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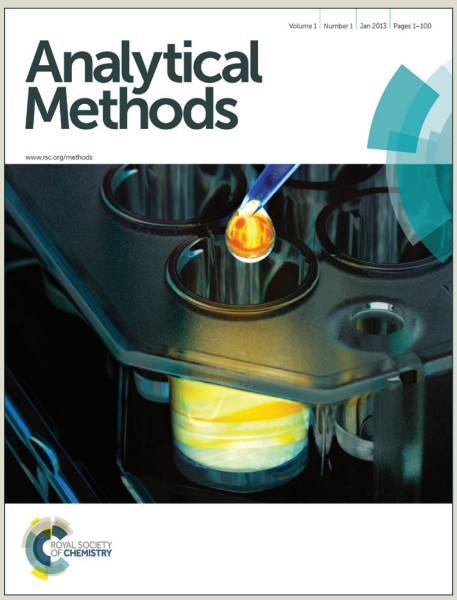

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Separation of camptothecin from Camptotheca acuminate sample using cloud point extraction

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The micelle-mediated extraction and cloud point preconcentration has been successfully applied for separating camptothecin from Camptotheca acuminate. The camptothecin was firstly extracted from Camptotheca acuminate sample by aqueous non-ionic surfactant Triton X-114 solution under ultrasonic assisted. And then a small volume of surfactant-rich phase was obtained by preconcentrating camptothecin, and used for high performance liquid chromatography analysis. Various experimental conditions were investigated to obtain highest extraction yield. The optimum technology parameters for micelle-mediated extraction process were as follows: Triton X-114 concentration was 7%, pH was 7, the ratio of liquid to solid was 125/1 (mL g^{-1}) and ultrasonic time was 40 min. The highest extraction recovery was obtained with 25% (m/v) sodium chloride and equilibration at 50 °C for 20 min. Compared with other extraction methods, this technique showed significant advantages, for instance, low cost and toxicity. In addition, it has the ability to preconcentrate the CPT with the enhancement factor of 5 and obtains higher extraction yield. In this paper, the kinetic of cloud point extraction was also discussed, and the process could be described mathematically by a second-order kinetic model. The limit of detection of camptotheca was 0.7 μg mL^{-1}. The precision of the proposed method was expressed as relative standard deviation at 5.4% (n=5). Finally, the method was successfully applied to extract camptotheca from camplotheca acumiate fruit, bark and leaf, and the extraction yields of camptotheca obtained were 0.1157%, 0.0926% and 0.0612%, respectively. The recoveries of CPT were in the range of 92.3%-93.6%.

Keywords: high performance liquid chromatography, camptothecin, camptotheca acuminate, micellar extraction, cloud point preconcentration

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1. Introduction

Camptotheca acuminate is a kind of species indigenous tree in southern China. It produces antitumor alkaloids, most notably, camptothecin (CPT).[1] CPT is attracting considerable attention worldwide, because of promising antitumor characteristic, which was discovered in the 1960s during screening of plant extracts for antitumor activity. Its structure (Fig. 1) was determined by Wall et al.[2] CPT and its analogs have demonstrated effectiveness in killing various cancer cells such as small and non-small cells lung cancer, ovarian cancer, pancreatic cancer, myelomonocytic leukemia and relative disorders.[3] As a result, a lot of anticancer drugs, such as 9-nitrocamptothecin, irinotecan,[4] 9-aminocamptothecin, topotecan[5,6] and so on, are semisynthesised by CPT for curing cancers. So the extraction and analysis of CPT from Camptotheca acuminate became very important.

At present, many conventional methods have been used for the separation and purification of CPT, such as homogenate extraction,[1] maceration extraction,[7] soxhlet extraction,[8,9] heat reflux extraction,[10] ultrasound-assisted extraction,[3,11,12] microwave-assisted extraction,[13] Column chromatographic extraction,[14] and so on. But all these methods are relatively laborious and time-consuming. Furthermore, above methods except ultrasound-assisted extraction with the solution of sodium carbonate and ionic liquid need to employ large quantities of expensive and toxic organic solvents like methanol, ethanol and acetone.

