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PAPER

ILs-based microwave-assisted extraction coupled with aqueous two-phase for the extraction of useful compounds from Chinese medicine†

Xiao Lin, Yuzhi Wang,* Xiaojie Liu, Songyun Huang and Qun Zeng

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Ionic liquids-based microwave-assisted extraction (ILs-MAE) of medicinal or useful compounds from plants was investigated as an alternative to conventional organic solvent extractions. The extraction and the preconcentration of aqueous two-phase (ATP) systems have been integrated. Various operating parameters were systematically considered by single-factor and L_9 (3^4) orthogonal array experiments. 1-Butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF₄]) has been selected to extract *Apocynum venetum*. The extract was then converted to the top phase by [bmim][BF₄]/NaH₂PO₄ system which was suitable for the preconcentration. Reversed phase high performance liquid chromatography (RP-HPLC) with ultraviolet detection was employed for the analysis of hyperin and isoquercitrin in *Apocynum venetum*. The optimal experiment approach could provide higher detection limit of hyperin and isoquercitrin which were $3.82 \mu\text{g L}^{-1}$ and $3.00 \mu\text{g L}^{-1}$ in *Apocynum venetum*. The recoveries of hyperin and isoquercitrin were 97.29% (RSD = 1.02%) and 99.40% (RSD = 1.13%), respectively, from aqueous samples of *Apocynum venetum* by the proposed method. Moreover, the extraction mechanism of ILs-MAE and the microstructures and chemical structures of the herb before and after extraction were also investigated. The method exhibited potential applicability with other complicated samples.

Introduction

In recent years, Ionic liquids (ILs) have attracted much research interest thanks to their unique properties: negligible vapor pressure, excellent thermal stability, wide liquid range, tunable viscosity and miscibility with water and organic solvents, as well as good solubility and extractability for various organic compounds.^{1–3} ILs have been gaining great exposure for potential use as green solvents and possible replacements for traditional volatile organic solvents in many applications, including analytical applications, organic synthesis, catalysis and separation science.^{4–7} As a potential extraction agent, ILs can effectively improve the selectivity and the extraction efficiency of complicated samples such as traditional Chinese medicine.⁸

Apocynum venetum belongs to Apocynaceae, leaves of which are considered as a traditional Chinese medicine (TCM).⁹ *Apocynum venetum* is a perennial shrub widely distributed throughout the Mediterranean area and northwestern China. *Apocynum venetum* leaves (Apocynum) have been proved to exhibit a lot of activities, such as lower blood pressure, reduce blood lipids, antidepressant, sedation, hepatoprotective effect, bacteriostatic action, antioxidant and anti-aging effect and

anxiolytic properties.^{10,11} Recently, teas prepared from Apocynum have become a popular healthy beverage in many countries and are marketed as anti-aging nutritional supplements. The Apocynum appear to be free of alkaloids typical for the Apocynaceae family, but they are rich in flavonoids.¹² The dominant compounds in flavonoids of Apocynum, hyperin and isoquercitrin (as shown in Fig. 1) have been proven by pharmacological experiments to lower blood pressure and to exert an anti-inflammatory effect.

The quality control of hyperin and isoquercitrin in Apocynum is the principal factor impeding its international marketization. As a weighty step of quality control, sample pretreatment must be taken seriously because it can isolate and enrich the target analytes, thus improving the sensitivity of determination. Several common methods, such as marinated extraction (ME), heat-reflux extraction (HRE), soxhlet extraction (SE) and MAE, have

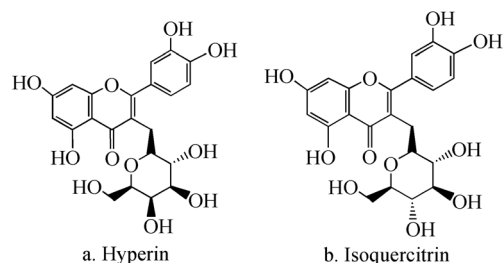


Fig. 1 The chemical structure of (a) hyperin and (b) isoquercitrin.

State Key Laboratory of Chemol Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, P. R. China. E-mail: wyzss@hnu.edu.cn; Fax: +86-731-88821848; Tel: +86-731-88821903

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been reported for the extraction of traditional Chinese medicine.^{13–15} Currently, microwave heating coupled with ILs provide an efficient alternative to traditional techniques with advantages of higher extraction efficiency, lower solvent, less pollution, faster extraction processes and power consumption, *etc.* Ionic liquids can efficiently absorb and transfer microwave energy as well. The results suggest that ILs-MAE provide an efficient alternative to conventional extraction techniques.^{16,17} However, the dissolution of the active ingredients of traditional Chinese medicine is limited. Ionic liquids-based aqueous two phase systems (ILs-ATPS) has been developed to overcome these deficiencies.

ILs-ATPS is one simple and environmentally friendly separation system, which is generated by mixing Ionic liquid with certain salt at high concentration. As the pioneer, Rogers and co-workers reported that some hydrophilic IL form ATPS when contacting with concentrated solutions of water-structuring salts for the first time in 2003.¹⁸ They proved that the aqueous biphasic system indicates adequate chemical potential use in separations applications. With the use of ATPS, one can simultaneously carry out purification, extraction and enrichment. Furthermore, these systems are suitable for the biological samples because each phase contains 70–90% water, which means that biomolecules will not be denatured.^{19,20} Li *et al.* presented an ionic liquid-based aqueous two-phase system as a new pretreatment strategy for the analysis of opium alkaloids in *P. papaveris*.²¹ Pei *et al.* studied IL-based ATPS and their results suggested that IL-based ATPS have the potential to offer new possibilities in the purification and separation of proteins.²² So, the combination of the ILs-based MAE technique with the use of ILs-ATPS proposed as an adequate alternative for the extraction and enrichment of compounds from herbs. A tiny volume of extract ionic liquid solution would conquer imprecise measurement because of rapid water vaporization. The detection limit can be correspondingly improved. Besides, it provides an efficient alternative to traditional techniques with advantages of higher extraction efficiency, faster extraction processes, lower solvent and power consumption, *etc.*²³

The present work reports the systematic optimization of the use of aqueous two phase IL solutions of microwave-assisted extraction. [bmim][BF₄] was selected to extract *Apocynum venetum*. With adding salts, such as NaH₂PO₄, [bmim][BF₄] was subsequently converted to the above phase to achieve phase separation and preconcentration of the extraction. The schematic diagram of the experiment is shown in Fig. 2. HPLC-UV

was employed for the analysis of hyperin and isoquercitrin in *Apocynum*. The conditions of affecting preconcentration are studied and the extraction mechanism is also investigated. ILs-based microwave-assisted extraction coupled with aqueous two-phase (ILs-MAE-ATPS) is successfully developed as a new pretreatment strategy for the analysis of hyperin and isoquercitrin in *Apocynum*.

