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Short communication

Determination of the active constituents in *Arnebia euchroma* (Royle) Johnst. by ionic liquid-based ultrasonic-assisted extraction high-performance liquid chromatography

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ABSTRACT

Shikonin and β,β′-dimethylacrylshikonin in *Arnebia euchroma* (Royle) Johnst. were extracted by ionic liquid-based ultrasonic-assisted extraction (IL-based UAE) and determined by high-performance liquid chromatography (HPLC). The dried powder of *A. euchroma* (Royle) Johnst. was mixed with a room temperature ionic liquid [C_6MIM][BF_4] to form a suspension, and then the ultrasonic extraction was performed in a water bath at ambient temperature. The calibration curve showed good linear relationship ($r > 0.9998$) in the concentration range of 1.75–140 μg/mL for shikonin and 2.15–1360 μg/mL for β,β′-dimethylacrylshikonin. The recoveries were between 69.79% and 82.35%. The IL-based UAE is free of volatile organic solvents, and consumes less sample, time and solvent, compared with regular ultrasonic and Soxhlet extraction. There was no obvious difference in the extraction yields of active constitutions obtained by the three extraction methods.

1. Introduction

Arnebia euchroma (Royle) Johnst., a boraginaceous medicinal plant, is beneficial to the wound healing, antiinflammation, antibacteria, antitumour, antidiabetes and antivirus [1]. It is an important traditional Chinese medicinal herb. The major pharmacological constituents in *Arnebia euchroma* (Royle) Johnst. are naphthoquinone pigments, among which shikonin and β,β′-dimethylacrylshikonin are chosen as "marker compounds" in the process of chemical evaluation or standardization of *A. euchroma* (Royle) Johnst. and its products [2].

The extraction of active constituents from medicinal plants was traditionally performed by solvent extraction or maceration extraction. Unfortunately, these methods are usually time consuming, laborious, and a large amount of hazardous and volatile organic solvents are required, although the methods are often effective. As the analytical technique has rapidly developed, there has been a trend towards less (organic) solvent consumption, short extraction time and miniaturization in the analytical extraction.

Compared with traditional and other modern extraction techniques, ultrasonic-assisted extraction (UAE) is proposed as an alternative method for sample pretreatment. Ultrasound can break plant tissue and accelerate the solvent penetrating through plant tissue. The benefit of using UAE in the direct extraction of organic compounds from solid matrices has already been demonstrated in recent years [3–7].

Room-temperature ionic liquids (RTILs) are melting salts of a kind which consist of organic cations and organic or inorganic anions. The RTILs emerge as possible "green" solvents [8,9] and have a wide utilization in synthesis [10], catalysis [11,12], separation [13] and electrochemistry [14] for their unique properties such as negligible vapor pressures, chemical and thermal stability, good solubility for both organic and inorganic molecules, and miscibility with water and organic solvents. In recent years, the RTILs have attracted increasing interest and are used more and more as attractive alternatives to environmentally unfriendly solvents in sample preparation [15–20].

In this work, based on the advantages of ionic liquid and ultrasound, IL-based ultrasonic-assisted extraction (UAE) was developed for the first-time on extract of active constituents from *A. euchroma* (Royle) Johnst. For the comparison, the UAE and Soxhlet extraction (SE) were also applied. The UAE is a standard method recommended in Chinese Pharmacopoeia [2]. The SE is widely accepted and applied for a long time. To evaluate the new extraction method, the SE is often adopted as a reference method.

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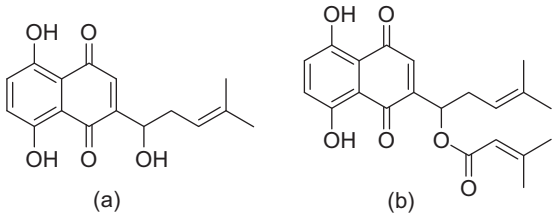

Fig. 1. Chemical structures of shikonin (a) and β,β′-dimethylacrylshikonin (b).

