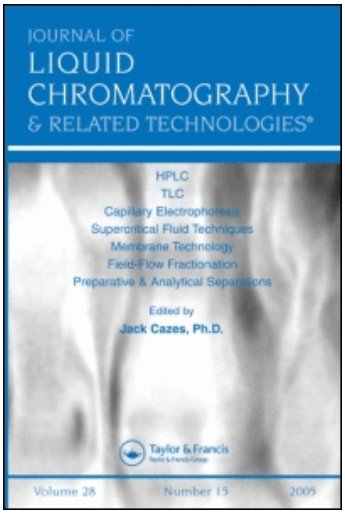

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Micellar Extraction and Preconcentration of Anthraquinone Derivatives from *Rhubarb* Prior to Their HPLC-DAD Determination

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Abstract: In this paper, the nonionic surfactant, oligoethylene glycol monoalkyl ether (Genapol X-080), was employed for the micellar extraction and preconcentration of the five anthraquinone derivatives from rhubarb prior to HPLC analysis. Various experimental conditions were investigated to optimize the extraction process. Under optimum conditions, i.e., 10% Genapol X-080 (w/v), liquid/solid ratio of 50:1 ($mL \cdot g^{-1}$), ultrasonic assisted extraction for 45 min, the extraction yield of anthraquinone derivatives reached the highest value. For the preconcentration of anthraquinone derivatives by cloud point extraction (CPE), the solution was incubated in a thermostatic water bath at 55°C for 45 min, and 0.24 $g \cdot mL^{-1}$ sodium sulfate was added to the solution to facilitate the phase separation and increase the preconcentration factor during the CPE process. Compared with commonly used organic solvents, 10% Genapol X-080 has the highest extraction yield of anthraquinone derivatives. The micellar extraction and preconcentration method established in this paper is demonstrated to be a simple and effective environment friendly alternative to conventional organic solvent extraction methods.

Keywords: Micellar extraction and preconcentration, Cloud point extraction, Genapol X-080, Anthraquinone derivatives, High performance liquid chromatography

INTRODUCTION

Rhubarb is a commonly used traditional Chinese medicinal herb. It is reported to have the pharmaceutical effect of purgation, purging heat, loosening the

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bowels, curing gastric and renal disorders, removing bacterial dysentery, removing heat from the blood, clearing toxins away, promoting blood circulation, and removing blood stasis. Furthermore, rhubarb has antitumor and antimutagenicity[1] action. A variety of constituents have been isolated from rhubarb, in which anthraquinone derivatives including aloe-emodin, rhein, emodin, chrysophanol, and physcion and their glucosides, are accepted as the most important active components.[1–3] Figure 1 shows the molecular structures of the five anthraquinone derivatives.

In Chinese Pharmacopoeia, anthraquinone derivatives are selected to be the marker constituents to evaluate the quality of rhubarb.[4] Methods commonly used for the determination of anthraquinone derivatives in rhubarb are high performance liquid chromatography (HPLC),[5–7] capillary electrophoresis (CE),[2,3,8,9] and thin-layer chromatography (TLC).[10] Inevitably, extraction and preconcentration of the analytes are required in these methods. But conventional extraction methods usually use large amounts of toxic organic solvents and need tedious procedures for the preconcentration of the analytes.[2–10] In order to minimize the use of organic solvents and simplify the operating procedure, other extraction methods should be established.

The special properties of some surfactants, such as their good solubilization capacity towards solutes of different nature and their unique cloud point behavior, have drawn increasing attention in recent years. When the temperature rises above the cloud point temperature, the solution separates into two phases: the small volume of surfactant rich phase and the large volume of aqueous phase. The small volume of the surfactant rich phase allows us to preconcentrate the analytes.[11] This methodology offers the advantages of simplicity, low cost, ability to concentrate solutes, easy disposal of surfactant, and low toxicity compared with classical organic solvents, etc.[11,12] The cloud point extraction (CPE) methodology has been successfully used for the extraction and preconcentration of species of widely differing character and nature, such as metal ions,[13–15] proteins and

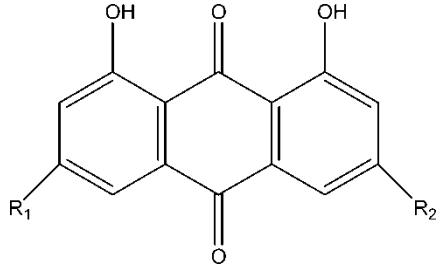

Figure 1. Chemical structures of the five anthraquinone derivatives in Rhubarb. Aloe-emodin: $R_1 = H$, $R_2 = CH_3OH$; Rhein: $R_1 = H$, $R_2 = COOH$; Emodin: $R_1 = OH$, $R_2 = CH_3$; Chrysophanol: $R_1 = H$, $R_2 = CH_3$; Physcion: $R_1 = CH_3O$, $R_2 = CH_3$.