Experimental

Apparatus

HPLC analysis was carried out on an Agilent Series 1260 liquid chromatograph, which contained a vacuum degasser, quaternary pump, manual injector and UV-Vis detector system. Chromatographic separation was performed on an Agilent Eclipse XDB-C18 (5 $\mu\text{m} \times 250 \text{ mm} \times 4.6 \text{ mm i.d.}$, Agilent, USA) and Chem Station software (Agilent, USA) were used for separation and Data collection. A UV-2450 UV-Vis Spectrophotometer (SHIMADZU, Japan) was used to determine the absorbance of the standard sample and ILs. An MDS- 2002AT pressure self-control microwave decomposition system equipped with a temperature sensor supplied from Sineo microwave chemical technology company LTD (Shanghai, China) and Auto Science AS-2060B Ultrasonic Cleaner (Tianjin, P.R.China) were used for the extraction. A TGL-16C High-speed centrifuge (Shanghai, China) was employed to accelerate the phase separation process. Samples were dried and ground by a 101-0E Ventilated drying oven (Beijing, China) and FW-100 plant pulverizer (Beijing, China). An S-4800 scanning electron microscope (Hitachi, Japan) was used to examine the surface microstructures of samples before and after extraction. FT-IR spectra were recorded in the range of 450–4000 cm^{-1} on a Spectrum One FT-IR spectrometer (PerkinElmer, USA). A Varian Inova 400 NMR Spectrometer (Varian, USA) was employed to record ¹H NMR and ¹³C NMR spectra of the synthetic ILs.

Reagents and materials

Raw herbs of *Apocynum* were collected directly from various production sites in China and commercial drugs were purchased in different drugstores. Before extraction, the materials were ground into powder with particle diameter of 0.2–0.9 mm by plant grinder. Hyperin (98%) and isoquercitrin (98%) were purchased from Chinese Medicine Research Institute (Nanjing, P.R.China).

N-Methylimidazole, 1-bromobutane, 1-bromooctane and anhydrous Sodium dihydrogen phosphate were all purchased from Aladdin Reagents Company (Shanghai, China) and were of 98% purity. Sodium fluoroborate was obtained from Jingchun Company (Shanghai, China) and Beijing Yili Company (Beijing, China). Methanol (HPLC grade), ethanol (analytical grade) and other reagents were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Deionized water was used throughout the experiments.

Synthesis and characterization of ILs

Two steps were used in the synthesis of ionic liquids. [bmim][BF₄] and 1-octyl-3-methylimidazolium tetrafluoroborate ([omim]

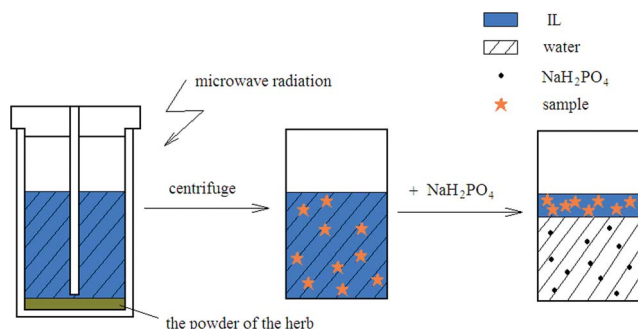


Fig. 2 The schematic diagram of the experiment.

[BF₄]) were prepared with minor modification,^{24,25} and the reaction conditions were optimized systematically in our work.

In the first step, 1-butyl-3-methylimidazolium bromide([bmim][Br]) was synthesized. In a one-necked 500 mL round bottomed flask equipped with a reflux condenser and magnetic stirrer under vigorous stirring, 118.68 mL (150.74 g, 1.1 mol) of freshly distilled bromobutane was added dropwise over 1 h to a solution of 79.24 mL (82.1 g, 1.0 mol) of 1-methylimidazole at room temperature. The mixture was then heated to 70 °C for 1 day and decanted from the hot solution in a separatory funnel, then washed three times with 100 mL of ethyl acetate, and then dried under reduced pressure at 70 °C for 2 days. [omim][Br] were prepared under the same procedures.

In the second step, [bmim][BF₄] was synthesized. In a one-necked 500 mL round bottomed flask equipped with a reflux condenser and magnetic stirrer under vigorous stirring, 49.38 g NaBF₄ was added to a solution of 98.56 g of [bmim][Br] in 30 mL acetone at room temperature for 2 days. Due to the different traits of NaBF₄, the [bmim][BF₄] of removed salt and retained salt in the synthesis of ionic liquids were prepared, respectively (as shown in Fig. 3). It is found that [bmim][BF₄] of retained salt is suitable for the experiment. [omim][BF₄] was prepared under the same procedure.

The synthetic routes of ILs is shown in ESI Fig. S1†. The synthetic ILs were dried for 24 h at 70 °C under vacuum before usage and their structures were confirmed by FT-IR, ¹H NMR and ¹³C NMR spectra, respectively (as shown in ESI Fig. S2 and Table S†).

ILs-based microwave-assisted extraction

The ILs-MAE experiments were performed on an MDS-2002AT instrument. 0.2 grams of herb medicine powders were accurately weighed and introduced into the 50 mL closed control vessel which was covered by a lid and a temperature sensor was inserted into it to detect and control the internal temperature. 10 mL of [bmim][BF₄] aqueous solution was poured into it. The pressure was set at 10 atm during 10 min. The initial temperature was set at 30 °C and then elevated step by step (a 2–5 °C increase every time) until it attained the experimental requirement (30–70 °C). Afterwards, the extracts obtained were centrifuged at 10 000 rpm for 20 min, and the supernatant liquids were used in the next step.

