using a popular traditional Chinese medicine, *Flos sophorae*, showcasing the tuneability of DESs as designer solvents to selectively and efficiently extract bioactive compounds from biomass. As a result, a solvent called PG-1 that was tailor-made from a 2:5 mixture of L-proline and glycerol using freeze-drying method, was more effective than methanol for extraction of quercetin, kaempferol, and isorhamnetin glycosides from *Flos sophorae*. With PG-1-based ultrasound-assisted extraction (UAE), operational conditions including the DES content in the extractant, extractant-to-sample solid ratio, and ultrasound irradiation time for UAE were statistically optimized using a central composite design combined with response surface methodology. The resulting extraction method in which 50 mg of sample powder was extracted by UAE for 45 min using 1.00 mL of aqueous solution containing 90% w/w PG-1 was found to be a greener and more efficient process than common extraction methods such as methanol-based UAE and heat reflux extraction that are generally environmentally harmful. Based on the antioxidant activity measured by DPPH assay, the tailor-made extractant exhibited additive activity arising from its component, L-proline. Recovery of extracted flavonoids from the DES, which was assessed from rutin, since it is the major flavonoid extracted, was 75% with the use of water as an anti-solvent, and could reach as high as 92% with the simple application of C18 solid phase extraction (SPE). In comparison, the recovery efficiency of the anti-solvent method was significantly reduced for the flavonoid glycosides from the real *Flos sophorae* extracts, while the efficiency of the SPE method was reasonably high (81-87%). The present study suggests that DESs are truly designer solvents that can be used as sustainable and safe extraction media for pharmaceutical and biochemical applications.

## 1. Introduction

Green chemistry is defined as the "design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances".[1] In this sense, solvents are an important challenge for green chemistry as they comprise the vast majority of waste mass, they are usually toxic and volatile and thus contribute to environmental pollution, and they are hazardous to human health.[2] Several types of solvents including ionic liquids (ILs) have been suggested as "green" solutions to replace volatile organic solvents. The negligible volatility and non-flammability of ILs allow them to be qualified as green solvents. However, many reports have recently addressed drawbacks to ILs including hazardous toxicity, poor biodegradability, and high cost for synthesis, which detract from their "greenness". Since being introduced by Abbott *et al*. in 2003,[3] deep eutectic solvents (DESs) have been recognized as a novel class of sustainable solvents. Because they are produced from the mixing of two or more naturally-occurring, inexpensive, and biodegradable components, DESs are generally safe, non-toxic, and thus are preferable to ILs, which have very similar physicochemical properties. As a result, DESs have grown in interest as attractive solvents in a variety of scientific and technological areas.[4-7]

One of the most important and extensive applications of sustainable solvents is in extraction processes, including sample pre-treatment for analytical method development, purification of fuels, and selective isolation and recovery of target compounds.[4] In particular, green extraction of natural products from traditional Chinese medicines (TCMs) and foods using sustainable solvents is considered an important area of pharmaceutical and biochemical research.[5, 8, 9] Compared to ILs,[10, 11] the number of reports on the use of DESs for extraction purposes is still low, although it is envisioned that

DESs will be extensively utilized as green extraction solvents in the near future.[4, 6]

One key advantage of ILs and DESs is their tuneability to have targeted functionality due to diverse possible combinations of starting components. From the point of view of extraction, DES selectivity for extraction and separation can be tailored by changing the nature and molar ratio of their hydrogen-bonding components.[7] In this study, we demonstrate for the first time the tuneability of DESs as designer solvents that selectively and efficiently extract bioactive compounds from biomass. For this purpose, a commonly used traditional Chinese medicine (TCM) called *Flos sophorae* was used because of its popularity in pharmaceutical use, high content of bioactive compounds, and ease of procurement. *Flos sophorae* is the dried flowers of *Sophora japonica* L. (Leguminosae) and is used as a hemostatic agent.[12, 13] It is known to be rich in various flavonoids. In particular, rutin, a glycoside of quercetin, is the predominant constituent,[12, 14] which is reported to possess anti-platelet, anti-tumor, and vasodilation activities.[15-17] Other minor flavonoids including quercetin, isorhamnetin, nicotiflorin, and narcissin have also been isolated from *Flos sophorae*.[14, 18] Besides these flavonoids, *Flos sophorae* is also known to contain triterpenoids such as betulinic acid, and isoflavonoids such as genistein and sophoricoside.[18, 19]

A series of methods have been reported for the efficient extraction of bioactive flavonoids from *Flos sophorae* and related TCMs derived from *S. japonica* L.[14, 20-22]. Because the solubility of flavonoids in water is generally low, various organic solvents including methanol, ethanol, acetone, and ethyl acetate have been commonly used as extraction solvents in combination with extraction methods such as heat reflux extraction (HRE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), and ultrasound-assisted extraction (UAE),[20, 23-25] all of which generally require large amounts of solvent and long extraction times. Recently, DESs have been suggested to be superior dissolution and extraction media for water insoluble compounds.[26] For example, the solubility of rutin in DESs was at least 50-fold higher than in water.[27] Nonetheless, the number of reports on the application of DESs to the extraction of natural products remains limited.[14, 15] In this study, we aimed to evaluate and maximize the potential and effectiveness of DESs as green extraction solvents to selectively isolate bioactive flavonoids from *Flos sophorae* as an example. After initial screening, DESs were tailored for the highest extraction efficiency, followed by statistical optimization of operational conditions using response surface methodology (RSM) to produce the most efficient process for flavonoid extraction from *Flos sophorae*. Finally, the efficiency of the optimized method, the effects of the tailored DESs on the bioactivity of the extracted compounds, and the recovery of extracted compounds from DESs were evaluated.