other biomaterials,[16–18] or organic derivatives of strongly differing polarity.[19–25] However, the majority of CPE applications have been reported to deal with analytes present in aqueous samples. CPE has rarely been reported to be used for the extraction and preconcentration of chemical constituents from plants or herbal materials.[26,27] In our previous work, we have studied the feasibility of employing a nonionic surfactant solution as an alternative and effective solvent for the extraction of tanshinones from Salvia miltiorrhiza[12] and isoflavone daidzein from Puerariae radix.[28]

In this paper, the nonionic surfactant, oligoethylene glycol monoalkyl ether (Genapol X-080) was employed to extract and preconcentrate anthraquinone derivatives from rhubarb. The micellar extraction and preconcentration method established in this work includes two steps: the first step is to extract anthraquinone derivatives from solid herbal materials into aqueous surfactant solutions; the second step is to preconcentrate the anthraquinone derivatives by phase separation based on the cloud point phenomenon of the surfactant. The extracted and preconcentrated anthraquinone derivatives were determined by HPLC-DAD. Various factors influencing the extraction of anthraquinone derivatives from solid herbal materials were evaluated. For the preconcentration of anthraquinone derivatives by cloud point extraction (CPE), the influences of incubation time, temperature, and the concentration of salt added were also investigated. Compared with commonly used organic solvents, 10% Genapol X-080 has the highest extraction yield of anthraquinone derivatives. Our work demonstrates that the micellar extraction and preconcentration method is a simple and effective environment friendly alternative to the conventional organic solvent extraction method.

EXPERIMENTAL

Plant Materials

Rhubarb, which had already been cut into pieces, was purchased from a local pharmaceutical store (Baoding, China). The dried plant materials were pulverized and sieved to produce samples with particle sizes of 40–60 mesh.

Chemicals and Reagents

Authentic standards of aloe-emodin, rhein, emodin, chrysophanol, and physcion were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Nonionic surfactant, oligoethylene glycol monoalkyl ether (Genapol X-080) was obtained from Fluka (USA) and used as received without further purification. Various concentrations (w/v) of aqueous surfactant solutions were prepared by weighing appropriate amounts of the surfactant, and by directly dissolving

the surfactant with double distilled water. All other reagents used in this work were of analytical grade.

Apparatus

All analyses were performed on a Shimadzu LC-6A liquid chromatograph (Shimadu, Japan) equipped with a solvent delivery pump, Shimadzu SPD-M6A photodiode array UV-VIS detector, and a 7125 injection valve with 20 μL loop. The chromatographic data were recorded and processed with the Shimadzu SPD-M6A software. A Supelcosil LC-18-DB chromatographic column (150 × 4.6 mm i.d., 5 μm) was used and the column temperature was controlled at 27°C.

A versatile plant pulverizer (Foshan, Guangdong, China) was used to make the plant materials into powder. A KQ-250 ultrasonic generator from the Kunshan Company (Jiangsu, China) was used to extract anthraquinone derivatives from the samples. Cloud point extraction was carried out in a water bath with a thermostat. Sieves (40 mesh, 60 mesh, Zhejiang, China) were used to sieve the rhubarb powder. A high speed centrifuge was employed to centrifuge the sample solutions (Model 800, Shanghai, China).

Procedures for the Extraction and Preconcentration of Anthraquinone Derivatives

Extraction Procedure

Rhubarb was accurately weighed and placed in a 10 mL centrifuge tube; Genapol X-080 solutions at different concentration levels were added. The tube was then capped and placed in the ultrasonic cleaning bath, employing the ultrasound energy to extract the anthraquinone derivatives from rhubarb. After ultrasonic assisted extraction (UAE), the rhubarb extracts were centrifuged for 10 min; the supernatant was filtered through a 0.45 μm membrane, and then injected into the HPLC system.

Preconcentration Procedure

To study the preconcentration of the extracted anthraquinone derivatives by phase separation of the aqueous surfactant solution, an appropriate amount of sodium sulfate was added to the sample solution and vortex dissolved for 2 min. The sample solution was then kept in a thermostatic water bath at the appropriate temperature until the solution completely separated into two distinct phases. The upper phase was the small volume of surfactant rich phase and the lower phase was the large volume of aqueous phase. After centrifugation for 10 min, the aqueous phase was sucked out using a syringe with

a long needle. When the needle was penetrated through the surfactant rich phase, it should be shaken gently several times to remove the sticky surfactant rich phase clinging to it. The oil like drops clinging to the needle went up to congregate with the upper surfactant rich phase layer. When the lower aqueous phase was all sucked out, the needle was washed with methanol and the methanol was vortex mixed with the surfactant rich phase in the tube to lower the viscosity of the surfactant rich phase. After filtration through a 0.45 μm nylon membrane, 10 μL of the solution was injected into the HPLC system for analysis.

