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Extraction of Shikonin with Surfactant-Assisted Ultrasonic from *Arnebia euchroma*

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An efficient surfactant assisted ultrasonic extraction technology was employed for extracting shikonin from *Arnebia euchroma* and the extraction process was optimized. Based on single-factor experiments, a four-factor-three-levels experimental design has been developed. The optimum conditions were carried out at 35 °C for 35 min at a solid-to-liquid ratio of 1:12 upon the addition of 0.0004 g L^{-1} surfactant. Shikonin contents could be determined readily by high-performance liquid chromatography within 20 min. The mean yield of shikonin was 30.64 mg L^{-1}. Moreover, the contact angle of the powder was 30.501° in methanol. The proposed method was reliable by repeatability, stability and recovery tests. The results indicated that addition of surfactant could significantly increase the extraction yield of shikonin.

Keywords: *Arnebia euchroma*, Shikonin, Ultrasonic, Contact angle, Surfactant.

INTRODUCTION

Arnebia euchroma is a well-known medicinal plant that distributes widely in Xinjiang (Uygur Autonomous Region, China). Pharmacological research showed that *Arnebia euchroma* can enhance inflammatory, antibacterial[1], antiviral and particularly antitumor activity. Moreover, it can also promote wound healing[2]. It has also been used as a complementary and alternative medicine for thousands of years. Shikonin is the major active constituent among *Arnebia euchroma*.

In previous research, the process of extracting of shikonin from *Arnebia euchroma* has been studying, such as supercritical fluid extraction[3], microwave assisted extraction[4] and ultrasound assisted extract[5], *etc*. Generally speaking, for the extraction of shikonin, these techniques are both advantages and disadvantages. Therefore, an appropriate extraction method is a key consideration.

In recent years, the development and application of ultrasonic or surfactant in extraction have become more and more widespread. Fu *et al.*[6] reported the method of surfactant assisted extraction. In this method, wettability play an important role in enhancing shikonin extraction yield because extraction was affected by flow behaviour of solvent. Dynamic contact angle measurement gives not only practical information on wetting but also valid indications for chemical composition[7]. The determination of contact angle is the most widely accepted method for determining the average wettability of a specific surface.

In this study, a new extraction procedure was developed to extract shikonin from *Arnebia euchroma* and the extraction technique was optimized. Most important of all, the surfactant assisted ultrasonic extraction technique was unitted into one process to enhance the extraction yield of shikonin. Moreover, the application of contact angle on the extraction of shikonin from *Arnebia euchroma* has not been reported.

EXPERIMENTAL

Arnebia euchroma (Royle) Johnst was from a commercial drug store (Rizhao, Shandong province, China) and stored in a darkened environment. The extract was prepared by surfactant-asisted ultrasonic extraction.

The shikonin standard was provided by Pharmacy Experimental Center of Jining Medical College. All surfactants were from the Experimental Center of Pharmacy (Jining Medical College, Shandong, China). Phosphoric acid used for the assay was of analytical grade while methanol was of HPLC grade.

The HPLC system comprised a LC- 6AD isocratic pump, automated isocratic controller and SPD-20A ultraviolet detector (523 nm) (Shimadzu, Kyoto, Japan). The analytical column was a reversed-phase Shim-pack CLC-ODS-18 (10 μm; 25 mm × 46 mm; 1.0 mL min^{-1}; Shimadzu).

A surface tension meter (DCAT21, Hamburg, Germany) was used to measure the contact angles. Ultrasonic cleaning systems (KQ-3000, Shanghai, China) and a SHZ-II rotary

evaporator (RE-5298, Shanghai, China) were used for the treatment of crude extract.

Calibration curve for shikonin: Working standard solutions of shikonin of 0, 0.012, 0.024, 0.036, 0.048 and 0.06 mg mL^{-1} were prepared by serially diluting the 0.06 mg mL^{-1} stock solution of the authentic reference compound with methanol. Five replicate injections of each standard solution were made on the HPLC and the mean values of peak area of shikonin were calculated.