2. Experimental

2.1. Chemicals and materials

The shikonin (purity ≥95%) and β,β′-dimethylacrylshikonin (purity ≥98%) were obtained from Chinese Drug Biological Product Qualifying Institute (Beijing, China). The chemical structures of the compounds are shown in Fig. 1. Chromatographic grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, Pennsylvania, USA). 1-Alkyl-3-methylimidazolium ionic liquids, including [C_6MIM][BF_4], [C_8MIM][BF_4], [C_4MIM][BF_4], [C_2MIM][BF_4], [C_6MIM][PF_6] and [C_8MIM][PF_6] were purchased from Cheng-jie Chemical Co., ltd (Shanghai, China). Water was purified with a distillator (Rong-hua Co., Ltd, Jiangsu, China) and filtered through a 0.45 μm membrane.

2.2. Apparatus

A 1100 series liquid chromatograph (Agilent Technologies Inc., USA) equipped with photodiode-array detector (DAD) was employed. The chromatographic separation of the analytes was carried out on a Zorbax Eclipse XDB-C8 column (5 μm, 4.6 mm × 150 mm, Agilent, USA). KQ-100DE ultrasonic generator (Kunshan, Jiangsu, China) and SH-36 mixer (Zhenghui, Shanghai, China) were used in the extraction step. RE-52AA vacuum rotatory evaporator (Yarong, Shanghai, China) was employed.

2.3. Sample preparation

Three kinds of *A. euchroma* (Royle) Johnst. samples (named as samples 1–3) cultivated in different areas were bought from local drugstores (Changchun, China). In the study, all experiments were performed on sample 1 except for the experiments mentioned in Section 3.3.3. The samples were cleaned with water, and dried thoroughly in the cabinet drier at 50 °C for 24 h. The samples then were triturated by a pulverizer, passed through a 110 mesh stainless steel sieve and stored in a desiccator.

2.4. Preparation of standard solutions

For each analyte, the standard stock solution was prepared by dissolving the analyte in acetonitrile, and stored at 4 °C. Working solutions were prepared by diluting the stock solutions with acetonitrile.

2.5. Preparation of spiked samples

The spiked samples were prepared by spiking the standard stock solutions into sample powders. To ensure the standard solution to be well distributed, a reasonable amount of methanol was added to moisten the sample powder and careful agitation was performed followed by an air-drying for 24 h at ambient temperature before sample analysis.

2.6. Chromatographic conditions

The flow rate of the mobile phase was maintained at 1 mL/min. The injection volume of the sample solution was 5 μL. The temperature of the column was controlled at 30 °C. The mobile phase consisted of water (A) and acetonitrile (B). Gradient program was as follows: 0–4 min, 35% B; 4–5 min, 35–22% B; 5–12 min, 22% B. The absorbance was measured at a wavelength of 516 nm.

2.7. Determination of extraction yield and recovery

$$\text{Extraction yield}(\mu g/mg) = \frac{\text{mass of analyte in sample powder}(\mu g)}{\text{mass of the sample powder}(mg)} = \frac{C(\mu g/mL) \times V(mL)}{\text{mass of the sample powder}(mg)}$$

where C was the concentrations of the analytes in the sample solution; V was the extraction solvent volume for IL-based UAE and the sample solution volume for SE and UAE.

$$\text{recovery} = \frac{\text{found yield}(\mu g/mg) - \text{original yield}(\mu g/mg)}{\text{spike concentration}(\mu g/mg)} \times 100\%$$

2.8. IL-based UAE

2.8.1. Selection of ILs

150 μL of [C_2MIM][BF_4], [C_4MIM][BF_4], [C_6MIM][BF_4], [C_8MIM][BF_4], [C_6MIM][PF_6] and [C_8MIM][PF_6] were placed in 2 mL centrifuge tube, respectively. 0.050 g of 110 mesh sample 1 powder was added into each tube. The ionic liquid and the sample powder were mixed by a rapid mixer. Then the tubes containing the homogeneous mixture were placed in the water bath of ultrasonic generator whose power was 100 W. The extraction was performed for 5 min at 20 °C. The suspensions were filtered through 0.45 μm membrane filter and the resulting solutions were referred to as the sample solutions. The experiment was performed in triplicate.

2.8.2. Extraction solvent volume

150, 200, 300, 400, 500 and 600 μL of [C_6MIM][BF_4] were placed in 2 mL centrifuge tube, respectively. 0.050 g of 110 mesh sample 1 powder was added into each tube. The following procedure was the same as that mentioned in Section 2.8.1.