## 2. Experimental

### 2.1. Materials and equipment

Dried *Flos sophorae* was purchased from a local TCM market (Kyungdong Market, Seoul, Korea). According to the distributor, Kwang Myeong Herbal Medicine, Ltd. (Busan, Korea), which guaranteed its quality and authentication, it was originally from China and imported as a standardized product under the permission of the Korean Food and Drug Administration (KFDA). *Flos sophorae* was ground using a laboratory blender (model RT-08) from Rong Tsong Precision Technology (Taichung, Taiwan) and was stored in glass bottles at -20 °C until analysis. Analytical standards, quercetin (≥ 95.0 %) and isorhamnetin (≥ 95.0 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rutin trihydrate (≥ 97.0%) and kaempferol (≥ 98.0 %) were obtained from Alfa Aesar (Ward Hill, MA, USA) and Shin-Jung Hi Tech Co., Ltd. (Seoul, Korea), respectively. Compounds for DES preparation including choline chloride (≥ 98.0 %), glycerol (≥ 99.0 %), xylitol (≥ 99.0 %), D-(+)-glucose (≥ 99.5 %), L-proline (≥ 99.0 %), citric acid (≥ 99.5 %), adonitol (≥ 99.0 %), D-(-)-fructose (≥ 99.0 %), sucrose (≥ 99.5 %), betaine (≥ 99.0 %), and DL-malic acid (≥ 99.0 %) were obtained from Sigma-Aldrich. Trifluoroacetic acid and hydrochloric acid were obtained from Sigma-Aldrich while HPLC-grade methanol and water were purchased from J.T.Baker (Phillipsburg, NJ, USA). Double distilled water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals were at least analytical grade.

Centrifuges (model 1580MGR and Gyrospin), an ultrasonic bath (PowerSonic 410), and a freeze dryer (model FD8508) were obtained from Gyrozen (Incheon, Korea), Hwashin Technology (Seoul, Korea), and Ilshin Biobase (Yangju, Korea), respectively.

### 2.2. Preparation of standard solutions

Standard stock solutions were prepared by dissolving each flavonoid in methanol at a concentration of 1.0 mg $mL^{-1}$ for rutin, quercetin and kaempferol and 0.10 mg $mL^{-1}$ for isorhamnetin, and they were stored at -20 °C. Standard working solutions were prepared by diluting the stock solutions with mobile phase to produce standard solutions at concentrations of 20, 40, 80, 160, and 320 μg $mL^{-1}$ for quercetin, 4, 16, 32, 64, and 128 μg $mL^{-1}$ for kaempferol, and 8, 16, 32, 64, and 100 μg $mL^{-1}$ for isorhamnetin.

### 2.3. Preparation of DESs

DESs were prepared using a freeze-drying method as previously described.[28] Briefly, each component was accurately weighed and combined in a 50 mL conical tube. After dissolving the mixed components in the smallest amount of double distilled water, the mixture was centrifuged at 2898 g for 10 min, followed by cooling at -80 °C for 90 min. The added water was removed by lyophilization for 18h or longer until a constant weight was reached. The resulting DESs prepared are listed in Table 1.

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

| Abbreviation | Component 1 | Component 2 | Mole ratio |
|---|---|---|---|
| ChG | Choline chloride | Glycerol | 1:1 |
| ChX | Choline chloride | Xylitol | 5:2 |
| ChGlu | Choline chloride | D-(+)-Glucose | 1:1 |
| PGlu | L-Proline | D-(+)-Glucose | 5:3 |
| CaGlu | Citric acid | D-(+)-Glucose | 1:1 |
| CaA | Citric acid | Adonitol | 1:1 |
| BM | Betaine | DL-Malic acid | 1:1 |

### 2.4. Flavonoid extraction from *Flos sophorae* and hydrolysis of the extract

For initial screening of the extraction solvent and method, precisely weighed 50 mg of *Flos sophorae* powder was added to 0.75 mL of extraction solvent in a 2 mL microfuge tube and was briefly vortexed. Extraction was performed by ultrasonic irradiation at ambient temperature for 20 min, followed by



centrifugation at 12300 g for 30 min. Parent flavonoid glycosides in the extract were non-enzymatically converted to their respective aglycon by acid hydrolysis, during which 150 μL of the extract mixed with 500 μL of 4 M hydrochloric acid in methanol was heated at 90 °C for 1 hr. The extraction yield was assessed based on the aglycon flavonoid levels determined by liquid chromatography-ultraviolet detection (LC-UV) analysis using the following equation: extracted amount = (mass of flavonoid aglycon, mg) / (mass of weighed *Flos sophorae* powder, g).
For HRE, which was performed to compare the extraction efficiency of the optimized DES-based UAE method, 10 g of sample powder was refluxed in 150 mL of methanol for 120 min, and was then subjected to acid hydrolysis as described above.

### 2.5. LC-UV analysis for quantification of extracted flavonoids

LC-UV analysis was performed using a PerkinElmer LC system (Norwalk, CT, USA) equipped with a PerkinElmer micro pump, a column oven (series 200), an auto-sampler (series275) and a photodiode array (PDA) detector (series 275). TotalChrom Workstation software was used for system operation and data collection. Hydrolyzed or unhydrolyzed flavonoid extracts and standards were chromatographed on a ZORBAX Eclipse Plus C18 column (5 μm, 4.6 mm × 150 mm) from Agilent (Santa Clara, CA, USA) at 30 °C. The mobile phase consisted of water containing 0.1% trifluoroacetic acid (eluent A) and methanol (eluent B). The gradient program was as follows: 0-13 min, 40-70% B; 13-16 min, 70% B, 16-16.5 min, 70-100% B, and maintained for 2 min. The column was re-equilibrated to the initial conditions for 11.5 min before a subsequent injection. The acid hydrolysis extract was diluted six-fold with mobile phase and was filtered through a 0.45 μm membrane filter (Whatman, Piscataway, NJ, USA) before injection. The detection wavelength was 360 nm.[29-31]
Calibration curves were established for the three flavonoid aglycons by plotting the nominal concentrations of standard solutions *versus* peak areas. Linear regression equations were $y = 20225x - 192344$ ($R^2 = 0.9994$) for quercetin, $y = 16538x - 30968$ ($R^2 = 0.9996$) for kaempferol, and $y = 13592x - 71800$ ($R^2$=0.9994) for isorhamnetin. The linear ranges were 20-320 μg $mL^{-1}$ for quercetin, 4-128 μg $mL^{-1}$ for kaempferol, and 8-100 μg $mL^{-1}$ for isorhamnetin. The method precision was below 15.2% RSD, and the accuracy, obtained as the concentration ratio of back-calculated to nominal values, was between 95.1% and 120.1% in all concentration ranges.