Recently, the cloud point phenomenon has been used in the science of separation as an attractive alternative for extraction, purification, and preconcentration.[15] The cloud point extraction technique is based on the fact that most nonionic surfactants form micelles in aqueous solutions,[16] and when the surfactant concentration is increased above a certain threshold called critical micellar concentration, the surfactant molecules become associated to form molecular aggregates called micelles.[17] One of the most important properties of these organized structures is their excellent capacity to solubilize some compounds by the electrostatic and hydrophobic interactions or combination of both effects.[18] Another important property is that cloud point of aqueous solutions

of nonionic surfactants becomes turbid and then the solution can be separated into two phase when the temperature rises above the cloud point temperature: the large volume of aqueous phase and the small volume of surfactant-rich phase,[19] which allows us to preconcentrate the target analytes.[19,20] In other words, with this method, the analytes are efficiently extracted into the separated surfactant-rich phase and highly concentrated, which brings great convenience for direct chromatographic analysis without further sample cleanup or evaporation.[21-23] Compared to the initial solution volume, the surfactant-rich phase volume is very small, thus a high enrichment factor can be obtained.[24] Moreover, this methodology offers a simple, safe, inexpensive, and nonpolluting approach for extraction/preconcentration and analysis of inorganic and organic analytes in environmental, food and biological samples.[25]

In this study a methodology of micelle-mediated extraction and cloud point preconcentration was developed to extract, preconcentrate and determine the CPT by using the non-ionic surfactant Triton X-114. The technique in this work mainly includes two steps. Firstly, CPT was extracted from Camptotheca acuminate into aqueous surfactant solution assisted with ultrasonic. Secondly, the target analyte was preconcentrated by phase separation based on the cloud point phenomenon of the surfactant. During the process, a variety of experimental conditions were investigated to evaluate and optimize the extraction and preconcentration process. At the same time, the mechanism of cloud point preconcentration was also discussed. Once the method was optimized and validated, it was applied to separate and preconcentrate CPT from camplotheca acumiate fruit, bark and leaf.

2. Materials and methods

2.1 Reagents

The Camptotheca acuminate samples were purchased from Huqiao (Haozhou, China). The standard of CPT was purchased from F.S. Biological Development (Baoji, China). Non-ionic surfactant Triton X-114 was purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium chloride, sodium hydroxide, hydrochloric acid, methanol, ethanol and acetone were of analytical grade and purchased

from Kermel (Tianjin, China). The chromatographic grade acetonitrile was obtained from Fisher (Pittsburgh, PA, USA). The water used was purified with a Milli-Q water purification system from Millipore (Billerica, MA, USA). The laboratory glassware was soaked in washing liquid for several minutes and rinsed with distilled water at least three times prior to use.

The dry samples of Camptotheca acuminate were powdered using a cyclone mill into a homogeneous size and then sieved (60 mesh). The stock standard solution of CPT (0.5 mg mL^{-1}) was prepared by dissolving an appropriate amount of this compound in chloroform- methanol (1:1, v/v) solution. The solution was stored in a refrigerator at 4 ^{o}C. The working standard solution was prepared daily by diluting the stock standard solution. Various concentrations of aqueous surfactant solutions were prepared by dissolving appropriate amounts of the surfactant in water.

2.2 Apparatus

Chromatographic analysis was performed on a LC-15C high performance chromatograph with ultraviolet detector (Shimadzu, Kyoto, Japan). A Hypersil ODS2 column (150 mm×4.6 mm, 5μm) was used as an analytical column (Elite, Dalian, China). A KQ5200E ultrasonic apparatus (Kunshan, China) at a constant power of 200 W was used for assisting extraction of CPT from Camptotheca acuminate. A DZKW-C thermostatic bath (Shanghai, Chain) was used to keep constant temperature during the experiment of enrichment. A SH-36 vortex mixer (Jintan, China) was used to mix the micellar solution. A TG 16-WS centrifuge (Changsha, Chain) was used to accelerate the phase separation process.