Comparison with Conventional Extraction Solvents

Extraction efficiency was compared between 10% Genapol X-080 and various organic solvents, such as methanol, ethanol, 50% methanol, chloroform, butanol, and ethyl acetate. For the comparison experiment, identical experimental conditions were used: samples amount, 0.1 g; extractant volume, 5 mL; and ultrasonic assisted extraction time, 45 min.

HPLC Analysis

The HPLC mobile phase was a mixture of methanol–0.1% phosphoric acid (75:25, v/v). The flow rate was 0.8 $mL \cdot min^{-1}$; the detection wavelength was set at 254 nm. Peaks in the chromatograms were identified by comparing the retention times and UV spectra with those of the authentic anthraquinone derivatives. Peak area was used for quantification.

RESULTS AND DISCUSSION

Analysis of Anthraquinone Derivatives by HPLC

To obtain satisfactory separation of the analytes, various proportions of methanol and 0.1% phosphoric acid were tested as mobile phases. As shown in Figure 2(A), when the volume ratio of methanol to 0.1% phosphoric acid was fixed at 75:25, the five anthraquinone derivatives were completely separated from each other, and other coexisting components in the extracts of rhubarb did not interfere with the detection of the anthraquinone derivatives.

Calibration graphs were obtained by plotting the peak area (y) versus weight (x). Calibration curves were linear from 0.0540 to 2.1600 μg for aloe-emodin ($Y = 61.4191X + 1.4501$, $r = 0.9980$, $n = 7$), from 0.1135 to 4.5400 μg for rhein ($Y = 70.3110X + 1.6070$, $r = 0.9988$, $n = 7$), from 0.0850 to 3.4000 μg for emodin ($Y = 56.4730X + 2.2201$, $r = 0.9984$, $n = 7$), from 0.1095 to 4.3800 μg for chrysophanol ($Y = 64.1160X +$