2.8.3. Sample amount

0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 g of 110 mesh sample 1 powders were placed in 2 mL centrifuge tube, respectively. 150 μL of [C_6MIM][BF_4] was added in each tube, respectively. The following procedure was the same as that mentioned in Section 2.8.1.

2.8.4. Size of sample particle

0.05 g of 20, 60, 80 and 110 mesh sample 1 powders were placed in 2 mL centrifuge tube, respectively. 150 μL of [C_6MIM][BF_4] was added in each tube, respectively. The following procedure was the same as that mentioned in Section 2.8.1.

2.8.5. Extraction time

0.050 g of 110 mesh sample 1 powder and 150 μL of [C_6MIM][BF_4] were placed in a 2 mL centrifuge tube and mixed by a rapid mixer. The tube containing the homogeneous mixture was placed in the ultrasonic water bath whose power was 100 w. The extraction was performed at 20 °C for 2, 5, 10, 20, 30 and 40 min, respectively. The following procedure was the same as that mentioned in Section 2.8.1.

2.9. SE

1.0 g of sample powder was placed in a thimble-holder of the Soxhlet extractor, and 100 mL of petroleum ether was added into the distilling flask of Soxhlet extractor. The extraction was carried out for 5 h. Then the extraction solvent was evaporated to dryness under reduced pressure at 40 °C. The residue was dissolved in 10 mL of acetonitrile. After filtration with a 0.45 μm membrane filter, the resulting solution was referred to as the sample solution.

2.10. UAE

According to Chinese Pharmacopoeia [2], 0.5 g of sample powder was put into an Erlenmeyer flask equipped with a stopper, in which 25 mL of petroleum ether was added accurately. The flask was weighed afterwards. Then ultrasonic extraction was performed for 30 min. After cooling, the flask was weighed again, and the loss weight was made up with petroleum ether. After shaken up, the resulting solution was filtered with filter paper. 10 mL of the filtered solution was evaporated to dryness under reduced pressure at 40 °C. The residue was dissolved in 10 mL of acetonitrile. After filtration with a 0.45 μm membrane filter, the resulting solution was referred to as the sample solution.

3. Results and discussion

3.1. Selection of ILs

The structures of ILs have significant influence on its physicochemical properties, which might greatly affect the extraction efficiency of the target analytes [21]. In order to evaluate the performance of the ILs, six kinds of ILs, including [C_2MIM][BF_4], [C_4MIM][BF_4], [C_6MIM][BF_4], [C_8MIM][BF_4], [C_6MIM][PF_6] and [C_8MIM][PF_6] were used as the extraction solvent to treat the sample 1. The experimental results are shown in Table 1. When the anion was BF_4^-, the extraction yield of the β,β′-dimethylacrylshikonin dramatically increased with the increase of alkyl chain length from ethyl to octyl. When the anion was PF_6^-, the decrease of extraction yield of the analyte might be due to high viscosity of the ILs. The effect of IL type on the extraction yield of shikonin was slight. Because the high viscosity of [C_8MIM][BF_4] made the handling difficult, [C_8MIM][BF_4] was not chosen and [C_6MIM][BF_4] was used in the following experiments.

3.2. Optimization of IL-based UAE

The effects of experimental parameters, such as amount of the ionic liquid, sample amount, size of the sample particle and extraction time on the extraction yields of the analytes in the sample 1 were investigated. The extraction yields decrease when the volume of [C_6MIM][BF_4] increases from 150 μL to 600 μL as shown in Table 1. Additionally, the volume lower than 150 μL made collection difficult. Therefore, 150 μL of [C_6MIM][BF_4] was used in this study. The effect of sample amount on extraction yields of target analytes was investigated from 10 mg to 60 mg. As shown in Table 1, the extraction yields increase when the sample amount increases from 10 mg to 50 mg, and when the sample amount is 60 mg, the extraction yield decreases. So 50 mg of sample powder was used in the work. The effect of size of the sample particle also obviously influenced the extraction yields. The sizes of sample particles from 20 mesh to 110 mesh was investigated. Results shown in Table 1 indicate that the smaller the sample particles, the higher the extraction yields obtained whereas, sample particles smaller than 110 mesh made filtration difficult. Hence, sample powder which passed through a 110 mesh sieve was used in this work. The effect of extraction time was examined from 2 min to 40 min. As shown in