2.3 Micelle mediated extraction-cloud point preconcentration

The extraction was conducted by using a 50 mL centrifuge tube in an ultrasonic bath for 40 min, which contained 0.2 g of powdered Camptotheca acuminate and 25 mL of 7% (v/v) Triton X-114 solution. Then the mixture was centrifuged at 5000 rpm for 5 min.

The supernatant obtained by above procedure was transferred into another 50 mL centrifuge tube. And the sodium chloride (5 g) was added into the tube and mixed vigorously for 3 min using a vortex mixer. Then the resultant cloudy sample solution was incubated in a water bath at 50 °C for

20 min. After that, separation of the aqueous and surfactant-rich phases was accomplished by centrifugation for 10 min at 5000 rpm. Finally, the aqueous phase was removed by a syringe, and the sticky surfactant-rich phase was obtained and diluted to 5 mL with methanol to reduce its viscosity.

2.4 HPLC analysis

Sample analysis was performed using liquid chromatography. The chromatographic mobile phase was a mixture of acetonitrile–water (30:70, v/v). The flow rate was 1.0 mL min^{-1} and the detection wavelength was set to 254 nm. Extraction solutions were finally filtered through 0.45 μm filter papers before high performance liquid chromatography analysis. Fig. 2 shows the Chromatograms of CPT standard (a) and Camptotheca acuminate sample (c). As can be seen from the result, the CPT can be separated with Triton X-114 and other compounds in Camptotheca acuminate.

3. Results and discussion

3.1 Optimization of extraction conditions

During optimizing the conditions for extraction of CPT into aqueous surfactant solution, the extraction process was evaluated by the extraction yield of CPT. Some extraction conditions including Triton X-114 concentration, pH, liquid/solid ratio and ultrasonic time were optimized.

3.1.1 Effect of Triton X-114 concentration. The theoretical preconcentration factor depends on the concentration of surfactant. Triton X-114 was chosen as the extract for its low cloud point temperature, low UV absorbance and high density.[24,26] The effect of the surfactant on the extraction in the concentration of 1.0% - 9.0% (v/v) was investigated. As shown in Fig. 3a, the extraction yield of CPT increased with the increase of surfactant concentration from 1.0% to 7.0%, and no obvious increase was observed when the surfactant concentration increased from 7.0% to 9.0%. So the best concentration of Triton X-114 found was to be 7.0% (v/v).

3.1.2 Effect of pH. For organic molecules, pH is perhaps the most critical factor regulating the partitioning of the target analyte in the micellar phase.[27] So it is necessary to optimize the pH for

significant extraction yield of CPT. In order to obtain the desired preconcentration efficiencies, the pH value was studied in the range of 2.0–10.0 adjusted by diluted HCl and NaOH. As shown in Fig. 3b, the extraction yield of CPT increased rapidly when pH of solution increased from 2.0 to 7.0 and decreased from 7.0 to 10.0. Hereby, pH of 7.0 was selected in further experiments.

3.1.3 Effect of liquid/solid ratio. The liquid/solid ratio is also an important factor with respect to increasing the extracted amount of CPT. Once the amount of solvent increased, the chance of bioactive components coming into contact with the solvent also increased, leading to higher extraction yield of the components.[28] Larger volumes of solvent not only decrease the economic feasibility, but also create unnecessary waste. A series of extractions were carried out with different liquid/solid ratios (25/1, 50/1, 75/1, 100/1, 125/1 and 150/1 mL g^{-1}). The results presented in Fig. 3c indicated that the extracted amounts of CPT increased with increasing liquid/solid ratio ranging from 25/1 to 125/1 (mL g^{-1}), then kept constant at the liquid/solid ratio over 125/1 (mL g^{-1}). As a result, the liquid/solid ratio of 125/1 (mL g^{-1}) was sufficient for economic considerations.