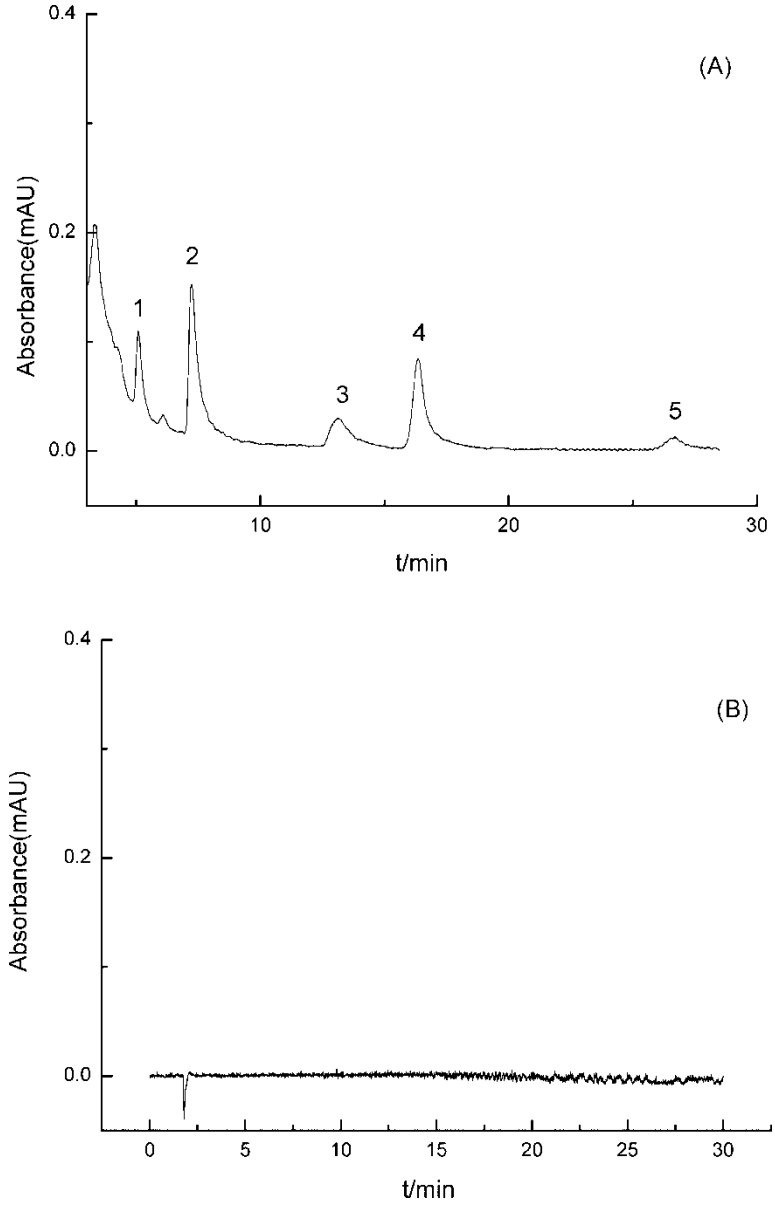

Figure 2. HPLC chromatograms of anthraquinone derivatives extracted from Rhubarb and the surfactant. HPLC conditions: Supelcosil LC-18-DB chromatographic column (150 × 4.6 mm i.d., 5 μm) controlled at 27°C; a mixture of methanol–0.1% phosphoric acid (75:25, v/v) as mobile phase at a flow rate of 0.8 mL · min^{-1}; the detection wavelength was set at 254 nm. (A) anthraquinone derivatives extracted from Rhubarb (B) Genapol X-080 (10%, w/v). 1. Aloe-emodin, 2. Rhein, 3. Emodin, 4. Chrysophanol, 5. Physcion.

2.4843, $r = 0.9985$, $n = 7$), and $0.0315 \sim 1.2600$ μg for physcion ($Y = 60.6972X + 0.5047$, $r = 0.9983$, $n = 7$).

Selection of the Surfactant

Most of the surfactants show high UV absorbance and give very broad peaks in the HPLC chromatogram, which will severely interfere with the determination of the anthraquinone derivatives. Genapol X-080 is a kind of polyoxyethylene glycol monoether type nonionic surfactant, which has eight oxyethylene units and tridecyl alkyl moities. With no aromatic moiety, Genapol X-080 does not absorb above 210 nm. Figure 2(B) shows the chromatogram of 10% Genapol X-080. It can be seen, that the elution of the surfactant did not interfere with the detection of the five anthraquinone derivatives. In this case, Genapol X-080 was chosen as the CPE surfactant for further studies.

Optimization of the Micellar Extraction Conditions

In order to optimize the micellar extraction of the five anthraquinone derivatives from the solid herbal materials, a number of experiments under different conditions were performed. The effects of the concentration of surfactant, granularity of the plant powder, extraction time, liquid/solid ratio, and pH on the extraction efficiency, were evaluated via yield of extracts (%, w/w):

$$\text{yield of extacts}(\%, \text{w/w}) = \frac{\text{amount of anthraquinone derivative extracted}}{\text{amount of herbal material}} \times 100\%$$

Effect of the Surfactant Concentration on the Extraction Efficiency of Anthraquinone Derivatives

Anthraquinone derivatives are known to be hydrophobic derivatives. Usually, they are extracted with organic solvents, such as chloroform and methanol; whereas, in our work, it was demonstrated that anthraquinone derivatives could be extracted by surfactant solution with certain concentrations. The ability of the aqueous nonionic Genapol X-080 solution in extracting anthraquinone derivatives may be related to the solubility enhancement effect of the surfactant micelles. It can be seen from Figure 3, that the yield of aloe-emodin, rhein and emodin increased sharply when the surfactant concentration increased from 0.5% to 5% (w/v) and, then, tends to remain fairly constant in the surfactant concentration range of $5 \sim 15\%$. For chrysophanol and physcion, the extraction efficiency was too low, so that the peak areas could hardly be accurately integrated when the surfactant concentration was below 5%. When the surfactant concentration increased from 5 to 10%, the extraction efficiency for chrysophanol and physcion increased sharply. It

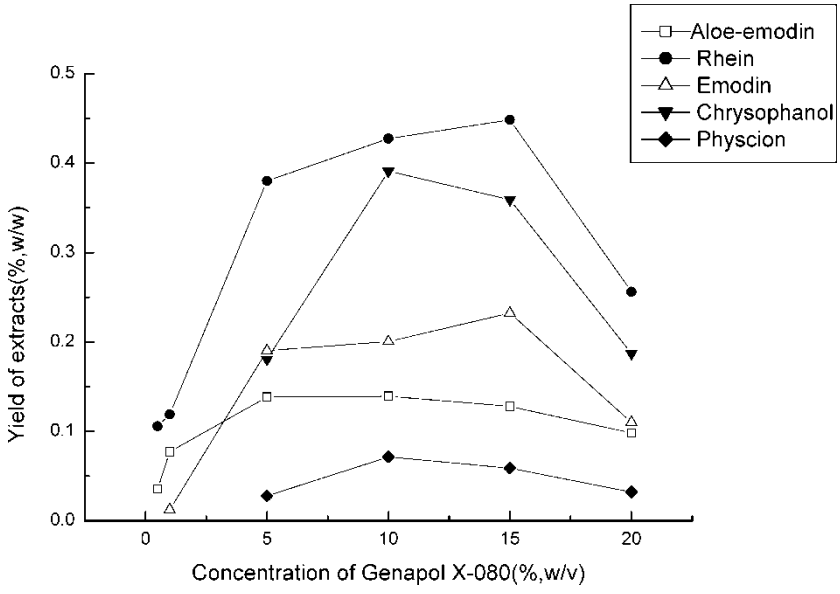

Figure 3. Effect of concentration of Genapol X-080 (10%, w/v) on the extraction efficiency of anthraquinone derivatives. Granularity: 40–60 mesh, ultrasonic-assisted extraction time: 45 min; liquid/solid ratio: 50:1 (mL/g).

