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Application of aqueous two-phase flotation in the separation and concentration of puerarin from *Puerariae* extract

Peng-yu Bi, Hui-ru Dong*, Yan-chao Yuan

Faculty of Science, Beijing University of Chemical Technology, Beijing 100029, PR China

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ABSTRACT

Aqueous two-phase flotation (ATPF), which has the advantages of both solvent sublation and aqueous two-phase extraction, is an effective separation technique for separating strongly polar compounds. A highly effective, economically applicable and environmentally safe method has been successfully developed for separating puerarin from *Puerariae* extract. In an ATPF system composed of polyethylene glycol (PEG)/ammonium sulfate, the effects of various solution pHs, polymer molar masses, concentrations of ammonium sulfate in aqueous solution, nitrogen flow rates, flotation time and initial volumes of the PEG phase were investigated. Under the selected optimal conditions, the separation efficiency of ATPF was more than 87%. The comparison results between aqueous two-phase extraction (ATPE) and ATPF showed that ATPF can effectively separate puerarin from a *Puerariae* extract with high concentration coefficient. At the same time, the flotation product was purified by high-speed counter-current chromatography (HSCCC), and the purity of the final product was more than 95%. The experimental results show that ATPF-HSCCC is an effective method for preparing higher purity puerarin from a *Puerariae* extract.

1. Introduction

Isoflavones are widely present in many different natural products with biological activity, and they play an important role in cancer prevention, inhibition of tumor initiation, reduction of oxidative damage and other health effects [1–4]. Recently, many techniques were developed for separating and purifying isoflavones from different plants, such as high-speed counter-current chromatography (HSCCC) [5–7], pressurized liquid extraction [8–10], and solid-phase extraction [11].

Aqueous two-phase extraction (ATPE) is an effective, environmentally friendly, economically viable separation method. It is widely applied for separation of proteins [12], amino acids [13], DNA [14], and other biomaterials. Furthermore, ATPE can also be used to extract some active compounds from natural products [15,16]. In our previous report [17], aqueous two-phase extraction was improved by combination with solvent sublation [18], and the new technique was named aqueous two-phase flotation (ATPF). Compared to aqueous two-phase extraction [17], ATPF gives a higher concentration coefficient and a reduced consumption of organic solvents. Therefore, it is more efficient, environmentally friendly and economically applicable in natural product extraction.

In this paper, ATPF was applied in the separation of isoflavones in natural products for the first time. Because of good surface activity, the puerarin in *Puerariae* was selected as the target compound for separation and concentration. In the ATPF process, puerarin can be adsorbed on the surface of bubbles, which float to the top of the aqueous solution (a mixture of *Puerariae* extract and ammonium sulfate) where they encounter a layer of PEG, in which the puerarin dissolves. Based on the optimal conditions, the puerarin in *Puerariae* was successfully separated and concentrated by ATPF with $(NH_4)_2SO_4$/PEG600 system. In order to obtain the high purity puerarin, the flotation product (PEG phase) was further purified by HSCCC.

2. Experimental

2.1. Apparatus

A Mettler Toledo 320-S pH meter (Mettler, Switzerland) was used to determine the pH of the solution. The ATPF apparatus was similar to the one mentioned in earlier papers [17,19]. An Agilent 1100 Series chromatograph (Agilent, USA) with a Diamonsil™ C18 Column (150 mm × 4.6 mm) was used to analyze the aqueous solution, the flotation product (PEG phase) and the purification product of HSCCC.

The HSCCC instrument [20] was used with a Model GS10AB multilayer coil planet centrifuge equipped with a PTFE (polytetrafluoroethylene) multilayer coil of 110 m × 1.6 mm I.D. with a

* Corresponding author. Tel.: +86 010 64944151.
E-mail address: donghr@mail.buct.edu.cn (H.-r. Dong).

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total capacity of 230 ml. The β values of the coil range from 0.5 at the internal terminal to 0.75 at the external terminal. It is designed and constructed in Beijing Institute of New Technology Application (Beijing, China).

2.2. Reagents

The *Puerariae* root was obtained from Hunan province, China. The purity of puerarin standard was more than 99.9% (China Medicines and Chemical Reagents Identification Institute, China). PEG200, PEG400, PEG600, PEG 1000, PEG2000 and PEG4000 were purchased from Fucheng Chemical Factory ($M_w \pm 2.5$–5%, Tianjin, China). Ammonium sulfate, phosphoric acid and all organic solvents used for HSCCC were all analytical grade (Beijing Chemical Factory, China). The methanol used for HPLC was chromatographic grade (Fisher, USA), and the water was deionized prior to use.

2.3. Preparation of aqueous phase and PEG phase

The fresh roots of Hunan *Puerariae* 10.00 g were extracted by 200 ml deionized water twice, each time 2 h. The *Puerariae* extract was transferred to a 1000-ml volumetric flask, and it was mixed with ammonium sulfate solution. The aqueous solution was used to optimize the ATPF parameters. For transfer convenience, the PEG phase contained 50% water (w/w).

