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Extraction of bioactive ginseng saponins using aqueous two-phase systems of ionic liquids and salts

Ai He, Bing Dong, Xueting Feng, Shun Yao*

School of Chemical Engineering, Sichuan University, Chengdu 610065, China

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ABSTRACT

In this research, two types of aqueous two-phase systems (ATPS) based on *n*-alkyl-tropinium and *n*-alkyl-quinolinium bromide ionic liquids (ILs) + salt + water have been developed and studied experimentally at different temperature. The binodal curves are fitted to nonlinear Merchuk relationship. For both investigated ILs, the longer the cation alkyl chain is, the greater its ability for ATPSs formation is; meanwhile the ability of the potential salts used for phase separation was compared and sorted, and high temperature was not beneficial to form these tropinium/quinolinium ILs-based ATPSs. On the basis of above results, the new method of extraction for ginsenosides from crude extracts of *Panax Ginseng* C. A. Mey using IL-ATPS was developed for the first time. Under appropriate conditions, a relatively high extraction efficiency (99.5%), partition coefficient ($K=651$) and selective enrichment for total saponins could be obtained. Furthermore, the target ginsenosides can be recovered from the IL-rich phase and the enrichment is conducive to improve their antioxidant activity. Above research is expected to provide meaningful reference for the separation of similar bioactive compounds.

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1. Introduction

The root of *Panax ginseng* C. A. Mey is a very famous traditional medicine in East Asian countries with immunopotential, cardio-protective, antidiabetic and anesthetic effects [1–3], which has been used to treat various human diseases for thousands of years. One of its main bioactive fractions is composed of dammarane-type triterpene saponins including ginsenosides Rg₁, Re, Rf, Rb₁, Rc, Rb₂, Rb₃ and Rd, etc. Generally, there are a large amount of coexisting non-saponins in the crude extracts of ethanol-water, which inevitably increase the burden of reprocessing for obtaining pure ginsenosides. These constituents have been found as vitamins, amino acids, proteins, peptides and (poly)saccharides, which have similar polarity, solubility and other properties with target saponins. Macroporous resin [4], solid phase extraction [5], expanded bed adsorption [6], hydrophilic interaction chromatography (HILIC) [7], centrifugal partition chromatography (CPC) [8], high-speed counter-current chromatography (HSCCC) [9] and supercritical CO₂ technology [10] have been employed to solve the separation problem of saponins. It is still expected for more and more efficient methods using in their

selective extraction and enrichment, which can provide researchers more new options.

Aqueous two-phase system (ATPS) is one of the new separation methods to extract and purify bioactive substances, which can be operated continuously in a mild condition and enlarged easily [11–13]. The selective partition of a target product between the two phases is the basis of a two-phase system separation. As a new class of purely ionic salt-like materials, ionic liquids (ILs) are low melting-point materials composed entirely of ions and have negligible volatility, low flammability, chemical stability, good environmental benignity and comprehensive solubility for organic compound. A large number of possible variations in cation and anion allow the fine-tuning of their properties [14–16]. So ILs can be effectively designed for various applications, especially for ATPS as a non-aqueous phase. Compared with traditional aqueous two-phase systems, IL-ATPS combines the advantages of both efficiently. Gutowski and his co-workers first used hydrophilic ionic liquid 1-butyl-3-methyl imidazolium chloride ([Bmim]Cl) and K₃PO₄ to form aqueous two-phase system with IL-rich upper phase and salt-rich lower phase [17]. This system could achieve high product purity as well as high yield, while maintaining the biological activity of the molecules. Up to now, versatile applications of ILs have emerged in ATPS for enrichment and purification of active ingredients from diverse natural products [18–23]. The results have already proven that the investigated ILs will not affect

* Corresponding author.

E-mail address: cusack@scu.edu.cn (S. Yao).

significantly structural integrity and activity of study objects. Moreover, as a kind of “green” solvent, ionic liquids could minimize environmental impacts resulting from the use of volatile organic compounds and could be recovered through the addition of a second hydrophobic IL or ion-exchange resins [24–26]. On the other hand, phase diagrams and phase equilibrium data are essential for the development, optimization, and scale-up of extraction process using ATPS [27]. Liquid-liquid equilibrium (LLE) data for imidazolium/pyridinium ionic liquids composed of 1-alkyl-3-methylimidazolium ($[C_n\text{mim}]^+$) or n -alkyl-pyridinium ($[C_n\text{Py}]^+$) as cation and BF_4^- , Br^- , Cl^- as anion have been measured by researchers [17,28–33]; and various salts including Na_2CO_3 , K_2CO_3 , K_3PO_4 , KOH , KCl , NaCl , K_2HPO_4 , and Na_2HPO_4 are frequently used to form IL-ATPS.

According to preliminary study, a series of ionic liquids with tropine/quinoline as cationic nucleus and bromide as anion were selected in the following IL-ATPS study. The former represents the series of saturated alicyclic ILs and the latter is the typical representative of planar conjugated ILs, and both of them are seldom applied in aqueous two-phase systems for separation of saponins. Moreover, their bromine salts were found to have better phase-forming ability according to preliminary experiments. So they were firstly synthesized and used to form ATPSs with K_3PO_4 , K_2CO_3 , K_2HPO_4 , $\text{K}_3\text{C}_6\text{H}_5\text{O}_7$, NaH_2PO_4 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ and water. Their phase diagrams, liquid-liquid equilibrium and tie-line data were measured for the first time, and obtained results are necessary for the optimization and design of extraction processes and provide essential basis for the prediction of phase composition when related data are not available. Finally, the new systems were used in extraction for ginsenosides from crude extracts of *Panax Ginseng* C. A. Mey. Appropriate conditions, extraction efficiency together with the results of separation and bioactivity assay were introduced in detail.