can be seen from Figure 3, when the surfactant concentration rises to 20%, extraction yields drop for all extracts. In addition, it was observed that in the CPE preconcentration experiment, the phase volume ratio (V_s/V_w) value increased with the increase of the surfactant concentration. Considering both the extraction efficiency and the phase volume ratio (the lower the V_s/V_w, the higher the preconcentration factor), 10% was chosen as the optimum surfactant concentration for the extraction of anthraquinone derivatives for further studies.

Effect of Ultrasonic Assisted Extraction (UAE) Time on the Extraction Efficiency of Anthraquinone Derivatives

The effect of ultrasonic assisted extraction time on the extraction efficiency of anthraquinone derivatives was studied by varying the extraction time from 5 to 90 min. The results indicated that the extraction efficiency of anthraquinone derivatives increased with the increase of extraction time, and reached the highest value for each compound when extracted for 45 min. When the extraction time was longer than 45 min, the extraction efficiency of derivatives was kept almost constant. So, in the following experiments, 45 min was selected for the extraction of the anthraquinone derivatives.

It has to be mentioned that if the herbal materials were extracted by the shake flask method at room temperature, the extraction efficiency was much lower. Even if extracted for 105 min, the yield of extracts only reached half of that of UAE for 45 min.

Effect of Liquid/Solid Ratio on the Extraction Efficiency of Anthraquinone Derivatives

The liquid/solid ratio is the proportion of the extractant volume to the mass of herbal material. It is one of the factors influencing the extraction efficiency of anthraquinone derivatives. As shown in Figure 4, the liquid/solid ratio of 50:1 ($mL \cdot g^{-1}$) was sufficient for each compound to reach the highest extraction efficiency, so it was employed in the following experiments.

Comparison of Genapol X-080 with Conventional Extraction Solvents

The extraction efficiency was compared between 10% Genapol X-080 and various commonly used polar and non polar organic solvents. Figure 5

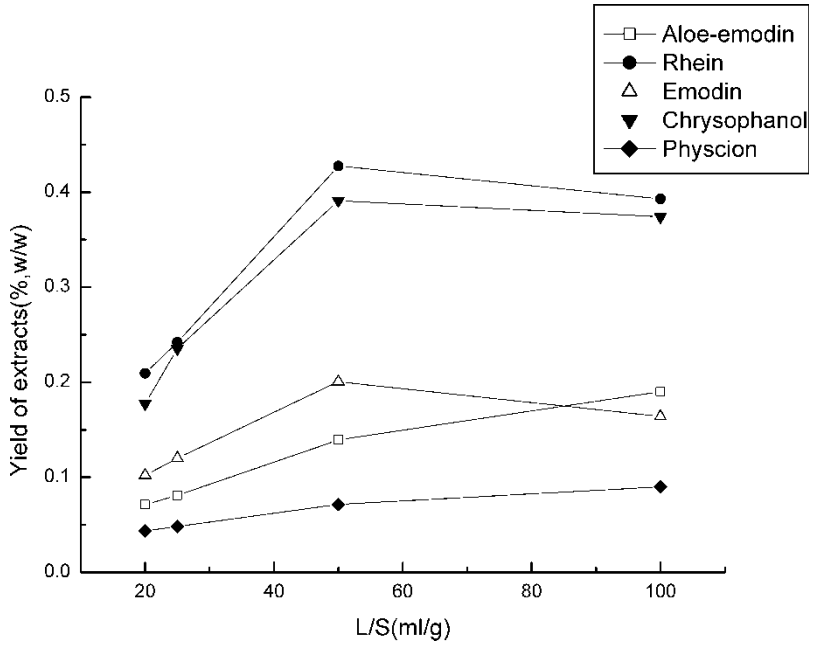

Figure 4. Effect of liquid/solid ratio on the extraction efficiency of anthraquinone derivatives. Concentration of Genapol X-080: 10%(m/v), granularity: 40–60 mesh, ultrasonic-assisted extraction time: 45 min.