3.1.4 Effect of ultrasonic time. Ultrasonic apparatus was used for assisting extraction of CPT from Camptotheca acuminate. So the ultrasonic time was also an important factor to study in this paper. The results illustrated in Fig. 3d showed that the extraction amounts of CPT dramatically increased as the ultrasonic time increase from 10 to 40 min, and there was no obvious increase observed after 40 min. Thus, 40 min was evidently selected as the optimal ultrasonic time.

3.2 Optimization of enrichment conditions

After the first micelle-mediated extraction step which is to extract CPT from Camptotheca acuminate into an aqueous surfactant solution was optimized, phase separation based on the cloud point phenomenon of the surfactant was performed. To optimize the cloud point preconcentration, we investigated the amount of sodium chloride and equilibration temperature. Moreover, the influence of equilibration time was also studied in the section of kinetics. During this part, the process of preconcentration was evaluated by the recovery of CPT.

3.2.1 Effect of the concentration of sodium chloride. The electrolytes play an important role in

the cloud point of non-ionic surfactant systems. When small amounts of inorganic salts are added to the system, a decrease in the cloud point temperature is noted.[29] Furthermore, the presence of salts may facilitate phase separation since they increase the density of the aqueous phase for most non-ionic surfactant.[26] This fact indicates that it is necessary to consider the effect of the concentration of NaCl during the process of cloud point preconcentration. The effect of NaCl on the extraction in the concentration of 0 - 30% (m/v) was investigated. The results in Fig. 4a indicated that the extraction recovery of CPT with NaCl was higher than that without NaCl. The extraction recovery of CPT kept constant while the concentration of NaCl was over 25%. So 25% was chosen as the effective extraction concentration of NaCl.

3.2.2 Effect of the equilibrium temperature. Theoretically, the optimal equilibrium temperature of the extraction occurs when the equilibration temperature is 15–20 ^{o}C higher than the cloud point temperature of surfactant.[30] So the influence of temperature on the extraction efficiencies of CPT was also investigated in this study. Fig. 4b shows the effect of temperature on the extraction of CPT: the recovery of CPT increased with temperature increasing from 40 ^{o}C to 50 ^{o}C. The temperature was set to 50 ^{o}C in the following experiments.

3.3 Kinetics of the cloud point extraction

In order to describe the kinetics of the process of the cloud point extraction, the effect of equilibrium time from 5 to 45 min on the recovery of CPT was investigated. As shown in Fig. 5a, the recovery of CPT increased when the equilibration time increase from 10 to 20 min, while remained constant from 20 to 45 min. Therefore, the equilibrium time of 20 min was chosen in this work.

Quantifying the changes in sorption with time requires an appropriate kinetic model and, traditionally, the pseudo-first-order equation and the pseudo-second-order equation[31,32] are to be applied to sorption kinetics, which are as follows:

pseudo-first-order equation: $\ln(q_{eq} - q_t) = \ln(q_e) - k_1 t$

pseudo-second-order equation: $\frac{t}{q_t} = \frac{1}{k_2 q^2{}_e} + \frac{t}{q_e}$

where q_{eq} (g L^{-1}) and q_t (g L^{-1}) refer to the absorbed of CPT at equilibrium and any time, respectively, k_1 (min^{-1}) and k_2 (L g $^{-1}$ min^{-1}) are the rate constant of pseudo-first-order and pseudo-second-order, respectively. q_e is the theoretic adsorption capacity of the pseudo-first-order kinetic model (g L^{-1}). For the pseudo-second-order model, the half life $t_{1/2}$ and initial rate of adsorption h are given by:

$$t_{1/2} = \frac{1}{k_2 C_0}$$

$$h = k_2 q_e^2$$

where C_0 is the initial concentration of CPT.