Reference extraction techniques

ILs-based heat extraction (ILs-HE), ILs-based maceration extraction (ILs-ME) ILs-based and ultrasound-assisted

extraction (ILs-UAE) were selected as the reference procedure for extraction of hyperin and isoquercitrin from the herbs performed. 0.2 g sample and 10 mL of specific IL aqueous solution (0.4 g mL⁻¹[bmim][BF₄] for Apocynum) were added in a erlenmeyer flask with a stopper. The mixtures were extracted with corresponding conditions and were measured (as shown in Table 1).

Preparation of phase diagrams

Phase diagrams were determined by the cloud-point method.²⁶ Briefly, 1.0 g [bmim][BF₄] was put into a 10 mL centrifugal tube. A salt solution of known concentration was then added to the test tube until turbidity and a two-phase system was formed in 5 min. The composition of this mixture was noted. Then, water was added dropwise to the tube to get a clear one-phase system and more salt was added again to afford a two phase system. The composition of this mixture was noted and so on.

Preconcentration

A 10 mL centrifuge tube was filled with the extract. NaH₂PO₄ was added into it and mixed thoroughly, and two clear phases were formed after about five minutes. Ten microlitres of the top phase was aspirated by a 50 µL microsyringe and directly injected into the HPLC system for analysis.

HPLC analysis and quantification

In order to achieve a good separation efficiency of hyperin and isoquercitrin from Apocynum, 15/85 acetonitrile–0.05% formic acid aqueous solution (v/v, 1 mL min⁻¹) was selected as the mobile phase for HPLC analysis. The column temperature was 30 °C and the injection volume was 10 µL. According to the report in the literature, UV-Vis was set at 254 nm.¹¹ Our work was correspondingly adjusted to 258 nm, which is shown in ESI Fig. S3†. In the experiment, the extraction content of hyperin and isoquercitrin were calculated by the method of H. Zeng *et al.*¹⁷

Results and discussion

Selection of extraction solvent

The selection of the most suitable solvent for extracting the analytes of interest from the sample matrix is a fundamental step in finding the optimal extraction method. According to the reported literature, [bmim][BF₄] and [omim][BF₄] could form two clear phases with adding salt.^{27,28} [bmim][BF₄] and [omim][BF₄] were used in this work and the experimental results are shown in Table 2. As seen from Table 2, comparing the results of ILs with that of water, methanol and ethanol water solution, the addition of ILs to water had significant effects on the extraction of hyperin and isoquercitrin in MAE. The probable reasons were that ILs increased the solubility of hyperin and isoquercitrin in solutions due to the multi-interactions of ILs, especially hydrogen bonding, polarity, π - π , π -n and ionic/charge-charge, between imidazolium cation and hyperin and isoquercitrin. Moreover, ILs changed the dissipation factor of solution and the transfer of energy from microwaves to sample, which affected the

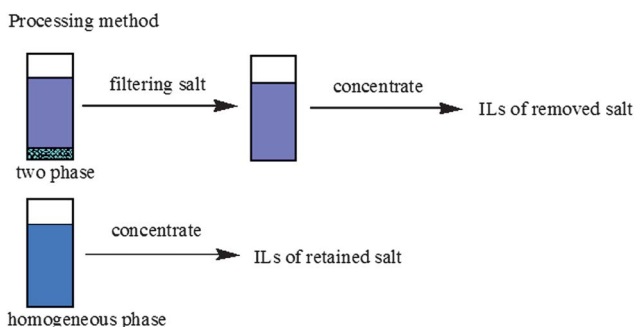


Fig. 3 Different processing methods after the second step of synthesis.

Table 1 Comparison of different extraction methods ($n = 3$)

Extraction techniques	Corresponding conditions ^a				Extraction yield (mean \pm SD, mg g ⁻¹) ^b	
	Concentration of IL (gmL ⁻¹)	Solid/liquid (g mL ⁻¹)	T (°C)	Time (min)	Hyperin	Isoquercitrin
ILs-MAE	0.4	0.02	60	10	2.4793 \pm 0.027	2.1299 \pm 0.021
ILs-UAE	0.4	0.02	25	60	2.3656 \pm 0.034	1.9003 \pm 0.030
ILs-HE	0.4	0.02	60	360	2.0651 \pm 0.021	1.6076 \pm 0.018
ILs-ME	0.4	0.02	25	2880	2.0396 \pm 0.022	1.6731 \pm 0.016

^a Those techniques were also carried out under optimized conditions. ^b Each value consisted of the mean and standard deviation (SD) of three independent experiments.

Table 2 The effect of different extraction solvents on extracting hyperin and isoquercitrin from Apocynum ($n = 3$)

Extraction solvent	Extraction yield (mean \pm SD, mg g ⁻¹) ^a	
	Hyperin	Isoquercitrin
[bmim][BF ₄]	2.226 \pm 0.019	2.010 \pm 0.017
[omim][BF ₄]	2.097 \pm 0.023	1.957 \pm 0.022
Methanol	1.721 \pm 0.017	1.791 \pm 0.019
Water	1.267 \pm 0.013	1.380 \pm 0.014
Ethanol solution (v/v = 80 : 20)	2.071 \pm 0.022	2.097 \pm 0.021

^a The value was mean \pm standard deviation (SD) of three independent experiments.

solvent and the sample warming rapidly and speed of energy transferring according to the mechanism of ionic conduction heating.

With the same anion of BF₄⁻, the changes of the alkyl chain length was evaluated. The results suggest that the cations of ILs influenced the extraction yields of hyperin and isoquercitrin to the same degree. The obtained extraction yields indicate that [bmim][BF₄] was more efficient than [omim][BF₄] in the ILs-MAE of Apocynum, which was likely related to the structures of ILs that determine their physical and chemical properties. The short alkyl chain was favorable for the formation of a large amount of micelles which could adsorb the organic molecules in the aqueous solution.

Moreover, comprehensively considering that methanol is volatile, flammable and harmful to humans and the environment, ionic liquid aqueous solutions were selected as a class of green and alternative solvents to displace volatile organic solvents like methanol in the ILs-MAE process. Therefore, [bmim][BF₄] aqueous solution was selected as the extraction solvent for hyperin and isoquercitrin from Apocynum.

