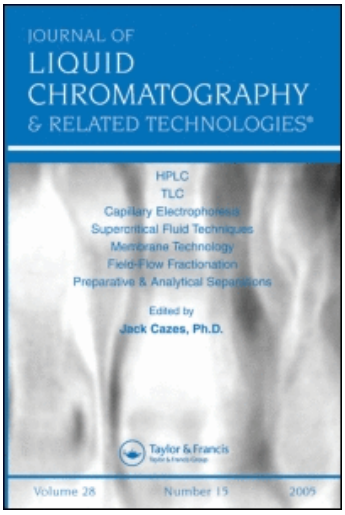

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Abstract: A new supercritical fluid extraction (SFE) pretreatment method for determination of tripterine in *Tripterygium wilfordii* Hook. F with high performance liquid chromatography is proposed in this paper. The optimized conditions of SFE were set as follows: n-butanol was selected as cosolvent and the ratio of n-butanol to the material was 1.0 mL/g material; the extraction temperature and pressure were 40°C and 35 MPa, respectively. The static and dynamic extraction times were both 15 min. Recovery of tripterine from SFE varied from 90.5 to 103.2% for samples with different content levels. During the validation of the method, the linearity and precision, stability, limit of detection and quantification were also determined, all of which satisfied the analytical requirements. Comparison of SFE with conventional soxhlet extraction shows SFE is more efficient in the extraction of tripterine, as it needs less organic solvent and extraction time. Besides, it does not need a further cleanup procedure before analysis by HPLC.

Keywords: *Tripterygium wilfordii* Hook. F, Tripterine, Supercritical fluid extraction, Soxhlet, High performance liquid chromatography

INTRODUCTION

Tripterygium wilfordii Hook. F (*T. wilfordii*) has been used as a traditional Chinese medicine for several hundred years. Tripterine (Fig. 1), a triterpenoid

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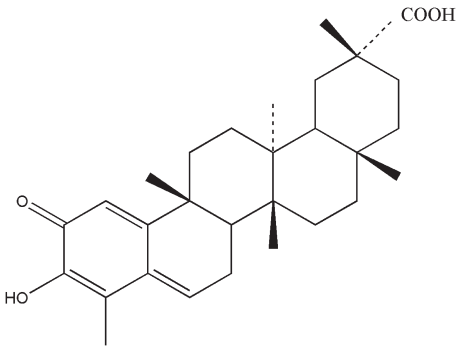


Figure 1. Chemical structure of tripterine.

in the roots of this plant, has a beneficial effect on systemic lupus erythematosus[1] and has also been reported as a potent pro-inflammatory cytokines like Interleukin-1 (IL-1β) release 1inhibitor.[2,3] The content of tripterine in *T. wilfordii* varies with different planting areas and different parts of the roots. It is necessary to have a rapid, sensitive, and reproducible method for determining the content of tripterine in the roots of *Tripterygium wilfordii* Hook. F.

For qualitative analysis of the content of some constituents in natural plant, the pretreatment procedures, including extraction and cleanup, are often the key steps. Ideally, an extraction procedure should be exhaustive with respect to the constituents to be analyzed.[4,5] A previously published method[6] for obtaining the analytical sample of tripterine involved soxhlet extraction followed by chloroform extraction and cleaning up over a silica gel column. This technique is time-consuming and thermal decomposition of tripterine is generally unavoidable. Analytical-scale supercritical fluid extraction (SFE), especially supercritical carbon dioxide extraction, is a promising technology in natural products research.[7] The main advantage with SFE is the possibility of obtaining clean extracts with reduced solvent consumption and extraction time.[8–11]

It has been observed in our early investigation[12,13] that tripterine existed in the SFE extract of roots of *T. wilfordii*. However, the optimum conditions were used for obtaining triptolide, but not for tripterine. The aim of this paper is to verify the possibility of using supercritical carbon dioxide extraction with cosolvent for the determination of tripterine.