### 2.6. LC-MS analysis for qualitative analysis of natural products

Ultra high performance LC coupled to quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) was performed for qualitative analysis of natural products existing in *Flos sophorae*. An Acquity UPLC system (Waters Co., Milford, MA, USA) was composed of a binary solvent delivery system, a cooling autosampler maintained at 4 ºC, and a column oven maintained at 45 ºC. Flavonoid standards and extracts of *Flos sophorae* in methanol or DESs were chromatographed on an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) from Waters (Milford, MA, USA). A linear gradient system was employed for elution using the mobile phase consisting of (A) 0.1% formic acid (FA) and (B) 0.1% FA in acetonitrile: 0-2.0 min, 10-12% B; 2.0-5.2 min, 12-30% B; 5.2-6.0 min, 30-45% B; 6.0-8.0 min, 45-100%, followed by washing the column with 100% B for 1.6 min. The flow rate was 0.35 mL $min^{-1}$. Hydrolyzed or unhydrolyzed extracts were diluted with the mobile phase to produce an overall 50-fold dilution of the original extracts, which significantly lowered the sample viscosity and reduced concentrations of the DES components. Diluted samples were filtered through a 0.45 μm membrane filter before injection.
Mass spectrometry was conducted with a Waters Acquity Xebo G2 Q-TOF tandem mass spectrometer (Waters Corp., Manchester, UK) equipped with an electrospray ionization interface in positive ion mode, which was controlled by Masslynx software (version 4.1, Waters Co., Milford, MA, USA). The instrument was calibrated by direct infusion of 5 mM sodium formate solution. The parameters were set as follows: capillary voltage, 3.0 kV; sample cone, 30 V; extraction cone, 4.0 V; source temperature, 120 °C; desolvation temperature, 300 °C; desolvation gas (nitrogen), 600 L $h^{-1}$; and cone gas, 0 L $h^{-1}$. Data were acquired from m/z 100 to 1500 Da and corrected during acquisition using an external reference (lock spray) composed of a solution of 2 μg $mL^{-1}$ leucine enkephalin (m/z 556.2771) infused at a flow rate of 20 μL $min^{-1}$. An $MS^E$ scan function was also applied for simultaneous detection of precursor ions and fragment ions at high and low collision energies in a single injection run. The high collision energy ramp ranged from 20 to 45 V. Detected compounds were identified based on accurate mass measurements as well as literature on *Flos sophorae* and related species.

### 2.7. Experimental design and statistical analysis

RSM was performed using the Design-Expert Ver. 8.0 (Stat-Ease Inc., Minneapolis, MN, USA). Central composite design (CCD) was used to find the optimal values for three independent variables: water content in the selected DES (A), liquid (extractant)-solid (sample powder) ratio (B), and ultrasonic irradiation time (C) at five levels (-α, -1, 0, +1, and +α). Whole experiments were composed of 20 experimental points that included six replicates of the center points. Experimental orders and levels of the variables are listed in Supplementary Table S1.
Statistical comparison was performed by GraphPad Prism 5.01 for Windows (GraphPad Software, San Diego, CA, USA) using a two-tailed t-test and one-way analysis of variance (ANOVA). The *p*-values < 0.05 were considered significant.

## 3. Results and discussion

### 3.1. Preparation of DESs

[23, 32]For DES preparation, several methods are available including evaporating, heating, and freeze-drying techniques.[32] In this study, freeze-drying method was employed because the procedure is simple[28] and it is safe for heat labile components such as amino acids.[5]
A number of hydrogen bond acceptors (HBAs) or hydrogen bond donors (HBDs) from renewable, inexpensive, and readily accessible resources were tested as potential DES components, including choline chloride, glycerol, xylitol, D-(+)-glucose, L-proline, citric

acid, adonitol, D-(-)-fructose, sucrose, betaine, and DL-malic acid. Based on the literature,[5, 26] more than 30 combinations were initially tested. Then, 12 of 18 combinations that appeared successful on initial tests were found to be stable as a clear, viscous liquid without solid precipitation or color change over the course of time (Supplementary Table S3).

In evaporating, heating, and freeze-drying methods, water is commonly used to dissolve individual components. It was reported that DESs produced by evaporating and heating methods contained small quantities (5-10% w/w) of water,[5] and these solvents were subsequently tested as an extraction media without consideration of their water content.[33, 34] All the stable DESs prepared by the freeze-drying method in this study were found to contain water at levels similar to the literature,[5] based on the increased weight of produced DESs in comparison to the summed weight of two individual components and Karl-Fischer titration (data not shown). Although hygroscopic properties were observed in several DESs, as reported,[28] additional water attraction to the formed DESs could be prevented by simple lid sealing during analysis (Supplementary Fig. S1). Therefore, for convenience, only the content of the two major components in DESs were considered in the subsequent extraction study.[33, 34]

### 3.2. Analytical method establishment for comparison of flavonoid extraction efficiency

For comparison of extraction efficiency, extracted amounts of major compounds can be measured. In this study, the LC-UV and UHPLC-Q-TOF-MS analyses of the methanol extracts of *Flos sophorae* without acid hydrolysis showed that rutin existed as the major compound, while other minor flavonoid glycosides including nicotiflorin and narcissin, which are a glycoside of kaempferol and isorhamnetin, respectively, were present at much lower levels (Supplementary Figs. S2 and S3, Supplementary Table S2).[22] Besides these flavonoids, betulinic acid and sophorabioside were also detected by the UHPLC-Q-TOF-MS; however, their levels were too low to quantify using LC-UV (Supplementary Figs. S2 and S3, Supplementary Table S). Accordingly, flavonoids were the target analytes of extraction as the predominantly existing bioactive compounds in *Flos sophorae*.

Flavonoid levels may be measured in their original form, *i.e.*, glycoside conjugates.[22] However, the various forms of the conjugates lead to too many peaks that significantly vary in retention time, which makes chromatographic separation very challenging using a conventional LC system.[20, 35] Moreover, the identification and quantification of each conjugate form in the extracts are often practically unfavorable because of the limited availability and extremely high cost of reference standards.[36] As a result, extracted flavonoids are usually converted to the corresponding aglycons by acid hydrolysis, which results in only a few major aglycons and, thus, much simpler chromatographic patterns.[35, 37] In the current study, the availability of nicotiflorin and narcissin standards was very limited. As a result, the extracted flavonoids were hydrolyzed and their aglycons were quantified for the comparison of extraction efficiencies.