Optimization of ILMAE conditions

The extraction conditions, including the concentration of [bmim][BF₄] aqueous solution, solid liquid ratio, radiating time of microwave and extraction temperature were optimized. We worked out the optimum levels of each factor by the single factor test. In addition, we arranged multi-factors through the use of the orthogonal test which is a scientific method.

Single factor experiments

Single factor test was performed by one factor varied with different levels while other factors are fixed. All results of single factor experiments are shown in Fig. 4. In the light of Fig. 4, it can be observed that when the concentration of [bmim][BF₄] aqueous solution was 0.4 g mL⁻¹ for isoquercitrin, the extraction yield reached the top. The yield of hyperin slowly increased as IL concentration increased from 0.4 to 0.6 g mL⁻¹. However, below 0.4 g mL⁻¹ of [bmim][BF₄] aqueous solution, the extraction yields of hyperin and isoquercitrin increased rapidly. That is because with the addition of [bmim][BF₄], both the solubility and the extracting capacity of the solvent were enhanced. At the same time, the capabilities of microwave absorption and microwave conversion were both increased. Furthermore, the interactions between [bmim][BF₄] and target element were strengthened. When the concentration of [bmim][BF₄] aqueous solution exceeded 0.4 g mL⁻¹, the extraction yields do not significantly increase.

The major cause was that the viscosity of the [bmim][BF₄] solution would be enhanced if the concentration increased and the diffusion capacity would be reduced. As a result, it was more difficult for [bmim][BF₄] to penetrate into the interior of sample matrixes. According to the above results, 0.4 g mL⁻¹ of [bmim][BF₄] aqueous solution was selected for extracting hyperin and isoquercitrin from Apocynum.

Different solid liquid ratios (from 0.02 to 0.1 g mL⁻¹) were used to extract hyperin and isoquercitrin from Apocynum. Fig. 4 shows that the maximum extraction yields of hyperin and isoquercitrin from the herb all occurred at the ratio of 0.02 g mL⁻¹ because of their largest driving force, which stemmed from its largest concentration gradient. Because the smaller the ratio of solid-liquid is, the more sufficient the contact between sample matrixes and [bmim][BF₄] aqueous solution is, as a result the more hyperin and isoquercitrin we obtained. Moreover, it was also probably due to the large volume of ILs aqueous solution that caused excessive swelling of the materials by water and absorbed microwaves efficiently with very little microwave energy absorbed directly by the materials. Thus, 0.02 g mL⁻¹ for Apocynum were adopted for the following work.

As shown in Fig. 4, it can be seen that the extraction yields of hyperin and isoquercitrin from Apocynum enhanced with the increase of the extracting temperature. The higher the extraction temperature is, the lower the viscosity of ILs and the more likely it is to have an opening of the cell matrix. The dissolution rates will be due to the increasing solubility of the solvents. Because

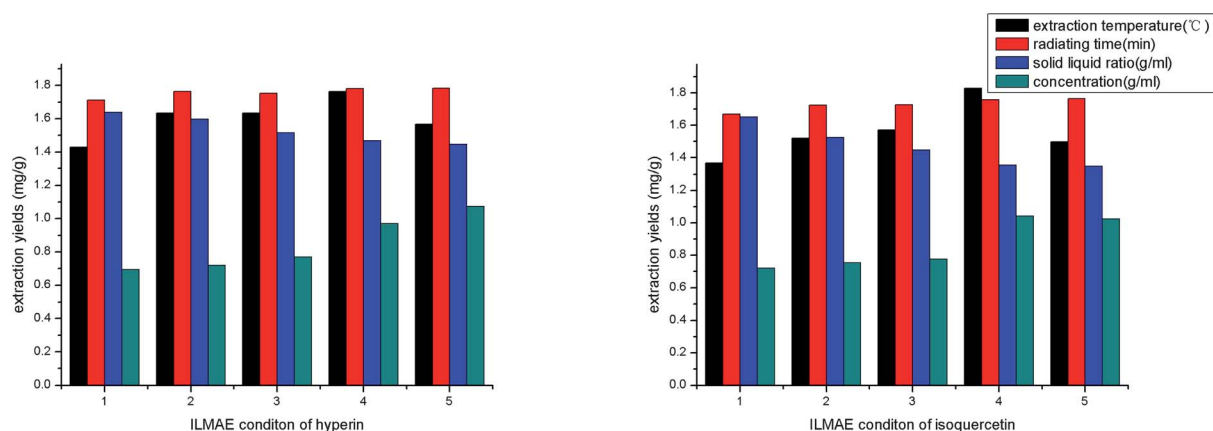


Fig. 4 The effect of microwave-assisted extraction conditions: extraction temperature (30, 40, 50, 60 and 70 °C), radiation time (5, 10, 15, 20 and 25 min), solid–liquid ratio (0.02, 0.04, 0.06, 0.08 and 0.10 g mL⁻¹), ILs concentration (0.05, 0.10, 0.20, 0.40 and 0.60 g mL⁻¹).

the increasing extracting temperature contributes to the reducing of the viscosity of ILs and the enhancing of the spread ability and solubility of ILs, it was beneficial to dissolve and extract hyperin and isoquercitrin. Above 70 °C, the yields of hyperin and isoquercitrin decreased, which may be due to the decomposition of some hyperin and isoquercitrin at high temperature. Thus, 60 °C were selected for further study of Apocynum.

It is necessary to select an appropriate irradiation time to guarantee completion of the extraction. In the light of Fig. 4, it can be seen that with the radiating time increasing, the extraction yields of hyperin and isoquercitrin were increasing rapidly, and after 10 min for Apocynum, it kept slightly increasing. Therefore, 10 min for Apocynum was enough to extract bulk hyperin and isoquercitrin from the sample powders.

Orthogonal design L₉ (3⁴) experiment

Orthogonal experiment of four factors and three levels was investigated.^{29,30} The levels setting values of four factors (A, B, C and D) used in the orthogonal arrayed design are shown in Table 3, and nine experimental trials were performed with the different array conditions as shown in Table 4. The analysis of variance (ANOVA) for the orthogonal test is shown in Table 5.