Determination of contact angle: Five portions of accurately weighed *Arnebia euchroma* sample powder were introduced into a 100 mL iodine flask with 50 mL pure water and different types surfactants (Tween-20, Tween-40, Tween-60, Tween-80 and sodium dodecyl sulfate) assisted ultrasonic extracts. The extraction yields were calculated by compraring extracts to the original sample powder.

The most popular method for measuring contact angle is Wilhelmy plate method which a capillary of 2 cm inner diameter was used to stuff *Arnebia euchroma* powder. Before determination of contact angle, we should select a kind of solvent which can completely moistened solid sample powder in order to confirm the coefficient of capillary. On the basis of the instructions of apparatus, hexane was employed to measure constant number because its surface tension coefficient and viscosity are all known.

The contact angle can be evaluated from the following relationship: $h^2 = \frac{k\gamma_{LA}\cos\theta}{2\eta}t$, where h is the height of liquid rise, t is the determination time, η is the viscosity of solvent, k is the constant, γ_{LA} is the surface tension coefficient, θ is the contact angle of between solvent and sample powder.

Different concentrations of methanol, ethanol and chloroform were used to investigate the contact angle between the solvent and sample powder respectively. Equal quantity of sample powder was placed into test tube. And then we measured surface tensions. The relevant results were substituted into equation to calculate the contact angle. As is known to all, the smaller the contact angle is, the greater the dissolving capacity becomes.

Surfactant-assisted ultrasonic extraction (SAUE) and determination of shikonin content: Four factors (ultrasonication temperature, ultrasonication time, solid-to-liquid ratio, addition of surfactant) and three levels were investigated (Table-1). The extraction design of shikonin was finished by SAUE method. Briefly, the dried powder of the sample (5 g) was placed into a 100 mL iodine flask with 50 mL organic solvent and extracted twice. The extract was concentrated to dryness in a vacuum. The residue was dissolved in solvent, transferred to a 10 mL volumetric flask and diluted to 10 mL. All solutions were filtered with a 0.45 μm nylon membrane filter and injected into the HPLC system.

TABLE-1
FACTOR LEVELS OF ORTHOGONAL EXPERIMENTS

Level	Addition of surfactant (g)	Time (min)	Liquid/solid (mL/g)	Temperature (°C)
	A	B	C	D
1	0.002	15	8	25
2	0.003	25	10	35
3	0.004	35	12	45

RESULTS AND DISCUSSION

The determination method was carried out using a Shimpack CLC-ODS-C18 column with an isocratic solvent system comprising methanol and 0.025 mol L^{-1} aqueous solution of phosphoric acid (80:20, v/v). The surfactant-assisted ultrasonic extraction described here offers a new extraction method for shikonin along with a rapid and satisfactory separation of shikonin (Fig. 1).

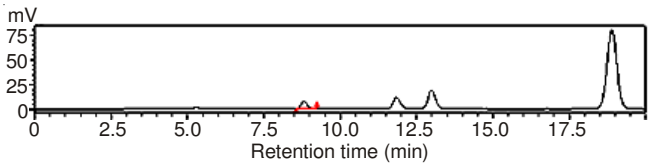

Fig. 1. Chromatogram of *Arnebia euchroma* extract (peak at 8.814 min = shikonin) and the other peaks are unknown compounds. Chromatographic conditions and the column used are described in the manuscript

A linear relationship was established a series of standard solutions of shikonin ranging from 0.012 to 0.06 mg mL^{-1}. The equation for calibration curves of shikonin was: Y= 2.0 E + 07X – 4839.0 where Y represents the peak area and X represents the concentration of analyte injected. This resulted in a correlation coefficient (r) of 0.9998 for shikonin. Shikonin contents were calculated by a comparison of the standard calibration curve of the extracts and the retention time of the authentic standard compound.

The precision and accuracy of the method were assessed by repeatedly injecting of shikonin standard solution three times at different concentration levels (0.012, 0.036 and 0.06 mg mL^{-1}) respectively. Inter-day (over 5 days) and intra-day precision and accuracy were then calculated. The mean determination per day and per standard was used to evaluate precision and accuracy. When the quantity of shikonin injected ranged from 0.24 to 1.2 μg, the relative standard deviation (RSD) for the intra-day calibration was 1.3-3.9 % and the recovery was 97.08-100.69 %. The RSD for inter-day calibration was 1.2-3.8 % and the recovery was 98.75-101.14 %, indicating a high degree of precision and accuracy for the method (Table-2).