Fig. 5b and Fig. 5c present the linearized forms of the pseudo-first-order model and pseudo-second-order model, respectively. The results showed that the pseudo-second-order equation was the more appropriate for the process of cloud point extraction. The specific results can be gained by applying of the pseudo-second-order model in this process, which are shown as follows:

y=13.5x+30.5 R^2 =0.9985 q_e = 0.556 g L^{-1}

K_2 = 6.0 L g $^{-1}$ min^{-1} $t_{1/2}$ =9.5 min h=0.03 g L^{-1} min^{-1}

3.4 Analytical performance

A calibration curve of CPT was obtained by plotting the peak-area versus the theoretical concentration of CPT. The linearity obtained was in the range of 1-250 μg mL^{-1}, and the regression equation and correlation coefficients were as follows:

A=6.87×$10^4$$C$+5.40×$10^2$, R= 0.9997

The result showed that a good correlation exists between the peak area (A) and the concentration (C) of CPT.

The sensitivity of the method was described by the limit of detection (LOD). The LOD defined as three times ratio of signal to noise was 0.7 μg mL^{-1}.

The precision of the proposed method was studied from five replicated experiments for real Camptotheca acuminate samples. The average extraction yield of CPT was 0.1157%, with the relative standard deviation (RSD) at 5.4% (n=5).

3.5 Application of the method and comparison of different methods

In order to demonstrate the applicability, the proposed method was used for the determination of CPT in Camptotheca acuminate fruit, bark and leaf. Furthermore, other methods including ultrasound -assisted extraction with different solvents of methanol, ethanol, acetone and water and soxhlet extraction with methanol were also used to extract the CPT. From the results shown in Table 1, the content of CPT is different in different Camptotheca acuminate parts. The extraction yields of CPT from Camptotheca acuminate fruit (0.1157%), bark (0.0612%) and leaf (0.0926%) achieved by the proposed method were all higher than those by other methods. The enrichment factor was calculated to be 5 considering that the initial extraction solvent volume was 25 mL and the final surfactant-rich phase volume after diluted with methanol was 5 mL. Moreover, the recovery of CPT from Camptotheca acuminate fruit, bark and leaf was studied with the spiked samples. The recoveries obtained were 92.3%-93.6% (Table 2).

4. Conclusions

The result obtained in this study indicates that the micelle-mediates extraction is a potentially powerful tool for the extraction of CPT from the traditional Chinese medicine of Camptotheca acuminate. And the procedure of cloud point preconcentration is a successful method to preconcentrate the CPT into the surfactant-rich phase. Compared with extracting by different solvents including methanol, ethanol, acetone and water, this method offers the advantages of safety, low cost, low toxicity, ability to concentrate solutes, easy disposal of surfactant, and higher extraction yield with a good precision. Moreover, the adsorption kinetics followed the mechanism of the pseudo-second-order equation for the process of cloud point preconcentration. Finally, this method was successfully applied to separate and preconcentracte CPT from Camptotheca acuminate

fruit, bark and leaf, and the yields of CPT were 0.1157%, 0.0612% and 0.0926%, respectively. This method provides the possibility of large-scale extraction and purification of active ingredients. The proposed method has its disadvantages such as the difficulty in removing surfactant after extracting tiny amounts of bioactive targets using this technique. However, some other studies using the method of dual-cloud point extraction solved this problem very well.[33-35] We will focus on this study in the future.

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Figure captions:

Fig. 1 The chemical structure of camptothecin

Fig. 2 The chromatograms of CPT standard (a), and Camptotheca acuminate sample (b)

Fig. 3 The effect of Triton X-114 concentration (a), pH (b), solid/liquid ratio (c), ultrasonic time (d) on the process of micelle-mediated extraction

Fig. 4 The Effect of NaCl concentration (a) and equilibration temperature (b) on the process of cloud point preconcentration

Fig. 5 The effect of equilibration time (a) and the linearized forms of the pseudo-first-order kinetic (b) and pseudo-second-order kinetic (c) for cloud point preconcentraction

Table 1

Comparison of different methods used for extraction of CPT (n=5)