As shown in Table 4, the *K* and *R* values were calculated and listed. *K* value is the average extraction yield of hyperin and isoquercitrin under each level of an investigating variable. According to the largest donating rule, the largest value of *K* is the optimized value. The *R* value is the range of *K* value. The optimum experimental conditions of ILs-MAE were as follows: the concentration of [bmim][BF₄] aqueous solution were 0.4 g mL⁻¹, the solid liquid ratio was 0.02 g mL⁻¹, the extracting

temperature was 60 °C and the radiating time was 5 min for hyperin and 15 min for isoquercitrin. The optimal conditions for extracting Apocynum from the herb by the MAE technique could be summarized as: the concentration of [bmim][BF₄] aqueous solution was 0.4 g mL⁻¹, the solid liquid ratio was 0.02 g mL⁻¹, the extracting temperature was 60 °C. Because the radiating time was not the significantly changed factor, 10 min could extract the hyperin and isoquercitrin almost completely. So, 10 min was the optimal radiating time.

In order to validate the optimum conditions of ILs-MAE, three samples of Apocynum extracted under optimum conditions, respectively, was determined. The mean extraction yields were higher than any group of the orthogonal experiment. It is indicated that the extraction yields of hyperin and isoquercitrin from Apocynum could be increased using the combination of four optimized factors under ILs-MAE. The *R* values and the analysis of variance summarized in Table 4 and Table 5 show that the solid liquid ratio had much more obvious effects on the extraction of hyperin and isoquercitrin from Apocynum than other factors.

Comparison of different extraction procedures

To evaluate the extraction efficiency of ILs-MAE with other conventional extraction techniques, the optimized ILs-UAE, ILs-HE and ILs-ME methods was used to extract hyperin and isoquercitrin from Apocynum. From the results shown in Table 1, we can find that the extraction efficiency of hyperin and isoquercitrin in the herb with ILs-ME which wasted most solvent and extracting time was the worst. ILs-HE can heat plant tissue conventionally and break cytoderm to accelerate dissolution of intracellular effective components. Owing to the character of ultrasound, ILs-UAE can improve extraction yields of active ingredients from herbs and save extracting time. However, ILs-MAE had high extraction efficiency compared with ILs-ME, ILs-HE and ILs-UAE due to its unique extraction mechanisms. Superheating, mass heating and fast heating were the unique extraction mechanism of MAE. So, MAE could possess higher extraction yields with less solvent consumption and shorter time compared with conventional extraction techniques.

Table 3 Factors and levels of orthogonal test

Factor	1	2	3
(A) ILs solution concentration (g mL ⁻¹)	0.2	0.4	0.6
(B) Solid/liquid ratio (g mL ⁻¹)	0.02	0.04	0.06
(C) Extraction time (min)	5	10	15
(D) Extraction temperature (°C)	50	60	70

Table 4 Arrangement and results of L_9 (3^4) orthogonal test^a

No.	Factor								Extraction yield (mean \pm SD, mg g ⁻¹) ^b	
	A		B		C		D		Hyperin	Isoquercitrin
1	1		1		1		1		1.667 \pm 0.022	1.644 \pm 0.020
2	1		2		2		2		1.784 \pm 0.025	1.699 \pm 0.024
3	1		3		3		3		1.439 \pm 0.013	1.352 \pm 0.013
4	2		1		2		3		1.970 \pm 0.026	1.886 \pm 0.024
5	2		2		3		1		1.804 \pm 0.018	1.663 \pm 0.017
6	2		3		1		2		1.829 \pm 0.017	1.699 \pm 0.016
7	3		1		3		2		2.196 \pm 0.014	1.935 \pm 0.013
8	3		2		1		3		1.814 \pm 0.011	1.712 \pm 0.010
9	3		3		2		1		1.596 \pm 0.017	1.464 \pm 0.014
	H		I		H		I		H: Hyperin, I: Isoquercitrin	
K_{1j} ^c	1.629		1.565		1.944		1.821		1.771	1.685
K_{2j}	1.868		1.749		1.800		1.691		1.783	1.683
K_{3j}	1.868		1.704		1.621		1.505		1.813	1.649
R_j ^d	0.237		0.184		0.323		1.498		0.035	0.043
O ^e	A ₃	A ₂	B ₁	B ₁	C ₁	C ₃	D ₂	D ₂		

^a H: hyperin; I: isoquercitrin. ^b Extraction yield values of the herb was comprised of mean and standard deviation of three independent experiments.

^c Logesh $K_{ij} = (1/3) \sum$ mean extraction yield of hyperin or isoquercitrin at factor j ($j = A, B, C, D$). ^d Logesh $R_{ij} = \max\{K_{ij}\} - \min\{K_{ij}\}$, j and i mean extraction factor and setting level here, respectively. ^e O means the optimum condition. The optimum combination of conditions for hyperin, is $A_3B_1C_1D_2$, while for Flos Sophorae, is $A_2B_1C_3D_2$.

Table 5 Analysis of variance (ANOVA) for orthogonal test^a

Source	Sum of squares		Degrees of freedom		F-value		P-value	
	H	I	H	I	H	I	H	I
A	0.114	0.055	2	2	39.161	22.987	^b	^b
B	0.157	0.151	2	2	54.143	63.026		
C	0.003	0.002	2	2	0.988	0.989		
D	0.102	0.055	2	2	35.220	23.013		
error	0.0029	0.0024	8	8				

^a H: Hyperin; I: isoquercitrin. ^b Significant ($P > 0.05$).

Preconcentration of hyperin and isoquercitrin from extract

Phase diagrams. Phase diagrams were used to characterize the phase systems, and NaH_2PO_4 was tested for the formation of ATPS with $[\text{bmim}][\text{BF}_4]$ (as shown in Fig. 5). The results show that ATPS can be formed by adding the appropriate amount of salt to aqueous solution of $[\text{bmim}][\text{BF}_4]$, while adding salt, such as $(\text{NH}_4)_2\text{SO}_4$, NaCl or KCl , can not drive $[\text{bmim}][\text{BF}_4]$ solution to separate into two phases. The distribution behaviors of drugs between the two phases were characterized by the extraction efficiency (E). The parameter E is defined as follows:

$$E = \frac{C_t V_t}{C_b V_b + C_t V_t}$$

C_b , C_t , V_b and V_t are the drug concentrations and volumes in the bottom and top phase, respectively. The extraction efficiency E is the ratio of herbs in the top $[\text{bmim}][\text{BF}_4]$ enriched phase and the total drugs added to the system. Fig. 5 shows the effects of $[\text{bmim}][\text{BF}_4]$ and NaH_2PO_4 concentration on the partition behavior of the extraction of Apocynum.