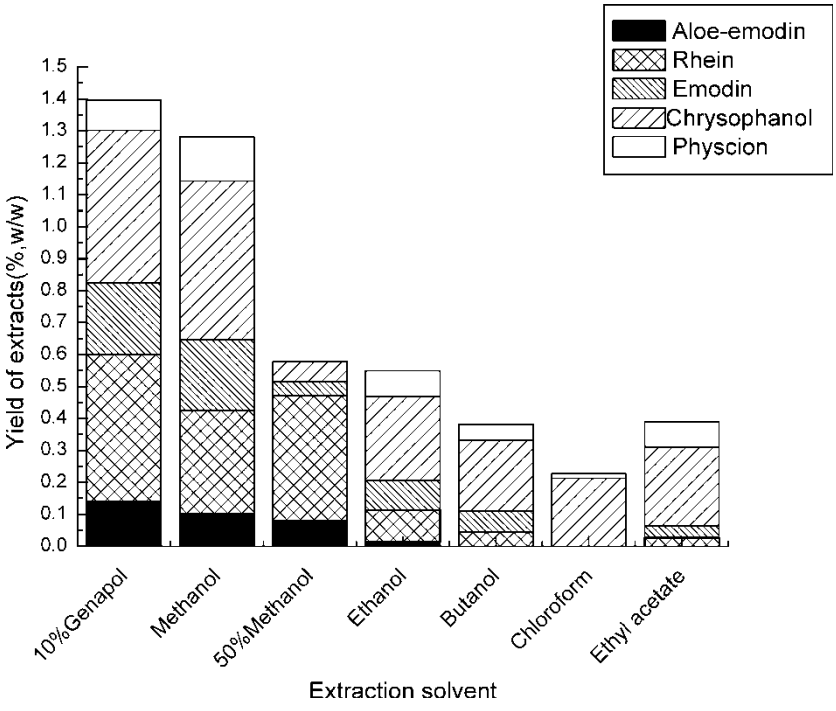

Figure 5. Comparison of the extraction efficiency between 10% Genapol X-080 (m/v) and other commonly used organic solvents. Solvent volume: 5 mL, rhubarb: 0.1 g, ultrasonic-assisted extraction time: 45 min.

shows that 10% Genapol X-080 has the highest extraction efficiency. Among the organic solvents, only methanol has similar extraction efficiency with 10% Genapol X-080. For physcion, methanol has higher extraction efficiency than 10% Genapol X-080. The reason why the extraction efficiency of 10% Genapol X-080 is higher than that of the polar and non polar organic solvents may be due to the solubility enhancing effect of the surfactant micelles.[12]

Optimization of the Preconcentration Conditions

As we have mentioned, the micellar extraction and preconcentration of anthraquinone derivatives from rhubarb includes two steps: the first step is to extract the anthraquinone derivatives from solid herbal materials into aqueous surfactant solution; the second step is to preconcentrate the anthraquinone derivatives by phase separation based on the cloud point phenomenon of the surfactant. Now that the first step has been finished, we will focus on the preconcentration of the anthraquinone derivatives.

From previous work, we know that adding an electrolyte into the solution and heating the solution above the cloud point temperature for some time will facilitate the separation of the two phases, and the volume of the surfactant rich phase decreases with the increase of the amount of electrolyte added.[12,26] In this paper, we studied the effect of the equilibration time and equilibration temperature, as well as the amount of electrolyte on the recovery of the anthraquinone derivatives during the cloud point preconcentration step.

Recovery of the anthraquinone compound was calculated using the following equation:

$$\text{Extraction recovery}(\%) = \frac{\text{Amount of analyte determined after CPE}}{\text{Amount of analyte determined before CPE}} \times 100\%$$

The Effect of Equilibration Time on the Recovery of the Anthraquinone Derivatives

The influence of equilibration time on the recovery of the anthraquinone derivatives was studied by varying the equilibration time between 30 and 90 min, and by keeping the equilibration temperature at 55°C and sodium sulfate of 0.28 $g \cdot mL^{-1}$. The extraction recovery depends on the time that the analytes need to interact with the micelles and get into their cores.[29] As shown in Figure 6, the recovery for each compound increases when the incubation time increases from 30 min to 45 min. Between 45 min and 75 min, the recovery remained fairly constant. After 75 min, the extraction recovery declined. Thus, 45 min was chosen as the optimum equilibration time for the cloud point preconcentration procedure.

The Effect of Equilibration Temperature on the Recovery of the Anthraquinone Derivatives

The dependence of the recovery of anthraquinone derivatives on the equilibration temperature is shown in Figure 7. When the incubation temperature increases from 50°C to 55°C, the extraction recovery of each compound increases significantly. Between 55°C and 70°C, the recovery does not change too much. Thus, 55°C was selected for further studies.