Extraction methods	Camptotheca acuminate fruit		Camptotheca acuminate bark		Camptotheca acuminate leaf	
	Extraction yield (%)	RSD (%)	Extraction yield (%)	RSD (%)	Extraction yield (%)	RSD (%)
Ultrasound extraction with methanol	0.0681	6.7	0.0456	8.6	0.0556	5.8
Ultrasound extraction with ethanol	0.0222	7.9	0.0207	9.5	0.0187	7.2
Ultrasound extraction with acetone	0.0598	10.3	0.0352	7.9	0.0453	10.6
Ultrasound extraction with water	0.0436	5.8	0.0318	8.9	0.0338	5.9
Soxhlet extraction with methanol	0.0843	5.7	0.0428	8.2	0.0621	6.3
Cloud point extraction with Triton X-114	0.1157	5.4	0.0612	6.3	0.0926	5.2

Table 2

The recovery of CPT from Camptotheca acuminate fruit, bark and leaf

Samples	Group	Amount of CPT in samples (%)	Standard addition value (%)	Measured value (%)	Recovery (%)	Mean recovery (%)
Camptotheca acuminate fruit	1	0.1157	0.10	0.2105	94.8	93.5
	2	0.1157	0.10	0.2083	92.6	
	3	0.1157	0.10	0.2089	93.2	
Camptotheca acuminate bark	1	0.0612	0.10	0.1564	95.2	93.6
	2	0.0612	0.10	0.1543	93.1	
	3	0.0612	0.10	0.1538	92.6	
Camptotheca acuminate leaf	1	0.0926	0.10	0.1869	94.3	92.3
	2	0.0926	0.10	0.1846	92.0	
	3	0.0926	1.00	0.1831	90.5	