2.4. Optimization of ATPF parameters

The ATPF parameters (pH, ammonium sulfate concentration in aqueous solution, PEG molecular weight, nitrogen flow rate, initial volume of PEG phase and flotation time) were optimized to yield the maximum isoflavone extraction. All the initial volumes of aqueous phase were 300 ml, and all experiments were performed at room temperature for three times. In order to accurately obtain the concentration of ammonium sulfate, the relationship between the volume concentration and the mass concentration was determined: $w = 0.0771C + 2.6067$ ($r = 0.9990$) [17], where C is the volume concentration (g/l) and w is the mass concentration (%). Using this equation, we can easily calculate the volume concentration of ammonium sulfate without using volumetric measurements.

2.5. Comparison experiments

In this work, ATPF was compared with ATPE. In the ATPE experiment, 300 ml of aqueous solution (Section 2.3) with pH 5.0 was extracted by 100 ml of PEG600 solution; in the ATPF experiment, 300 ml of aqueous solution (Section 2.3) with pH 5.0 was extracted by 10.00 ml of PEG600 solution with pH 5.0, and the nitrogen flow rate was 20 ml/min. The volume concentration of ammonium sulfate in all aqueous solution was 350 g/l. All separation processes were performed at room temperature.

2.6. HSCCC purification and HPLC analysis

The HSCCC solvent system utilized in the present work was prepared by a mixture of ethyl acetate/*n*-butanol/water (2:1:3, v/v). In the HSCCC purification, the multilayer coiled column was first entirely filled with the upper phase, and then the lower phase was pumped into the column at a flow rate of 2.0 ml/min. The apparatus was run at a revolution speed of 800 rpm. After hydrodynamic equilibrium was established in the column, 2 ml of the flotation product (PEG phase) was injected through the injection valve, and all the purification processes were performed at room temperature. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the elution profile.

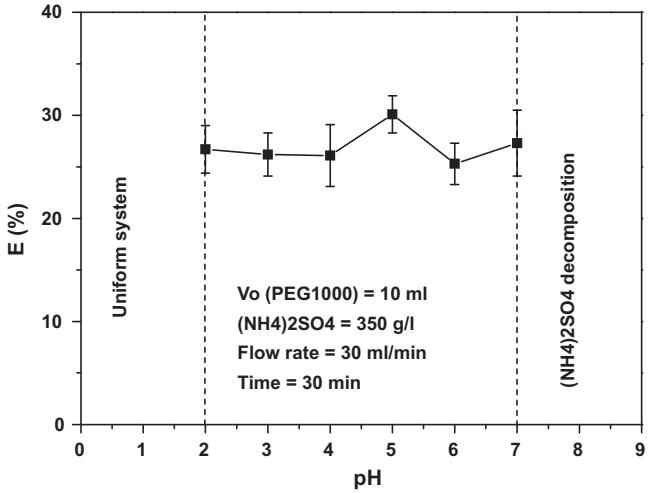

Fig. 1. Effect of pH on aqueous two-phase flotation.

Puerarins in the aqueous phase, the flotation product (PEG phase) and the purification product were determined by HPLC. In the HPLC analysis, the mobile phase was a mixture of 50 mM phosphoric acid and methanol (60:40, v/v). The flow rate was 1.0 ml/min, the detection wavelength was 254 nm, and the injection volume was 10 μl. All chromatographic analyses were performed at room temperature. The calibration curve for puerarin was obtained over the range 2.85–570 ng/ml. The regression equation was $y = 3.86 + 8.02x$ ($R^2 = 0.9999$), where y is the peak area and x is the concentration (ng/ml), and this was used to quantitatively analyze the separation efficiency, the distribution ratio and the purity of the final product.

3. Results and discussion

3.1. Optimization of ATPF parameters

For calculating the separation efficiency, two assumptions were made: firstly, both the aqueous phase and the PEG phase are completely homogeneous; secondly, the volume of aqueous phase does not change before and after flotation.

The separation efficiency (E), the concentration ratio (D) of puerarin between PEG and aqueous phases, and the concentration coefficient (α) were defined as follows:

$$E = \left(1 - \frac{C_w}{C_{wi}}\right) \times 100\% \quad (1)$$

$$D = \frac{C_{PEG}}{C_w} \quad (2)$$

$$\alpha = \frac{C_{PEG}}{C_{wi}} \quad (3)$$

where C_w is the puerarin concentration of the aqueous phase at time t, C_{wi} is the initial concentration of puerarin in the aqueous phase, and C_{PEG} is the puerarin concentration in the PEG phase at time t.

In liq–liq extraction, a suitable pH of aqueous solution is very helpful to increase the dissolving ability of the target compound in the organic phase. Solvent sublation, a special extraction technique [18], always has a best pH range for aqueous solution [19,21–23]. But aqueous two-phase system is different from the common liq–liq extraction system. The PEG phase can dissolve many strong polar compounds [17], so the effect of pH on aqueous two-phase systems is not very significant. As shown in Fig. 1, the separation efficiency of pH 5 is only slightly better than other values,