The contact angles between solvents and samples were obtained by analyses of yields. From the Table-3 we can see that sodium dodecyl sulfate (SDS) was found to maximize the yield of shikonin. However, sodium dodecyl sulfate remained undissolved during the entire study, so the optimum surfactant was Tween-20 instead of sodium dodecyl sulfate.

It takes for 15 min or so to determine surface tension and its constant is 0.0000011. The contact angle of the powder was 30.501° in methanol, smaller than any other contact angle of solvent. Therefore, we regarded methanol as the optimum extraction solvent. The optimum extraction method was carried out at 35 °C for 35 min at a solid-to-liquid ratio of 1:12 upon the addition of 0.0004 g L^{-1} surfactant (Table-4). The retention time of shikonin was 8.814 min.

The mean content of shikonin was 30.64 mg L^{-1} by the optimum extraction method. The repeatability of HPLC was 1.12 %, thereby indicating a high degree of reproducibility. To verify that a surfactant can increase the yield of shikonin for a certainty, a comparative study was conducted using three

TABLE-2
WITHIN-DAY AND FIVE-DAY PRECISION AND ACCURACY OF THE ASSAY FOR THE DETERMINATION OF SHIKONIN IN STANDARD SOLUTIONS

Injected quantity (μg)	Mean measured quantity ± SD (μg)		Relative standard deviation (%)		Recovery (%)	
	Within-day	Between day	Within-day	Between day	Within-day	Between day
0.24	0.233±0.009	0.237±0.009	3.9	3.8	97.08	98.75
0.72	0.725±0.02	0.7282±0.02	2.8	2.7	100.69	101.14
1.20	1.194±0.015	1.21±0.014	1.3	1.2	99.5	100.83

SD = Standard deviation; Means of values were calculated from the regression equation of shikonin standard for five determinations.

TABLE-3
EFFECTS ON THE EXTRACTION YIELDS FOR DIFFERENT SURFACTANTS

Types of surfactant	Critical micelle concentration (g/L)	Extraction yield (%)
Tween-20	6.0×10^{-2}	0.99
Tween-40	2.3×10^{-2}	0.91
Tween-60	2.8×10^{-2}	0.90
Tween-80	1.4×10^{-2}	0.88
Sodium dodecyl sulfate	8.6×10^{-3}	1.205

TABLE-4
RESULTS OF ORTHOGONAL EXPERIMENTS

No.	Factor				Yields (mg/L)
	A	B	C	D	
1	1	1	1	1	20.47
2	1	2	2	2	19.70
3	1	3	3	3	21.44
4	2	1	2	3	20.65
5	2	2	3	1	25.61
6	2	3	1	2	29.29
7	3	1	3	2	28.79
8	3	2	1	3	25.32
9	3	3	2	1	23.71
K1	20.50	23.30	25.00	23.30	
K2	25.20	23.50	21.40	25.90	
K3	25.90	24.80	25.30	22.50	
R	5.40	1.50	3.90	3.40	

aliquots of the sample powder: 5.003, 5.006 and 5.009 g. They were all extracted using only ultrasonication assisted solvent instead of surfactant-assisted ultrasonic extraction. A diluted solution of the extract was injected into the HPLC system and the mean content (19.37 mg L^{-1}) was clearly inferior to that of the control group (30.64 mg L^{-1}).

Conclusion

Under the critical micelle concentration condition, surfactant could improve the extract yield of shikonin remarkably, particularly surfactant assisted extraction and ultrasonic extraction was employed in the same process. We proposed a Wilhelmy plate method to analyze the contact angle between solvent and sample powder. The determination results of contact angle provided strong evidence for dissolving capacity of shikonin in solvent and what'more, it provides a reference method for modern extraction technology.

By optimizing the extraction process, the extraction yield of shikonin from *Arnebia euchroma* could reach 30.64 mg L^{-1}. Acceptable reproducibility, high accuracy and good linear relationships between the peak area and concentrations could be attained using the method described here. This is expected to help future studies of *Arnebia euchroma.*

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