2. Experimental

2.1. Reagents and materials

Tropine was purchased from Wenhua Chemical Co. Ltd (Zhengzhou, China); commercial cation-exchange resins were provided by Aladdin Chemical Co. Ltd (Shanghai, China), and their H/Na type was transformed through the reported method [34]; quinoline, n -ethyl/propyl/butyl/amylyl/hexyl bromide and the other salts/reagents were purchased from Kelong Chemical Co. Ltd (Chengdu, China). Except for chromatographic grade acetonitrile used for quantitative analysis, all reagents and solvents were analytical pure grade and were used without further purification if not stated otherwise. Crude powders of Ginseng extracts were self-made, which was obtained by the extraction of dried roots of *Panax ginseng* C. A. Mey (source area: Jilin province of China, provided by Sengfu Biotech Co. Ltd, Xi'an, China) with 90% ethanol and concentration under vacuum according to previous study [35]. Standard compounds of Ginseng saponins (Rg_1 , Re , Rb_1 and Rd) and 2,2-diphenyl-1-picrylhydrazyl were purchased from Mansite Bio-Technology Co., Ltd (Chengdu, China), and all of their purity was above 98.5%. Deionized water was obtained by the UPRI-5T water purification system (0.4 mm filter) from Ulupure Technology Co. Ltd (Chengdu, China).

2.2. Synthesis for two series of ionic liquids

Based on the previous study [36], tropine (7.05 g, 0.05 mol) was dissolved in 70 mL ethyl acetate (for n -ethyl, propyl, butyl bromide) or toluene (for n -amylyl bromide), and then n -alkyl bromide (0.06 mol) was added into the solution. The reaction system was

refluxed at 323 K under vigorous stirring for 12 h (for n -ethyl, propyl bromide), or 348 K for 24 h (for n -butyl bromide), or 353 K for 24 h (for n -amylyl bromide). At the end of the reaction, the mixture was filtered under reduced pressure to remove the solvent and the residual alkyl bromide. The product was washed with ethyl acetate/toluene for several times, and then the white solids were dried under vacuum at 323 K and recrystallized to give n -alkyl-tropinium bromide.

Similarly, quinoline (6.45 g, 0.05 mol) and n -alkyl bromide (0.055 mol) were mixed in a 100 mL one-necked flask and reacted in dark under vigorous stirring for 2 days at 333 K (for n -ethyl, propylbromide) or 343 K (for n -amylyl, hexyl bromide). Then the reaction products were dissolved in ethyl acetate to extract the unreacted quinoline and n -alkyl bromide for several times. Finally, the purple solids were dried under vacuum at 313 K for several days and n -alkyl-quinolinium bromide ionic liquids were obtained after recrystallization [37].

The purities of above ILs were finally analyzed by a high-performance liquid chromatography (HPLC) system, which was composed of a LC6A isocratic pump (Shimadzu, Japan), a 2000ES evaporative light scattering detector (Alltech, USA) and an in-line Rheodyne injection valve with a 20 μL sample loop. The LC column was a Waters symmetry C18 column (150 mm \times 3.9 mm, I.D., 5 μm), and mobile phase was acetonitrile-water solution (12:88 \sim 27:73, V/V; no buffer added) with the flow rate of 1 mL/min. As the result, their purities were determined as 98.2 \sim 99.0% mass fraction. In order to eliminate the influence of water and obtain accurate data, these ILs were thoroughly dried at 105 $^\circ\text{C}$ for 8 h before the following measurements.

2.3. Phase diagrams

The binodal curves were determined by the titration method (cloud point method) [38]. An analytical balance with a standard uncertainty of ± 0.0001 g was used to weigh all the samples. IL aqueous solution of known mass fraction was placed into the glass vessel and stirred vigorously, and the salt solution of known mass fraction was added dropwisely until the mixture became turbid or cloudy. The maximal standard uncertainty of the IL and salt in the compositions was estimated to be ± 0.003 in mass fraction. Then drops of deionized water were added continuously into the vessel to form a clear one-phase system, and the procedure was repeated until the system cannot become turbid or cloudy again. Finally, the vessel was immersed in a jacketed glass vessel, and the temperature of the system was kept at 298.15 K, 308.15 K and 318.15 K using a CY20A water thermostat (Boxun Industry & Commerce Co., Ltd., Shanghai, China) in extrinsic cycle pattern with a standard uncertainty of ± 0.05 K. The composition of the mixture for each cloud point on the binodal curve was calculated by mass using an electrical analytical balance. The data were fitted according to the empirical nonlinear equation developed by Merchuk [39]:

$$w_1 = a \exp(bw_2^{0.5} - cw_2^3) \quad (1)$$

where w_1 and w_2 are the mass fractions of ILs and salt, respectively; and a , b , c are fitting parameters.

2.4. Tie-lines

After binodal curves are determined, a mass fraction of ILs and salt was chosen which could form two phases for each ILs, salts and temperature. Then related systems of this mass fraction were built for different ILs, salts and temperature. Corresponding vessels filled with these systems were placed into the thermostatic bath ($T = \pm 0.05$ K) at a certain temperature for 12 h until equilibrium.

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