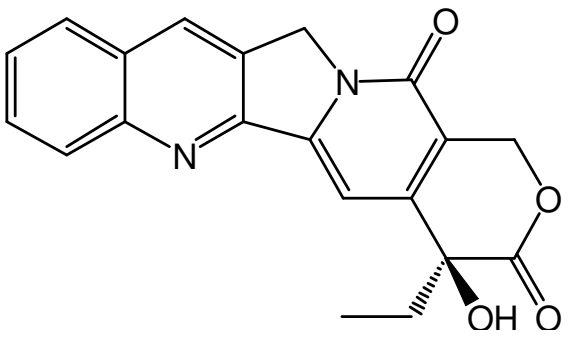

Fig. 1

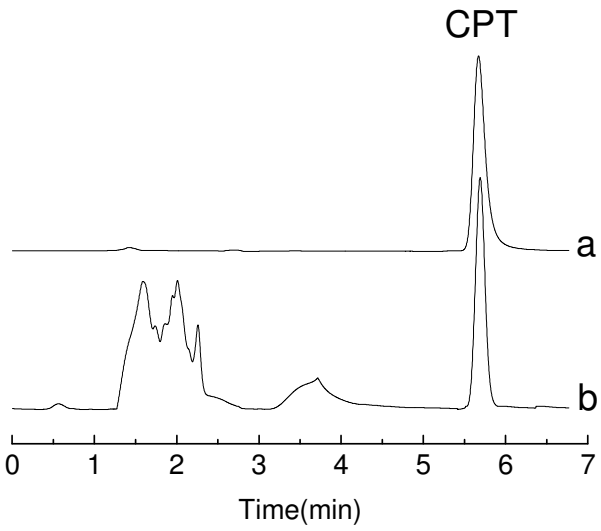

Fig. 2

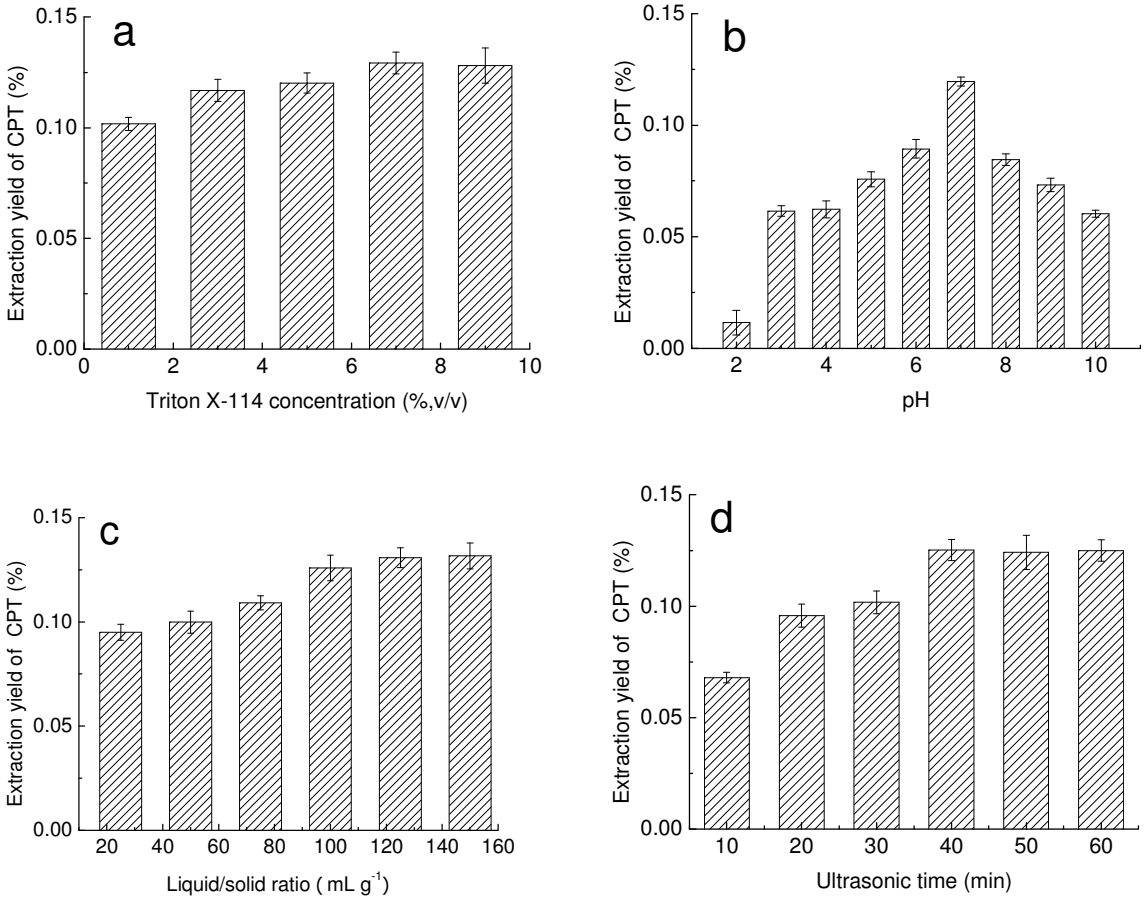