The Effect of the Amount of Sodium Sulfate on the Recovery of the Anthraquinone Derivatives

It has been reported that the addition of an inert salt can facilitate the separation of the two phases for some nonionic surfactant systems, since it increases the density of the bulk aqueous phase.[11] In this paper, sodium sulfate was chosen as the modifier. The study of the influence of the ionic strength on the extraction recovery was carried out by varying the concentration of Na_2SO_4 between

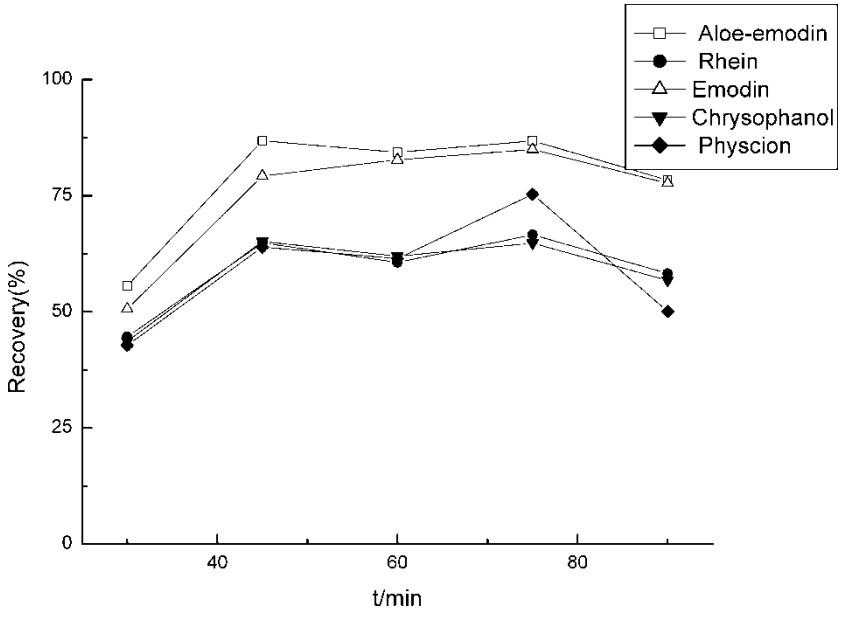

Figure 6. Effect of equilibration time on the recovery of the anthraquinone derivatives. Equilibration temperature: 55°C, sodium sulfate: 0.28 g · mL^{-1}.

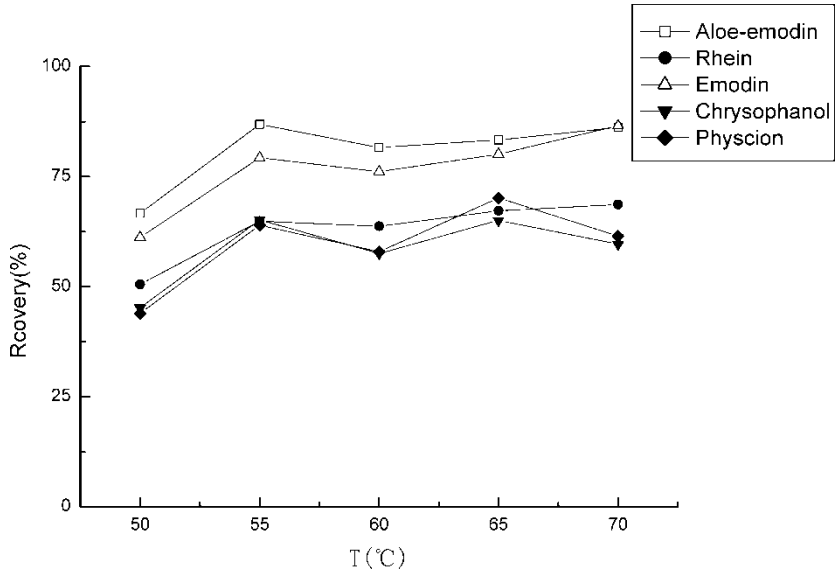

Figure 7. Effect of equilibration temperature on the recovery of the anthraquinone derivatives. Equilibration time: 45 min, sodium sulfate: 0.28 g · mL^{-1}.

0.20 g · $mL^{-1}$and 0.36 g · mL^{-1} at the constant equilibration temperature of 55°C for 45 min. It was observed, that the time for complete separation of the two phases was longer than 400 min when the amount of sodium sulfate added was less than 0.12 g · mL^{-1}. The result shows that the addition of Na_2SO_4 facilitates the separation between the surfactant rich phase and the aqueous phase. With the increase of the salt concentration, the micelle size and the aggregation number are increased and the critical micellar concentration remains constant. In addition, analytes may become less soluble in the solution at higher salt concentrations and, thus, contribute to higher recoveries. That is, the inert salt increases the extraction recovery by decreasing the solubility of the organic species in the aqueous phase. The result obtained from Figure 8 indicates that the CPE at salt concentration of 0.24 g · mL^{-1} gives the optimum extraction recovery of the anthraquinone derivatives. No significant improvement of extraction recovery was observed above this salt concentration. So, 0.24 g · mL^{-1} Na_2SO_4 should be chosen for the effective extraction of anthraquinone derivatives.