Efficiency of acid hydrolysis can be influenced by types of extraction solvents. In fact, during our preliminary experiments using various kinds of DESs, incomplete hydrolysis was detected in several DES extracts with the use of conventional hydrolysis conditions.[23] This led us to modify the acid hydrolysis conditions, resulting in stronger acidic conditions than reported ones. Using the optimized conditions as described in the Experimental section, rutin hydrolysis efficiencies in methanol and a DES (90% PG-1 as a sample solvent, which was selected as the optimal solvent in section 3.5) were compared. After hydrolysis, rutin peaks disappeared in both hydrolyzed products, while quercetin peaks appeared with similar peak areas ($p$ = 0.560, n = 3; Supplementary Fig. S4). Recovery for rutin hydrolysis, which was measured as the ratio of peak areas of quercetin in the DES to methanol after hydrolysis, was found to be close to 100% (102.4% ± 3.6%). These results suggest that the optimized hydrolysis conditions were efficient enough to overcome the potential effects of different types of extractants. In fact, no unhydrolyzed glycosides were observed in any acid hydrolyzed extracts regardless of extraction solvents during the entire study.

Acid hydrolysis of the extracts produced simpler chromatograms, which usually displayed three distinct peaks of hydrolyzed aglycons at quantifiable levels – quercetin, kaempferol, and isorhamnetin (Supplementary Fig. S2) and this was consistent with the literature.[20] Based on the literature and our UHPLC-Q-TOF-MS analysis, it was concluded that rutin, nicotiflorin, and narcissin were hydrolyzed to quercetin, kaempferol, and isorhamnetin, respectively (Supplementary Table S2 and Supplementary Fig. S3). These three aglycons from the acid hydrolyzed extract were quantified to evaluate the extraction efficiency throughout the study.

### 3.3. Selection of initial extraction conditions

Aqueous alcohols at various water contents are general solvents for extraction of plant materials. In particular, methanol and its aqueous solutions are the most commonly used solvents for the extraction of flavonoids.[23, 35] Comparison of 70% methanol and 100% methanol revealed that 100% methanol was similarly or more effective for flavonoid extraction, although the differences were not significant ($p \geq 0.1858$, n = 3; Supplementary Fig. S5). Thus, 100% methanol was employed as the control extraction solvent for comparing extraction efficiencies of various DESs, while water was tested as the most sustainable solvent.[38]

Numerous extraction methods including stirring, heating,[9] stirring+heating,[33] and UAE[39, 40] can be employed for extraction using DESs; however, the most popular HRE method is not compatible with DESs due to their low volatility. In this study, UAE was selected in the initial screening procedure because it is generally simple, fast, and effective, and can be applied regardless of solvent type.[39, 41]

### 3.4. Screening of DESs for high extraction efficiency

Among the 12 successfully produced DESs (Supplementary Table S3), five solvents containing D-(-)-fructose or sucrose were excluded from further study because heat treatment during the acid hydrolysis caused browning of the extracted phase and prevented accurate assessment of the flavonoid extraction yield. As a result, seven different DESs (Table 1) were screened for extraction efficiency. Although DESs containing glycerol or choline chloride such as ChG and ChX were relatively fluid, all of the DESs produced were still viscous. Accordingly, all of the DESs were tested for screening as mixtures that were prepared by mixing the produced DES with additional water at 7:3 (w/w) for easy handling.[6, 33]

Quercetin extraction efficiencies of the tested DESs varied greatly depending on the type of DES and gave results between those of methanol and water (Fig. 1). The lowest efficiency for water demonstrated the necessity of using solvents of different polarity from water to extract flavonoids. Among the seven types of DES, ChG, ChX, and PGlu exhibited similarly higher yields compared with other solvents for quercetin extraction. On the other hand, extraction yields for isorhamnetin and



kaempferol were less affected by solvent type. While water was still the poorest solvent for these two flavonoids, ChX and PGlu produced higher extraction efficiencies for isorhamnetin compared to methanol, although these differences were not statistically significant.

### 3.5. Optimal solvent design for flavonoid extraction

Based on observations that among the three choline chloride (Ch)-based solvents, ChG and ChX were much more efficient than ChGlu, it was hypothesized that Ch was not an effective component, but that glycerol (G) and xylitol (X) were important for flavonoid extraction (Fig. 1). Similarly, a comparison of the three glucose (Glu)-based DESs, ChGlu, PGlu, and CaGlu, led us to hypothesize that L-proline (P) was responsible for high extraction efficiency (Fig. 1). Accordingly, we attempted to tailor new DESs with enhanced efficiency by combining the more effective components. Since L-proline is categorized as a HBA and while glycerol and xylitol are HBDs,[7] L-proline was combined with glycerol and xylitol at various molar ratios from 5:1 to 1:5, resulting in 10 different DESs being successfully produced as displayed in Table 2. DESs containing L-proline and glycerol (PG) were formed in molar ratios of 1:2.5-1:4.5, while DESs composed of L-proline and xylitol (PX) were produced in ratios of between 1:1 and 1:4. This is the first report of DES formation between L-proline and glycerol or xylitol suggesting the possibility of producing a wide variety of DESs from many more sustainable components than previously reported.

The 10 DESs were tested for flavonoid extraction (Fig. 2). Except for PG-5, PX-4, and PX-5, all of the other DESs exhibited similar or higher quercetin extraction efficiencies compared to initial DESs ChG, ChX, and PGlu. Efficiencies of PG-1, PG-2, PG-4, PX-2, and methanol were significantly higher than those of middle-level group solvents such as PG-3 ($p < 0.05$) (Fig. 2a). In the case of kaempferol and isorhamnetin, extraction efficiencies did not vary greatly among the DESs, although PG-1 was significantly more efficient than solvents in the lower level group, such as PX-2 ($p < 0.01$) (Fig. 2b). Between PG-1 and PG-4, PG-1 was selected as the final extraction solvent due to its higher average efficiency with lower variation than PG-4, although the differences were not significant. Further extraction conditions were optimized in the following procedure.