EXPERIMENTAL

Plant Material

Roots of *T. wilfordii* without bark were purchased from Jiangxi Province, Guangxi Province, and Hunan Province, P. R. China. The whole roots of *T.*

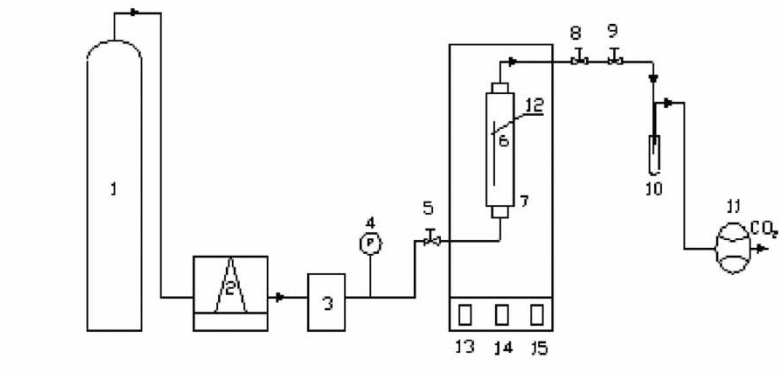
wilfordii were purchased from Hunan province, P. R. China. The plants were identified by Dr. Hongquan Duan (College of Pharmacy, Tianjin Medical University, P. R. China). They were powdered into particle sizes between 120 μm and 830 μm prior to extraction.

Chemicals and Reagents

The standard samples of tripterine (purity, 98.32%) were separated and purified in the laboratory of College of Pharmacy, Tianjin Medical University, P. R. China, and identified by mass spectrometric analysis. The carbon dioxide (purity, 99.95%), silica gel (diameter, 75 μm–150 μm), diatomaceous earth (AR), chloroform (AR), n-butanol (AR), and methanol (CR) were purchased from domestic reagent companies. Water used in all the experiments was doubly distilled and deionized.

Apparatus and Procedure for SFE

The extraction was performed using the Spe-ed SFE instrument (Applied Separations Inc., Allentown, PA, USA), which is shown schematically in Fig. 2. Liquid CO_2 was pressurized with an air-driven pump (3) and then charged into the cartridge (6) to the desired pressure. The pressure was controlled to an accuracy of about 1% over the measuring range. The cartridge, which was



1.CO_2-cylinder; 2.liquid-cooled bath; 3.air-driven pump; 4.pressure gauge; 5.in-let valve; 6.cartridge; 7.thermotank; 8.out-let valve; 9.restrictive micrometer valve; 10.galss vial; 11.wet-test meter; 12.thermocouple; 13.thermotank temperature indicator; 14.cartridge temperature indicator; 15.restrictive micrometer valve temperature indicator

Figure 2. Schematic of analytical-scale supercritical fluids extraction apparatus.

packed with powdered raw materials and cosolvent, was heated with a thermotank; its temperature was indicated and controlled by a thermocouple (12) to within $\pm 1\%$. When both the desired pressure and desired temperature were reached, the static extraction was performed for a desired period of time. Then, dynamic extraction was performed by opening the restrictive micrometer valve (9) to make supercritical CO_2 with dissolved compounds passing through it and was subsequently expanded to ambient pressure. The extract was precipitated in a glass vial (10) at ambient pressure and temperature. A calibrated wet-test meter (11) at known temperature and pressure measured the total amount of CO_2. The collected extract was then concentrated until dryness under reduced pressure. The resulting extract was purified over a silica gel column and then analyzed with HPLC.

Conventional Extraction Methods

In the soxhlet extraction, an amount of 8 g of powdered whole roots was loaded into the soxhlet extractor and extracted with 200 mL methanol for 5 h at 90°C. About 3 g diatomaceous earth was added to the resulting methanolic solution and the solution was concentrated under reduced pressure until dryness. The resulting diatomaceous earth-extract power was ultrasonically extracted with 50 mL chloroform (three times, 3×50 mL, total) at a temperature of 35°C and ultrasonic power of 200 W. The combined chloroform solution was filtered and evaporated with a rotary evaporator. The resulting extract was purified over a silica gel column and then analyzed with HPLC.