Table 1
Effect of experimental parameters on the yield of analytes.[a]

Analyte	IL	Yield (μg/mg)	V (μL)	Yield (μg/mg)	m (mg)	Yield (μg/mg)	d (mesh)	Yield (μg/mg)	t (min)	Yield (μg/mg)
Shikonin	[C_2MIM][BF_4]	0.26	150	0.35	10	0.26	20	0.12	2	0.29
	[C_4MIM][BF_4]	0.26	200	0.31	20	0.27	60	0.19	5	0.35
	[C_6MIM][BF_4]	0.35	300	0.29	30	0.29	80	0.29	10	0.35
	[C_8MIM][BF_4]	0.29	400	0.28	40	0.31	110	0.35	20	0.32
	[C_6MIM][PF_6]	0.23	500	0.26	50	0.35			30	0.31
	[C_8MIM][PF_6]	0.22	600	0.25	60	0.33			40	0.30
β,β′-dimethylacrylshikonin	[C_2MIM][BF_4]	0.97	150	2.21	10	1.88	20	0.87	2	2.15
	[C_4MIM][BF_4]	2.11	200	2.13	20	2.00	60	1.24	5	2.21
	[C_6MIM][BF_4]	2.21	300	2.06	30	2.09	80	1.81	10	2.21
	[C_8MIM][BF_4]	2.58	400	1.88	40	2.10	110	2.21	20	2.20
	[C_6MIM][PF_6]	2.28	500	1.78	50	2.21			30	2.18
	[C_8MIM][PF_6]	1.83	600	1.74	60	2.10			40	2.16

[a] IL, Type of ILs; V, Extraction solvent volume; m, Sample amount; d, Size of sample particle; t, Extraction time.