### 3.6. Selection of extraction method

UAE was employed as the extraction method in the initial screening of DESs due to its simplicity. Based on previous reports involving IL- and DES-based extraction, three other common extraction methods, stirring, heating, and stirring+heating were compared with UAE under the same conditions with PG-1 used as the extraction solvent (Fig. 3). Similar to the solvent screening above, the extraction efficiency for quercetin varied more depending on the extraction method than kaempferol and isorhamnetin did. While stirring alone was inefficient, it improved the extraction efficiency of the heating method for quercetin and isorhamnetin (heating vs. heating+stirring, $p < 0.05$ for both compounds). Considering its higher efficiency for all flavonoids and simplicity, UAE was selected over the heating+stirring method as the extraction method and further extraction conditions were optimized as described below.

**Table 2** List of DESs produced from combination of L-proline with glycerol or xylitol.

| Abbreviation | Component 1 | Component 2 | Molar ratio | Abbreviation | Component 1 | Component 2 | Molar ratio |
|---|---|---|---|---|---|---|---|
| PG-1 | L-Proline | Glycerol | 1:2.5 | PX-1 | L-Proline | Xylitol | 1:1 |
| PG-2 | L-Proline | Glycerol | 1:3 | PX-2 | L-Proline | Xylitol | 1:1.5 |
| PG-3 | L-Proline | Glycerol | 1:3.5 | PX-3 | L-Proline | Xylitol | 1:2 |
| PG-4 | L-Proline | Glycerol | 1:4 | PX-4 | L-Proline | Xylitol | 1:3 |
| PG-5 | L-Proline | Glycerol | 1:4.5 | PX-5 | L-Proline | Xylitol | 1:4 |

**Fig. 1** Extraction yields (mg of aglycon per g of *Flos sophorae* powder) of DESs for the three flavonoids. (a) Quercetin; (b) kaempferol (white bar) and isorhamnetin (black bar). In cases where the kaempferol level in the extract was too low to be accurately quantified using the established calibration curve, which is equivalent to 1.87 mg $g^{-1}$ in the powder, an approximate quantification was used (indicated with a cross). Error bars indicate the SEM (n = 3).

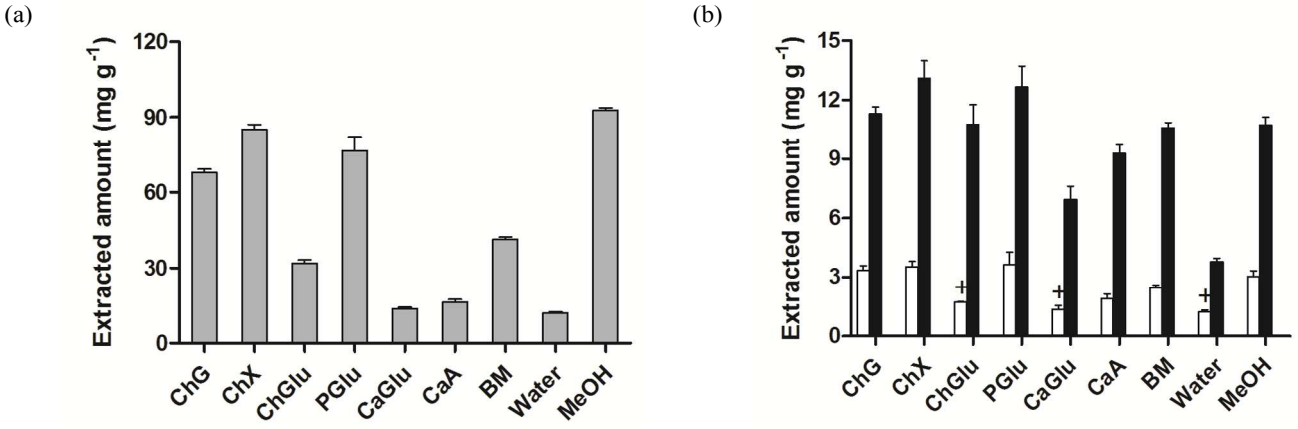



**Fig. 2** Extraction yields (mg of aglycon per g of *Flos sophorae* powder) of PG and PX series for the three flavonoids. (a) Quercetin; (b) kaempferol (white bar) and isorhamnetin (black bar). In cases where the kaempferol level in the extract was too low to be accurately

quantified using the established calibration curve, which is equivalent to 1.87 mg $g^{-1}$ in the powder, an approximate quantification was used (indicated with cross). Extraction efficiencies that were significantly higher in comparison to those of PG-3 (a) or PX-2 (b) were indicated with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Error bars indicate the SEM (n = 3).

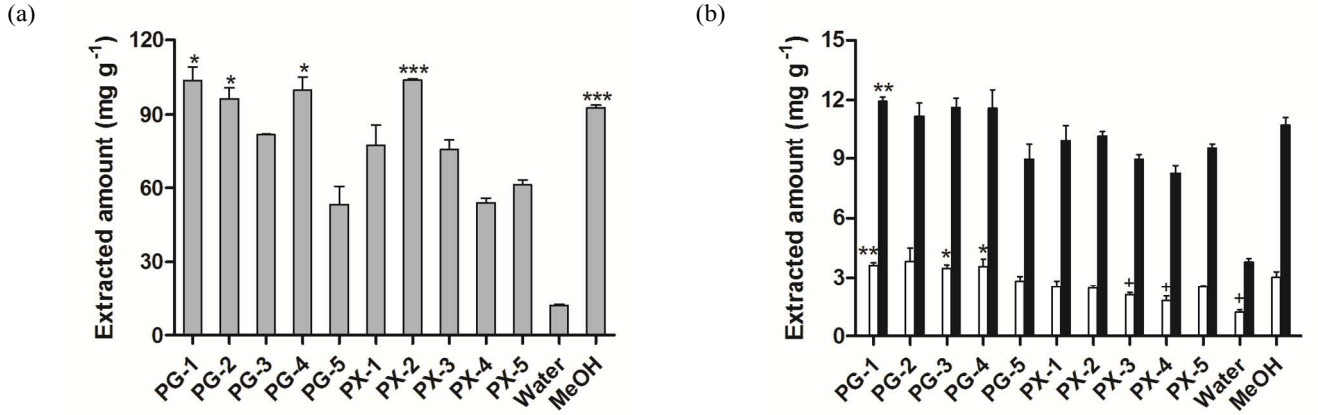
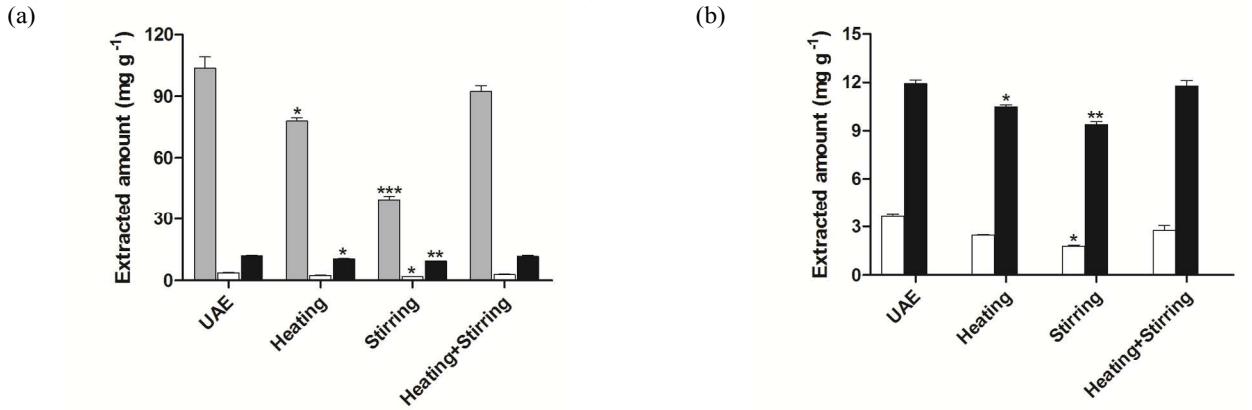