Cleanup Procedures

A cleanup procedure with a silica gel column was employed before HPLC analysis. The extract was dissolved in 1 mL chloroform and loaded to the column (ϕ10 mm $\times$ 200 mm) containing about 4 g silica-gel. About 50 mL chloroform was used to elute the low polar impurities. Then tripterine was eluted with 100 mL chloroform-methanol mixtures (95:5, v/v). The effluent mixtures were evaporated with a rotary evaporator. The residue was dissolved in methanol and filtered for HPLC analysis.

HPLC Analytical Conditions

In the HPLC analysis (LabAlliance series III pump; Model 500 UV detector), the column was Agilent TC-C18 (250 mm $\times$ 4.6 mm, 5 μm, Agilent Technology). The mobile phase was methanol-1% acetate solution mixtures (87:13, v/v) and its flow rate was set as 1.0 mL/min. The detection wavelength was 425 nm. The injection volume was quantified with 20 μL storage loops

(Rheodyne, 7725i, made in U.S.A). A series of seven standards in methanol (7.36, 18.4, 36.8, 73.6, 110.4, 147.2, and 184 μg/mL) served for calibration. The retention time of tripterine was about 15 min when the analysis was carried out at ambient temperature (Fig. 3).

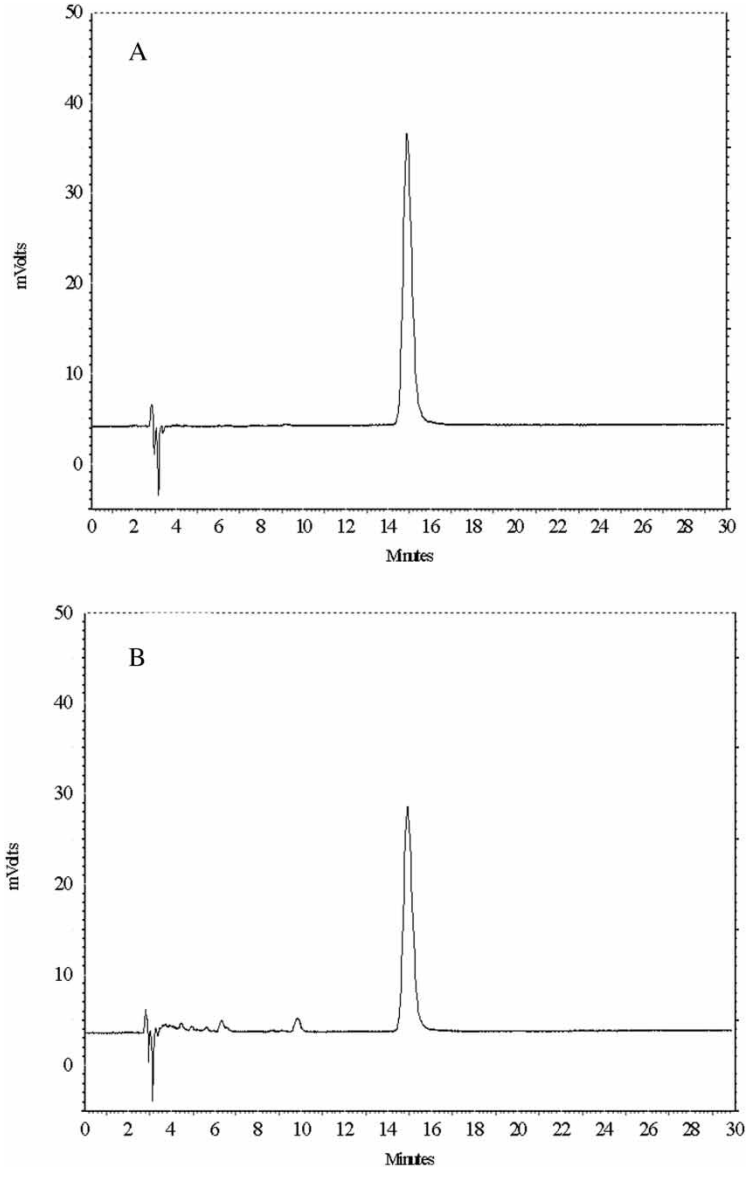


Figure 3. HPLC chromatogram of tripterine standard (A) and sample after column chromatography (B).