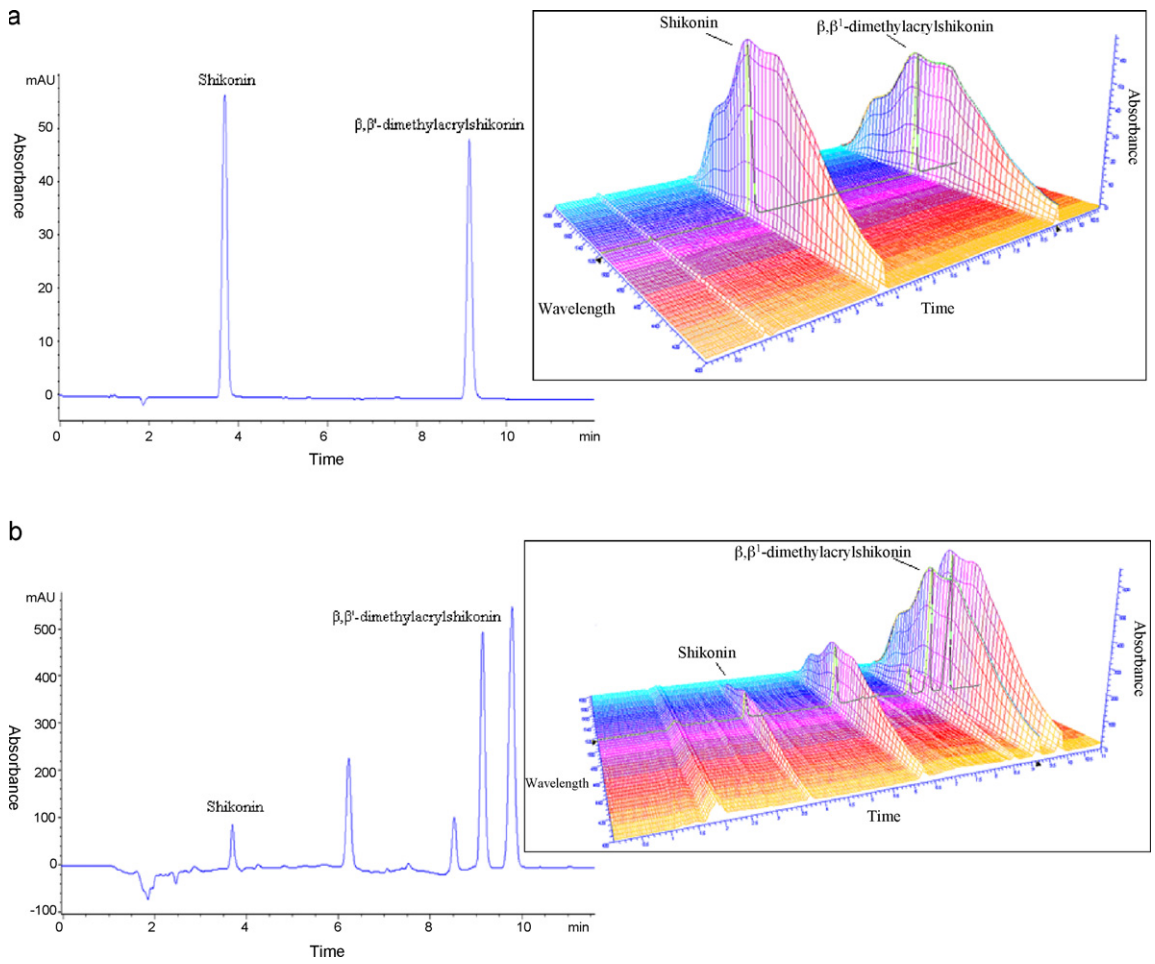

Fig. 2. Chromatograms of the standard solution (a) and the extract of sample 1 (b). Inset shows absorption spectra of the eluents. The concentrations of shikonin and β,β′-dimethylacrylshikonin in standard solution are 83.4 μg/mL and 82.1 μg/mL,and the extraction yields for the two analytes in the extract of sample 1 are 0.35 μg/mg and 2.23 μg/mg, respectively.

Table 1, the extraction yields are highest when the extraction time is 5 min. As a result, 5 min was chosen in this work.

3.3. Method validation

The target compounds were identified by comparing their retention times and absorption spectra with those of the authentic standard analytes. The spectral data for each chromatographic peak are helpful in the identification of species. Chromatograms with spectra as inserts are shown in Fig. 2. It can be seen from Fig. 2 that the retention time was 3.69 min for shikonin and 9.14 min for β,β′-dimethylacrylshikonin.

3.3.1. Linearity

The calibration curves were constructed by plotting the peak areas versus the concentration of the analytes. The working solutions for shikonin at five concentration levels (1.75, 8.75, 35, 70 and 140 μg/mL) and β,β′-dimethylacrylshikonin at five concentration levels (2.15, 42.5, 170, 680 and 1360 μg/mL) were used to prepare the calibration curves. The relationships between the analyte concentrations (C) and measured peak areas (A) were expressed as regression equations: $A = 5.0279C - 7.0232$ for shikonin and $A = 4.9092C - 9.606$ for β,β′-dimethylacrylshikonin. Good linearities were obtained in a range of 1.75–140 μg/mL with the correlation coefficient of 0.9998 for shikonin and 2.15–1360 μg/mL with the correlation coefficient of 0.9999 for β,β′-dimethylacrylshikonin.

3.3.2. Limits of detection and quantification

The limits of detection (LODs) were the concentrations of analytes that yielded a signal-to-noise ratio of 3. The LODs for shikonin and β,β′-dimethylacrylshikonin were 0.15 μg/mL and 0.20 μg/mL,

Table 2
Extraction yields and precision for the analytes.

Sample	Analyte	Extraction yield (μg/mg)	Intra-day RSD (%, $n = 3$)	Inter-day RSD (%,$n = 3$)
1	Shikonin	0.35	2.29	2.90
	β,β′-dimethylacrylshikonin	2.21	0.61	0.74
2	Shikonin	0.21	3.34	3.02
	β,β′-dimethylacrylshikonin	1.71	0.60	0.73
3	Shikonin	0.28	4.35	4.95
	β,β′-dimethylacrylshikonin	2.53	0.97	1.34

Table 3
Effect of IL volume on recoveries of the analytes.[a]

Analyte	Original (μg/mg)	Added (μg/mg)	Found (μg/mg)			Recovery (%)		
			150 (μL)	200 (μL)	300 (μL)	150 (μL)	200 (μL)	300 (μL)
Shikonin	0.35	0.17	0.49 (2.45)	0.49 (3.79)	0.48 (4.97)	82.35	82.35	76.47
Shikonin	0.35	0.35	0.62 (2.03)	0.63 (4.58)	0.62 (3.82)	77.14	80.00	77.14
β,β′-Dimethylacrylshikonin	2.21	1.17	3.08 (1.02)	3.11 (3.64)	3.10 (2.18)	74.36	76.92	76.06
β,β′-Dimethylacrylshikonin	2.21	2.35	3.85 (1.69)	3.98 (3.78)	3.97 (3.94)	69.79	75.32	74.90

[a] The values in the brackets are RSDs (%, $n = 3$).