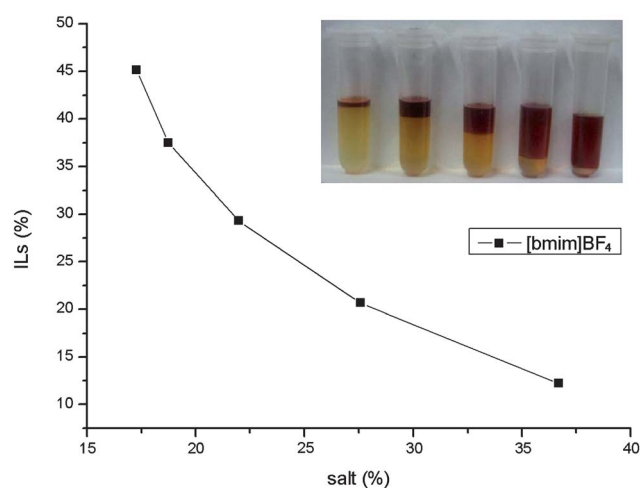


Fig. 5 Phase diagrams for the $[\text{bmim}][\text{BF}_4]$ /salt/water systems at 25 °C. In the region above the binodal curve, the system is separated into two phases, below the binodal curve, the system is a homogeneous phase.

Effect of [bmim][BF₄] concentration. The concentration of [bmim][BF₄] cannot be the significant factor affecting extraction yield, but it can significantly affect preconcentration efficiency. More [bmim][BF₄] could improve the yield of extraction, but it also implies inefficient preconcentration to the top phase. Conversely, less [bmim][BF₄] will lead to a higher concentration in the top phase. However, quite a small amount of IL caused the preconcentration to become difficult and insufficient, likely reducing accuracy and reproducibility. Therefore, the effect of [bmim][BF₄] concentration on the preconcentration concentrations in ILs phase was investigated from 0.2 to 0.6 g mL⁻¹. As shown in Fig. 6, we can see that the concentration of preconcentration decreased with increasing [bmim][BF₄] concentration. Hence, 0.2 g mL⁻¹ ILs aqueous solution was considered optimal. The average preconcentration efficiencies of hyperin and isoquercitrin in this range were 66.4% and 65.7%, respectively.

Method validation. To evaluate the proposed ILs-MAE-ATP approach, some parameters such as linearity, recovery, reproducibility, precision and stability were determined under the above optimized conditions.

Hyperin and isoquercitrin were evaluated by its corresponding chromatograms and retention time in comparison to that of the standard hyperin and isoquercitrin. The chromatograms of standard hyperin and isoquercitrin and the extracts of Apocynum are shown in Fig. 7 and ESI Fig. S4†. From Fig. 7, we can see that the retention time was 17.73 min and 19.38 min for hyperin and isoquercitrin from Apocynum, and [bmim][BF₄] aqueous solution did not show any interference for the analysis of hyperin and isoquercitrin.

A series of standard solutions of hyperin and isoquercitrin at six levels in the concentration were prepared and analyzed by HPLC. Each of them was performed in triplicate. The standard curves were obtained using the linear regression method. The results are shown in Table 6, which describes the good linearity of hyperin as a range from 0.001 to 0.06 mg mL⁻¹, and

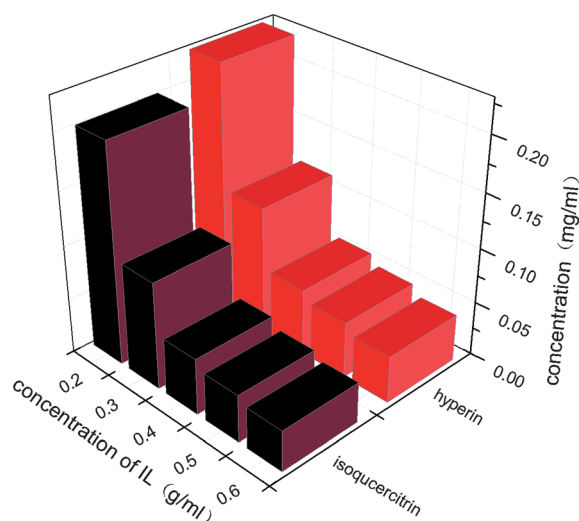


Fig. 6 The effect of [bmim][BF₄] concentration and the extracts concentration in the top phase.

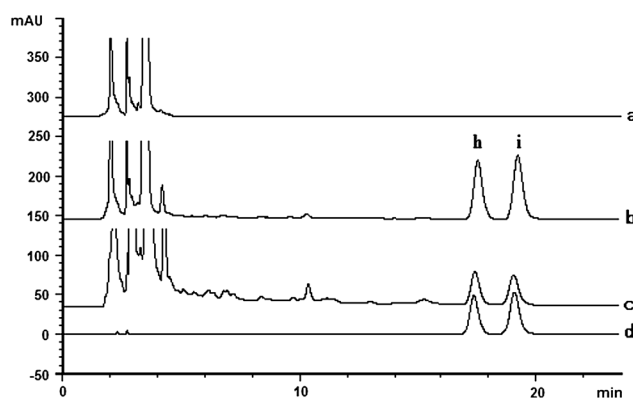


Fig. 7 The chromatograms of (a) [bmim][BF₄] aqueous solution; (b) mixed solution of the extraction of Apocynum and the standard solution of hyperin and isoquercitrin; (c) the extraction of Apocynum; (d) the standard solution of 0.07 mg mL⁻¹ hyperin and 0.06 mg mL⁻¹ isoquercitrin.

isoquercitrin ranged from 0.001 to 0.07 mg mL⁻¹. The linearity of hyperin and isoquercitrin monitored at 258 nm were $Y = 33\,529.925X - 0.6266$ ($r = 0.9997$) and $Y = 57\,371.481X - 24.0976$ ($r = 0.9999$), separately. The LODs were 3.82 $\mu\text{g L}^{-1}$ and 3.00 $\mu\text{g L}^{-1}$ which were evaluated on the basis of a signal-to-noise ratio of 3.