Preconcentration Factor of the CPE Method

In this paper, the preconcentration factor (C_F) was calculated as the ratio of the volume of the original sample solution to that of the obtained surfactant rich phase.

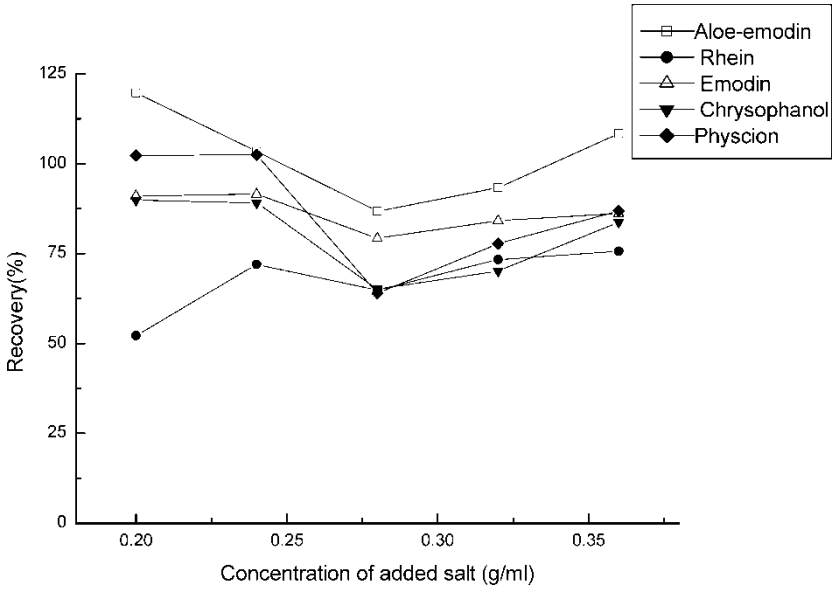

Figure 8. Effect of concentration of salt added on the recovery of the anthraquinone derivatives. Equilibration time: 45 min, equilibration temperature: 55°C.

Table 1. Reproducibilty of the established method for the extraction and preconcentration of anthraquinone derivatives

	Reproducibility of the method RSD (%) (n = 5)				
	Before CPE		After CPE		Determined contents (μg/mg)
Compounds	Retention time	Peak area	Retention time	Peak area	
Aloe-emodin	1.16	4.19	1.34	6.74	1.39
Rhein	1.38	3.43	1.69	11.49	4.61
Emodin	2.76	5.45	1.31	4.08	2.24
Chrysophanol	1.89	6.14	0.97	5.64	4.78
Physcion	2.32	3.84	1.06	3.03	0.95

Method statistics.

For the convenient measurement of the volume of the surfactant rich phase, a larger volume of 10% Genapol X-080 solution was used and the following experiment was carried out: 0.2 g rhubarb was accurately weighed and placed into a 50 mL centrifuge tube, 25 mL 10% Genapol X-080 solution was added. Under the optimized cloud point extraction and preconcentration conditions, a surfactant rich volume of 2 mL was obtained, the preconcentration factor (C_F) is 12.5.

Method Validation

The repeatability of the HPLC profile was determined by injecting the same processed sample five times on the same day. The RSD of retention time was 1.10% for aloe-emodin, 0.90% for rhein, 0.68% for emodin, 0.62% for chrysophanol, and 0.71% for physcion, respectively (n = 5).

The RSD of peak area was 1.13% for aloe-emodin, 1.48% for rhein, 0.93% for emodin, 1.41% for chrysophanol, and 4.30% for physcion, respectively (n = 5).

The reproducibility of the established CPE method was determined by processing five replicates of samples. The RSD values for retention time and peak area before and after CPE preconcentration are shown in Table 1.

CONCLUSIONS

The micellar extraction and preconcentration method described in this paper employed environmental friendly extractants and simplified the extraction and preconcentration procedure. Various factors influencing the extraction

and preconcentration efficiency were evaluated. The reproducibility of the established CPE method was acceptable. Compared with commonly used organic solvents, 10% Genapol X-080 solution has the highest extraction efficiency. The present work further demonstrated that micellar extraction and preconcentration method is a potentially powerful tool for the solubilization, purification, and preconcentration of active ingredients from herbal medicines. Furthermore, this method will be highly valuable in large scale extraction and purification of active ingredients from herbal materials.

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