Table 4
Comparison of IL-based UAE, UAE and SE.

	IL-based UAE	UAE	SE
Extraction yield of shikonin (mean±SD[a], μg/mg)	0.35 ± 0.01	0.32 ± 0.01	0.33 ± 0
Extraction yield of β,β′-dimethylacrylshikonin (mean ± SD[a], μg/mg)	2.21 ± 0.01	2.41 ± 0.01	2.26 ± 0.01
Sample amount (mg)	50	500	1000
Solvent type	$[C_6MIM][BF_4]$	Petroleum ether	Petroleum ether
Volume of solvent (mL)	0.15	25.00	100.00
Time (min)	5	30	300

[a] Standard deviation ($n = 3$).

respectively. The limits of quantitation (LOQs) were the concentrations of analytes that yielded a signal-to-noise ratio of 10. The LOQs for shikonin and β,β′-dimethylacrylshikonin were 0.50 μg/mL and 0.68 μg/mL, respectively.

3.3.3. Applicability and precision

To evaluate the applicability and precision of the proposed method three samples (samples 1–3) obtained from different cultivated areas were analyzed. The results (Table 2) indicate that the contents of shikonin and β,β′-dimethylacrylshikonin are in the range of 0.20–0.35 μg/mg and 1.63–2.53 μg/mg, respectively. The differences in target compound contents in these samples were due to the difference in cultivated area, growth conditions and picking period.

The precision of the proposed method was presented by relative standard deviation (RSD). The intra-day precision was obtained by analyzing the samples three times in one day and the inter-day precision was obtained by analyzing the samples once each day over three consecutive days. The intra-day and inter-day RSDs for shikonin and β,β′-dimethylacrylshikonin are between 0.6% and 4.35% and between 0.73% and 4.95%, respectively. Therefore, the reproducibility of the proposed method was acceptable.

3.3.4. Accuracy

To evaluate the accuracy of the proposed method, spiked samples were analyzed (Table 3) and the analytical results obtained by different methods were compared (Table 4). The recoveries were from 69.79% to 82.35%. It can be seen that the recoveries are related to the kinds of the analytes. Because $[C_6MIM][BF_4]$ has different capabilities to extract shikonin and β,β′-dimethylacrylshikonin, the recoveries of shikonin are higher than those of β,β′-dimethylacrylshikonin. The effect of extraction solvent volume on the recoveries was investigated. Results shown in Table 3 indicate that the effect of the solvent volume on the recoveries is not obvious.

3.4. Comparison of IL-based UAE, SE and UAE

In order to evaluate the performances of IL-based UAE, UAE and SE were also applied. The results are shown in Table 4. From those results, it can be seen that there is no significant difference in extraction yields obtained by the three methods. The results indicate that the accuracy of the proposed method is satisfactory. Compared with the conventional UAE and SE, the green solvent $[C_6MIM][BF_4]$ was used and only a small volume of the solvent (0.15 mL) was used in the proposed method. The extraction time of the proposed method (5 min) was much shorter than those of UAE (30 min) and SE (300 min). Considering the expenditures of sample amount, extraction time and extraction solvent, IL-based UAE should be a comparatively satisfactory method.

4. Conclusion

A green and effective method IL-based UAE has been developed for simultaneous extraction of shikonin and β,β′-dimethylacrylshikonin in *A. euchroma* (Royle) Johnst. The calibration curve showed good linear relationship ($r > 0.9998$) in the concentration range of 1.75–140 μg/mL for shikonin and 2.15–1360 μg/mL for β,β′-dimethylacrylshikonin. The RSDs were lower than 5%. The recoveries ranged from 69.79 to 82.35%. Compared with UAE and SE, the proposed method consumes less sample, time and solvent. The IL-based UAE shows a promising prospect in the extraction of the active constituents in the natural products.

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