The recovery study was tested by the standard-addition method. Some amounts of hyperin and isoquercitrin standard were added in 0.2 g of Apocynum powders, and then they were extracted and preconcentrated. At each addition, three samples were prepared and each sample was analyzed three times. The results are shown in Table 7 and show that the mean recoveries for hyperin and isoquercitrin from Apocynum were 97.29% (RSD = 1.02%) and 99.40% (RSD = 1.13%), respectively.

The reproducibility study was carried out on three repeated extractions with optimum ILs-MAE method, which were determined with 3.21% and 2.95% of RSD respectively. The results show accredited reproducibility.

The precision of the chromatographic determination was evaluated by the analysis of 0.07 mg mL⁻¹ of hyperin and 0.06 mg mL⁻¹ of isoquercitrin standard solution for five replicated times by HPLC-UV detection. The obtained RSDs were 0.7532% and 0.5971%, respectively, which indicated good precision of this analysis method.

The stability study was carried out on hyperin and isoquercitrin in extract every 2 h for 5 times. RSDs obtained were 0.4971%, 0.9617%, respectively, which indicates that the proposed method was credible.

Sample analysis. Raw herbs of Apocynum were collected from various production sites in china, and commercial drugs were purchased in different drugstores. They were extracted by the optimized ILs-MAE conditions respectively and were determined by means of HPLC-UV detection. The quantitative results are summarized in Table 8. It can be seen that the extraction efficiency of hyperin and isoquercitrin in Apocynum from different places extracted by the optimum ILs-MAE method were different greatly, which is identical with that in Apocynum from different places and factories.

Table 6 The calibration equations, correlation coefficients (*r*), linear ranges and limits of detection (LOD) of the proposed method

Analyte	Samples ^a	Calibration equations ^b	<i>r</i>	Linear ranges (mg mL ⁻¹)	LOD (μg L ⁻¹)
Apocynum	Hyperin	$Y = 33\,529.925X - 0.6266$	0.9997	0.006–0.06	3.82
	Isoquercitrin	$Y = 57\,371.481X - 24.0976$	0.9999	0.007–0.07	3.00

^a Calibration equations of three herbs were respectively acquired under optimized condition. ^b *Y* was peak area, *X* was concentration of standard solution as mg mL⁻¹.

Mechanism of ILs-MAE

Study of kinetic mechanism. The extraction efficiency and chemical change of the target compounds were the factors influencing the ILs-MAE process. In order to understand the effect of microwave irradiation time on the extraction efficiency of hyperin and isoquercitrin from the herb, the ILs-MAE kinetic mechanism was investigated. The results are shown in ESI Fig. S5†, which indicates the extraction process as follows. The extraction yields of hyperin and isoquercitrin from the herb enhanced rapidly in the initial 10 min, and increased or unchanged slackly after 10 min for Apocynum. There are two possibilities that may lead to these results. One was that the microwave could extract hyperin and isoquercitrin of the herb completely after 10 min. The other possibility was that the sample structures may be altered by the loss of glucose or acetyl, which decreases the extraction yields. Using 0.4 g mL⁻¹ [bmim][BF₄] aqueous solution according to the time, 10 min of MAE time was sufficient to obtain high extraction yields of hyperin and isoquercitrin from Apocynum.

Structural changes after extraction

As shown in Fig. 8 and ESI Fig. S6†, the microstructure and chemical structure of samples before and after extraction procedures were investigated by SEM and FT-IR spectroscopy. In Fig. 8a, the disruption of ILs-MAE on the surface and cell wall structures was obviously better than in Fig. 8b–e. The microwave radiation can arrive in the internal vascular and glandular cells within the herb material by freely passing through the ‘penetrable’ solvents. The structure of the cell was affected by microwave radiation due to the sudden temperature rising and the internal pressure increasing. Then the target of the cells was accordingly shifted to the surrounding solvent to be dissolved. The mechanism of ILs-MAE is different from the ILs-UAE, which is produced by the mechanical vibration waves. Due to the ‘cavitation effects’, the herbs could be broken down into small pieces. From Fig. 8g and h, it is obvious that

the particle sizes have been reduced after ILs-MAE and ILs-UAE procedures, while the ILs-HE and ILs-ME relatively maintain the large particles, as shown in Fig. 8f and i. ILs-MAE and ILs-UAE were both based on an explosion at the cell level, so they had high values. In Fig. 8c, the surface of the herbs was slightly ruptured by ILs-HE, and the destroyed appearance was similar to the ILs-MAE technique. The solvent could permeate and dissolve under high temperatures. The yields of hyperin and isoquercitrin extracted by the ILs-HE technique cannot achieve those extracted by the ILs-MAE and ILs-UAE techniques. Comparing Fig. 8d with Fig. 8e, the fiber structure is swelled. For ILs-ME, the leaching of solute entirely depended on the solvent solubilization. Although the process of ILs-ME spent more solvent and longer extraction time, it leads to little destruction on the microstructure of the sample and the lowest efficiency.

As FT-IR spectra can provide useful information for identifying the presence of certain functional groups or chemical bonds in a molecule or an interaction system, it was applied here to investigate the changes in chemical structures of Apocynum before and after extraction by various methods. The IR spectroscopy used in this part has identified the presence of certain functional groups or chemical bonds in compounds and detected subtle differences in chemical structures. Fig. S6† shows the FT-IR spectra of Apocynum raw material and its extracts using four methods. It can be seen that the absorption peaks of 3420 cm⁻¹, allocated to OH group, and the absorption peaks in the fingerprint regions at 1432, 1164 and 1058 cm⁻¹ attributed to cellulose structure almost did not obviously changed after ILs-MAE, ILs-UAE, ILs-HE or ILs-ME with [bmim][BF₄] aqueous solution, which indicates that the chemical structures of carbohydrate compounds, including cellulose, hemicellulose and insoluble starch, were unbroken before and after extraction. Those results probably suggest that water segregated the strong interactions between ILs and carbohydrate compounds of matrixes.^{1,31,32} Therefore, the chemical bonding interactions between ILs and sample matrixes was not obvious. Additionally, after being extracted by different