**Fig. 3** Comparison between UAE and other extraction methods using PG-1. Quercetin (gray); kaempferol (white); isorhamnetin (black). Extraction efficiencies that were significantly different from those of the heating+stirring method were indicated with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Error bars indicate the SEM (n = 3).




### 3.7. Optimization of the operational conditions using RSM

Statistics-based optimization of variables using RSM can be advantageous over the classical one-variable-at-a-time (OVAT) approach because it allows for the evaluation of interacting effects between variables and variable optimization in overall scope from fewer experiments.[42] In previous studies on IL- or DES-based extractions,[9, 11, 20] several independent, quantitative variables were usually investigated for optimization, including the DES content of the extraction solvent, liquid (extractant)-solid (sample power) ratio, extraction time, and extraction temperature. In this study, the extraction solvent type and extraction method, which can critically affect the extraction efficiency, were selected as PG-1 and UAE, respectively, as described above. Subsequent preliminary experiments for UAE condition optimization revealed that the extraction temperature had a tendency to rise with increasing irradiation (extraction) time and that the extraction efficiency was enhanced as the ultrasonic irradiation power was increased (data not shown). As a result, the irradiation power was set at the maximum value (330-450 W), and the following three variables were chosen for RSM-based optimization: PG-1 content in the extraction solvent (A; 49.6-100.0% w/w), extraction solvent volume per 100 mg of sample powder (B; 0.66-2.34 mL), and ultrasonic irradiation time (C; 4.8-55.2 min). Extraction yields for the three individual flavonoids were investigated as the response function of the CCD method, which is one of the most popular choices to define optimum values in multi-level design.[42] The experimental orders and levels of coded and uncoded variables are summarized in Supplementary Table S1.

The variables and response were processed to build a different quadratic multiple regression model for each flavonoid. The model quality was evaluated in terms of the square of correlation coefficient ($R^2$) and the lack-of-fit by the analysis of variance (ANOVA) at the 95% confidence level (Supplementary Table S4). The resulting $R^2$ values were 0.8678 or higher in the three models, indicating that the experimental data were in relatively good agreement with predicted extraction yields for each model. All of the $F$-values for the lack-of-fit of these models were insignificant ($p \geq 0.2432$), which supported that these models were sufficient to accurately represent the experimental data. The models were expressed as second order polynominal quadratic equations for the extraction yield ($Y$) and coded factors ($A$, $B$, and $C$) as follows:

$$Y_{\text{quercetin}} = 110.60 + 19.09A + 9.22B + 16.51C - 3.56AB - 0.084AC - 1.82BC - 5.80A^2 - 9.55B^2 - 7.35C^2$$

$$Y_{\text{kaempferol}} = 4.17 + 0.54A + 0.73B + 0.19C - 0.17AB - 0.068AC + 0.051BC - 0.34A^2 - 0.23B^2 - 0.41C^2$$

$$Y_{\text{isorhamnetin}} = 12.67 + 1.63A + 1.96B + 0.61C - 0.29AB - 0.35AC + 0.18BC - 0.95A^2 - 0.37B^2 - 0.78C^2$$



Statistical analysis (Supplementary Table S4) and 3D response plots (Fig. 4) illustrate the significant variables affecting extraction yields of the flavonoids and the interaction effects between the variables. In all three models, the PG-1 content of the extractant (variable A) and the liquid-to-solid (L-S) ratio (variable B) led to significantly different extraction yields. In general, the extraction efficiency increased with higher PG-1 content and higher L-S ratio for all flavonoids (Fig. 4. On the other hand, extraction time (variable C) had a mild positive effect on quercetin extraction efficiency and an insignificant effect on extraction efficiencies of kaempferol ($p$ = 0.1896) and isorhamnetin ($p$ = 0.0922).

The quercetin extraction model yielded optimal conditions with A = 89.8% w/w, B = 2.00 mL per 100 mg of *Flos sophorae* powder, and C = 45 min. Under these optimum conditions, the predicted extraction yield was 127.3, 4.5, and 14.3 mg $g^{-1}$ for quercetin, kaempferol, and isorhamnetin, respectively. Using the models for kaempferol and isorhamnetin, optimal conditions were found at A = 87.2% w/w, B = 1.96 mL, and C = 38 min, at which the predicted yield was 126.1, 4.7, and 14.7 mg $g^{-1}$. Because the two sets of optimal conditions were not significantly different and they yielded very close predicted values, the conditions were optimized for quercetin, in which 50 mg of sample powder was extracted by UAE for 45 min using 1.00 mL of PG-1 solution containing 90% w/w PG-1. Extraction experiments performed under these conditions (n = 3) yielded 126.7 mg $g^{-1}$quercetin, 3.7 mg $g^{-1}$ kaempferol, and 13.3 mg $g^{-1}$ isorhamnetin. These experimental values fell well within the 95% prediction intervals (PI) given that 95% PIs for quercetin, kaempferol, and isorhamnetin were 105.0-149.5 mg $g^{-1}$, 3.0-5.9 mg $g^{-1}$, and 10.8-17.8 mg $g^{-1}$, respectively.