Fig. 3

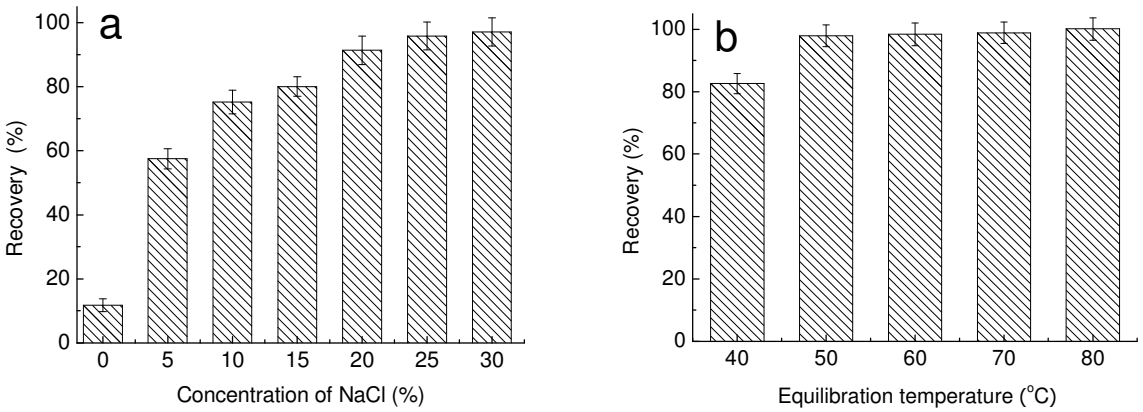

Fig. 4

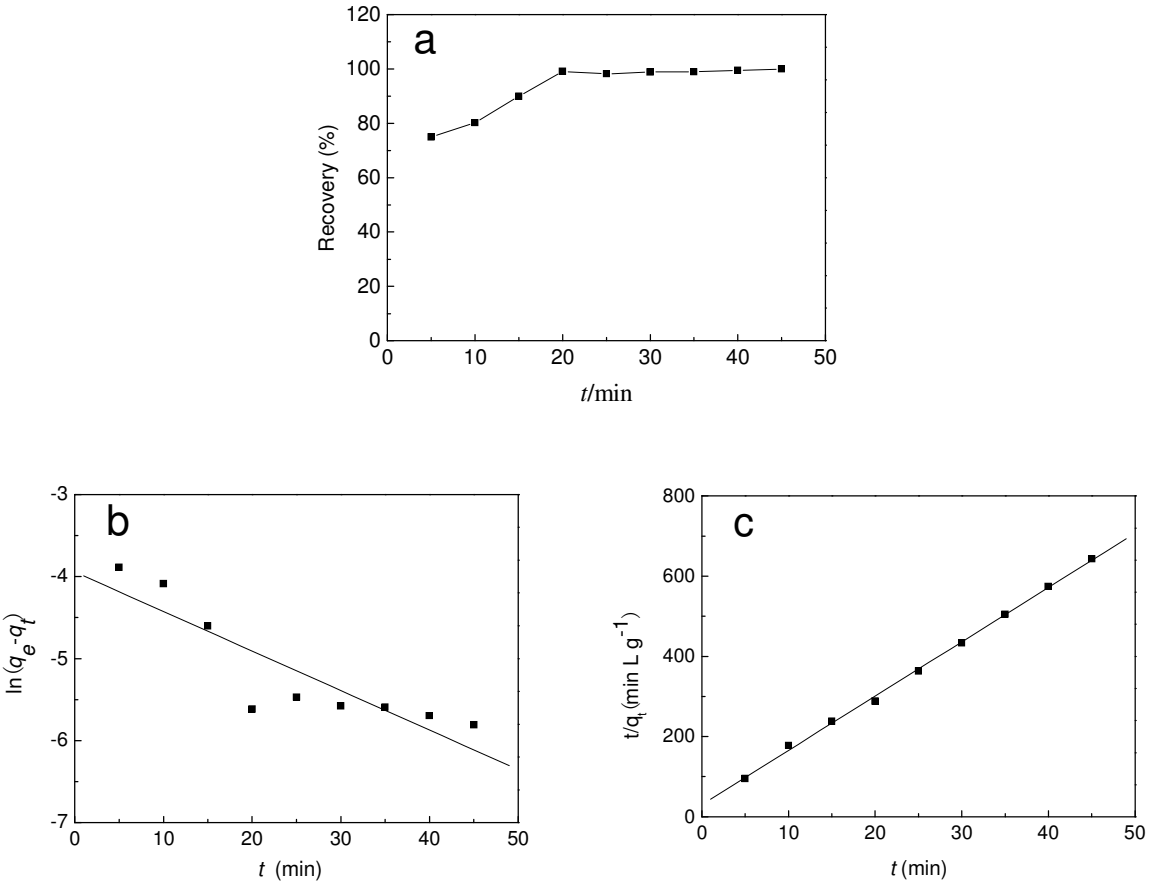

Fig. 5