Table 7 Recovery of hyperin and isoquercitrin from Apocynum (*n* = 3)

Samples	No.	Herb (g)	Content (mg)	Added (mg)	Found (mg)	Recovery (%)	Mean recovery (%)	RSD (%)
Hyperin	1	0.2002	2.1356	2.3	4.2697	96.26	97.29	1.02%
	2	0.2001	2.1297	2.7	4.7452	98.25		
	3	0.2001	2.1205	2.9	4.8227	97.36		
Isoquercitrin	1	0.2002	2.4702	2.6	4.9744	98.11	99.4	1.13%
	2	0.2001	2.4683	2.4	4.8747	100.13		
	3	0.2001	2.4693	2.7	5.1672	99.96		

Table 8 Extract value of some samples from different Origin and manufacturers ($n = 3$)

Origin or manufacturers	Type	Extraction yield (mean \pm SD, mg g ⁻¹)	
		Hyperin	Isoquercetin
Changsha, Hunan	Powder of the herb	2.4793 \pm 0.027	2.1299 \pm 0.021
Haozhou, Anhui	Powder of the herb	2.1139 \pm 0.021	1.7893 \pm 0.017
Cangzhou, Hebei	Powder of the herb	2.9536 \pm 0.033	2.4711 \pm 0.025
Xian, Shanxi	Powder of the herb	—	-3.3407 \pm 0.014
Linfen Jianmin Pharmaceutical Factory	Tablet	—	-1.0991 \pm 0.018
Jin Hui Xing Pharmaceutical Limited Company	Tablet	—	0.8996 \pm 0.010

techniques, the CH₂ asymmetric and symmetric vibrations occurred at 2918 and 2852 cm⁻¹, the C=O stretching vibration peak at 1735 cm⁻¹ and an aromatic C–H out-of-plane vibration in the lignin at 832 cm⁻¹. The information from IR spectra show that their signal intensities decreased. The absorption intensity of the residue of the Apocynum after processing by ILs-MAE appeared to be lower than those after processing by ILs-ME, ILs-HE and ILs-UAE. It reflects the advantage of using microwave technique.

Conclusion

In this work, aqueous two-phase ILs-MAE were successfully used in the procedure of the extraction and the preconcentration of hyperin and isoquercitrin from Apocynum instead of traditional volatile organic solvents. The optimal conditions of this approach were successively identified by single-factor and orthogonal experiments. With the adding of the salts, [bmim][BF₄] was subsequently converted to the above phase to achieve phase separation. The yield of hyperin and isoquercitrin in the

top phase were improved greatly. The optimal ILs-MAE-ATP approach could provide higher detection limit of hyperin and isoquercitrin which are 3.82 μ g L⁻¹ and 3.00 μ g L⁻¹ in Apocynum with RSD 1.33% within the highest preconcentration efficiency of 66.4% and 65.7%. The structure of ILs, especially the anions have a significant impact on the extraction efficiencies of the target. The enhanced extraction yields by ILs-MAE were primarily based on the damage of sample microstructures in the course of ILs-MAE. The kinetic mechanism curves of ILs-MAE method were probably related to the components and the interactions among the components of herb medicines, and longer time in MAE was not good for the extraction of hyperin and isoquercitrin. Due to its good reproducibility and precision, the proposed green and effective aqueous two-phase ILs-MAE method is a promising prospect in the extraction and the preconcentration of natural products. Thus ILs-based microwave-assisted extraction coupled with aqueous two-phase (ILs-MAE-ATPS) was successfully developed as a new pretreatment strategy for the analysis of hyperin and isoquercitrin in Apocynum.

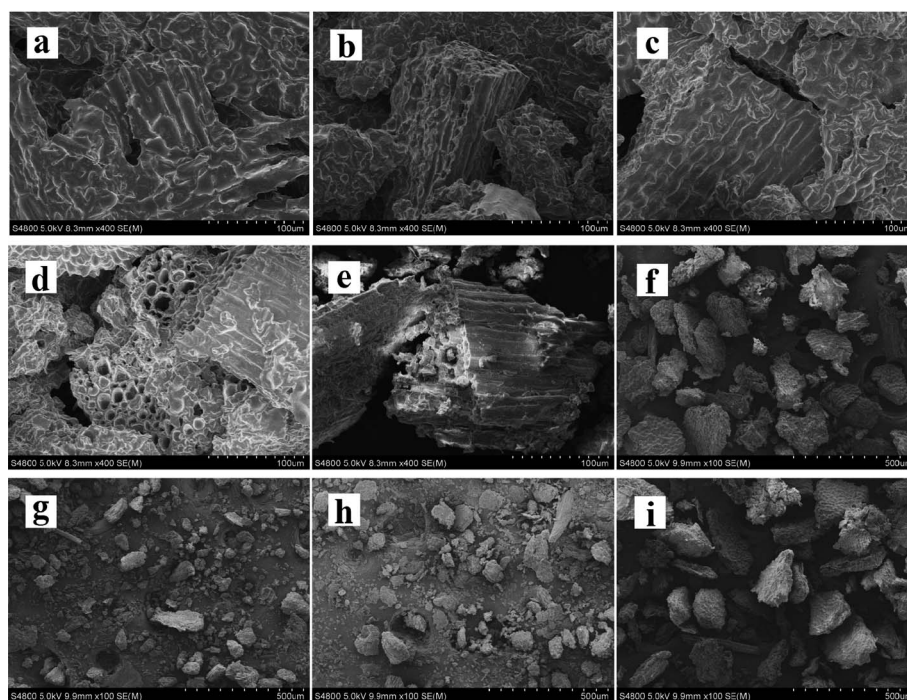


Fig. 8 Scanning electron microscope pictures of Apocynum (100 μ m, 5.0 kV) after ILs-MAE (a), ILs-UAE (b), ILs-HE (c), ILs-ME (d) and before (e); Scanning electron microscope pictures of Apocynum (500 μ m, 5.0 kV) after ILs-HE (f), ILs-MAE (g), ILs-UAE (h), ILs-ME (i).

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