### 3.8. Evaluation of the DES-based extraction method for extraction efficiency and bioactivity, and the recovery of extracted flavonoids from DES

Extraction efficiency of the optimized method was compared with two other common extraction methods (Fig. 5). While the methanol-HRE was more efficient than methanol-UAE for quercetin extraction ($p$ < 0.05), the latter method was better than the former method for kaempferol ($p$ < 0.01) and isorhamnetin extraction ($p$ < 0.001). Nonetheless, the optimized method based on PG-1 exhibited the highest extraction yields for all three flavonoids. Compared with conventional extraction methods employing toxic organic solvents such as methanol, the current extraction method is definitely a green and safe method with enhanced efficiency. The current extraction method provided extraction yields up to 14% w/w for total flavonoid aglycons, while the maximum yield of the HRE method was 11% w/w.

Given that the extracted flavonoids are bioactive compounds, the current method has potential usefulness in the pharmaceutical industry or related fields. Because the major compounds of *Flos sophorae* are flavonoids, and thus their most representative activity is antioxidant activity, the effects of the DESs on antioxidant activity were evaluated using DPPH radical photometric assay as described in the Supplementary Information. Extraction efficiencies were compared between the diluted solutions of the original methanol extract in methanol and 90% PG-1. Antioxidant activities measured as radical scavenging activities (RSAs), were 14% and 78% for the methanol- and DES-diluted extracts, respectively (n = 3). Intriguingly, the discrepancy in these two values was very close to the innate activity of 90% PG-1 (63%). L-Proline is known to have reactive oxygen species (ROS) scavenging activity[43] and the RSA was positively correlated with the L-proline content,[44] whereas glycerol had no antioxidant activity.[45] Our DPPH assays performed separately on L-proline and glycerol also displayed results consistent with the literature above. In addition, a very similar difference in the antioxidant activity was observed for rutin dissolved in methanol and 90% PG-1: RSAs of rutin in methanol and 90% PG-1 were 17% and 81%, respectively. Taken together, it was concluded that the antioxidant effect of the DES was attributable to its component, L-proline, which had an additive effect on the antioxidant activity of the *Flos sophorae* extract.

In IL/DES research, the recovery of extracted compounds from extracts is fairly challenging due to the negligible vapor pressure of ILs and DESs.[33] Several approaches have been reported to recover extracted compounds, including the use of supercritical carbon dioxide, recrystallization, and the application of anti-solvents.[6, 26] In the present study, recovery of extracted flavonoids from the DES was assessed upon rutin, since it was the major flavonoid extracted. Water, which was added 20-fold and incubated at 0 °C for 2 hr, appeared to be the most efficient anti-solvent among tested solvents with the recovery at 75%. On the other hand, the recovery was increased up to 92% with simple application of solid phase extraction (SPE) on a reversed-phase (C18) cartridge (see the Supplementary Information for the SPE procedure). Flavonoid recovery was also tested on the real PG-1 extracts of *Flos sophorae*. Efficiency of the anti-solvent method was significantly reduced for the quercetin, kaempferol, and isorhamnetin glycosides (below 50%), while the SPE method was reasonably efficient with the recovery of 81, 87, and 87% for quercetin, kaempferol, and isorhamnetin glycosides, respectively.

**Fig. 4.** Response surface plots of the models for quercetin (a, b, c), kaempferol (d, e, f), and isorhamnetin (g, h, i).

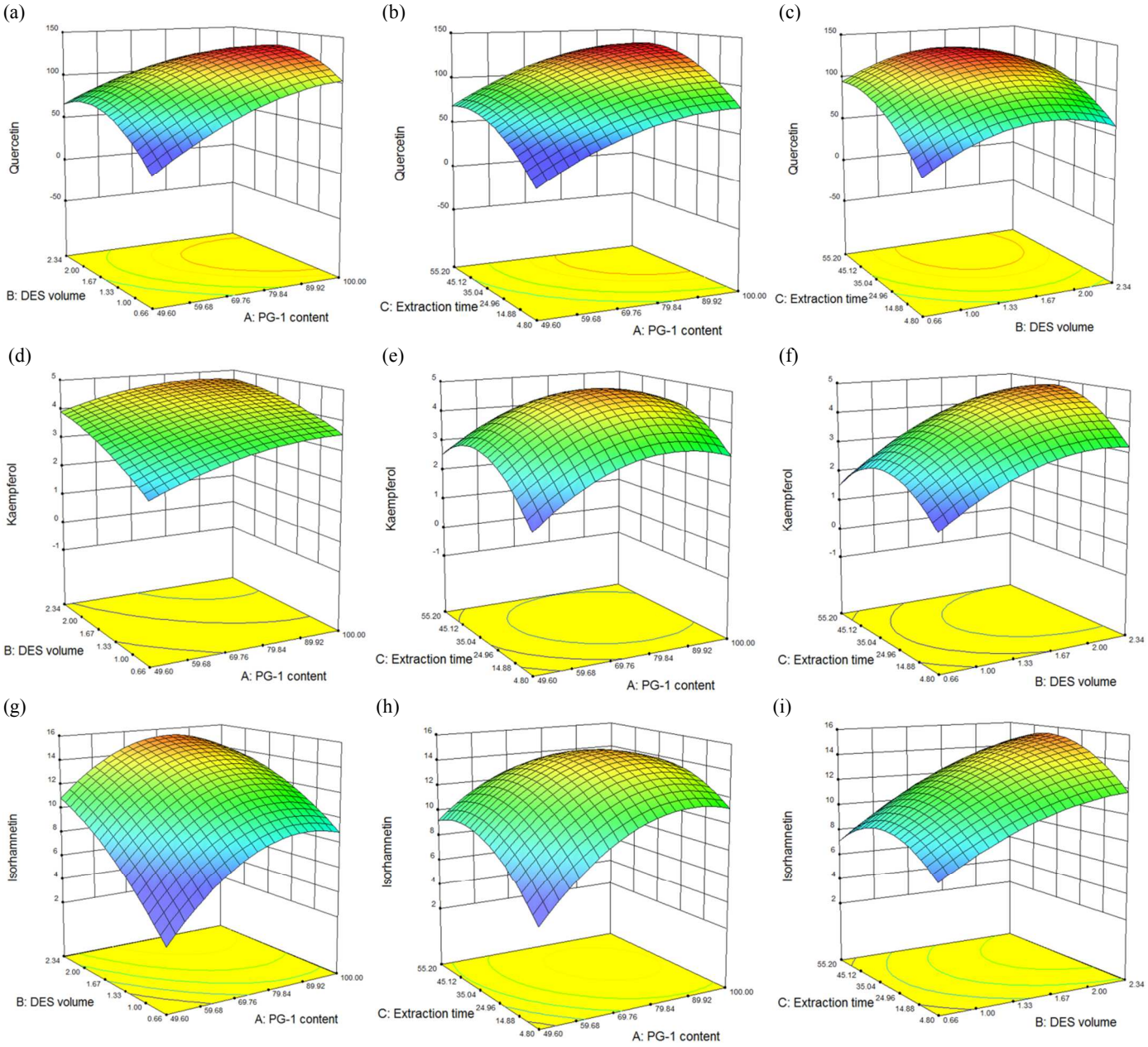
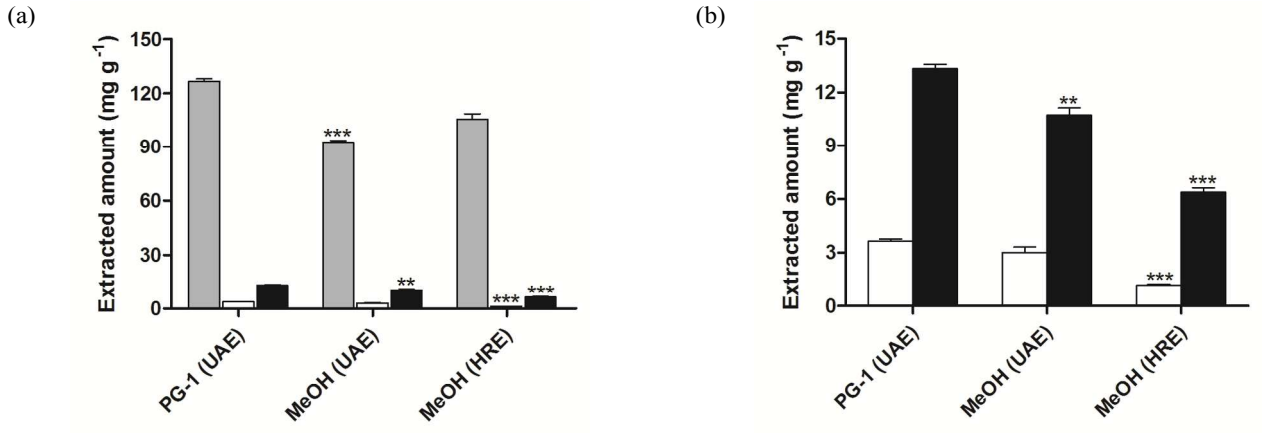



**Fig. 5.** Comparison between PG-1 using UAE and conventional extraction method. Quercetin (gray); kaempferol (white); isorhamnetin (black). Extraction efficiencies that were significantly different from those of PG-1 using the UAE method were indicated with * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). Error bars indicate the SEM ($n = 3$).




## Conclusions

A very efficient, green extraction method using a DES was described for flavonoid extractions from *Flos sophorae* as an example. The current study provided for the first time a practical example showcasing the tuneability of DESs as designer solvents that selectively and efficiently extract bioactive compounds from biomass. The solvent, PG-1, which was formed from a 2:5 mixture of natural, inexpensive, and safe components, L-proline and glycerol, was tailor-made to provide higher extraction efficiency than methanol for the extraction of quercetin, kaempferol, and isorhamnetin glycosides. Subsequent optimization of the operational conditions using RSM further improved the extraction efficiency up to 14% w/w for total flavonoid aglycons, which was significantly better than conventional methanol-based UAE or HRE. Based on antioxidant activity measured by DPPH assay, this DES could be an advantageous extractant with individual components that can additively enhance the bioactivity of the extracts. The recovery of extracted flavonoids from the DES was plausible using an anti-solvent strategy and a SPE technique. This study suggests that DESs are truly designer solvents that can be utilized as sustainable and safe extraction media for pharmaceutical and biochemical applications.

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## Notes and references

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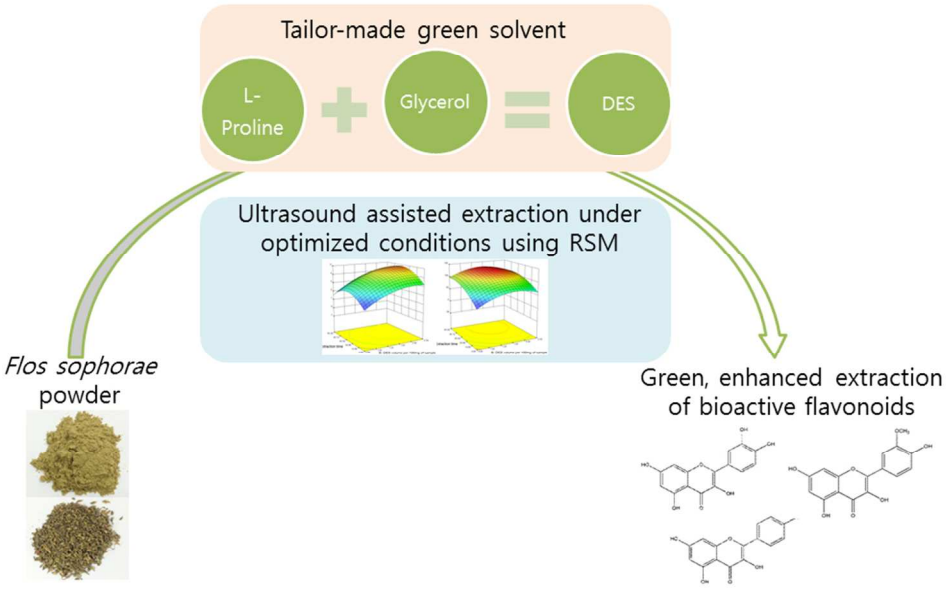


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