RESULTS AND DISCUSSION

Optimization of SFE Parameters

In the SFE experiments, a cartridge of 32 mL with 14.40 mm inner diameter and 195 mm length was used. About 8 g powdered whole roots and an appropriate amount of cosolvent were loaded into the cartridge. The static and dynamic extraction times were both 30 min. The other process parameters were optimized by investigating the effect of a variety of cosolvents, extraction temperatures, pressures, extraction times, and the ratio of cosolvent to the material on the yield of tripterine, as shown in Fig. 4.

Cosolvents of different polarities, acetone, ethanol, ethyl acetate, and n-butanol, were added to the *T. wilfordii* power in the ratio of 0.6 mL/g material. The extraction temperature and pressure were set to 50°C and 30 MPa, respectively. It can be seen from Fig. 4 that the maximum efficiency of extraction was observed when n-butanol was used as the cosolvent.

The effect of extraction temperature on the yield of tripterine was further investigated, where n-butanol was selected as cosolvent in the ratio of 0.6 mL/g material and the extraction pressure was kept at 30 MPa. The results reveal that a lower extraction temperature is beneficial for the extraction of tripterine. The temperature can affect the density of the supercritical fluid, which has a positive effect on the solubility of analytes.[14,15] When the extraction temperature was increased, the density of the supercritical fluids decreased. In addition, high temperature can bring the potential risk of tripterine degradation. So, the extraction temperature of 40°C is preferred, considering the yield of tripterine.

Extraction pressures ranging from 15 MPa to 35 MPa were also investigated, where n-butanol was used as cosolvent in the ratio of 0.6 mL/g material and the extraction temperature was 40°C. The results showed that the yield of tripterine reaches a maximum at a pressure of 35 MPa. When the extraction pressure is changed from 30 MPa to 35 MPa, there is only a

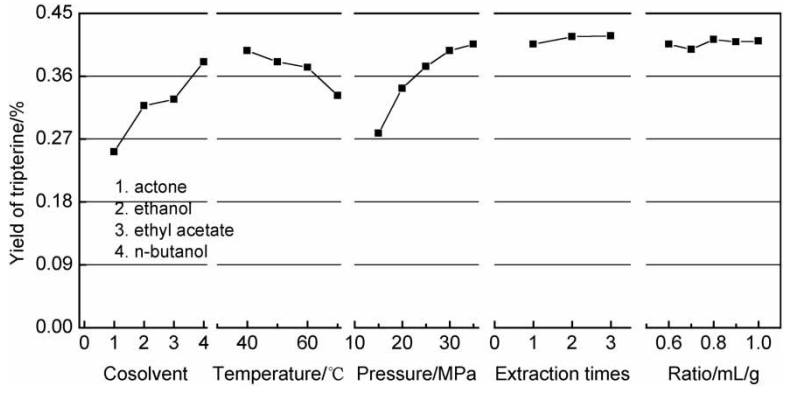


Figure 4. Optimization of supercritical carbon dioxide extraction with cosolvent.

small increase of the yield of tripterine. So, it can be concluded that the extraction pressure of 35 MPa is adequate for the extraction of tripterine.

The extraction times were studied with n-butanol being used as cosolvent, and the extraction temperature and pressure were 40°C and 35 MPa, respectively. There is no obvious increase in the yield of tripterine when the same extraction procedures are repeated with the same sample for two or three times. Under the assumption that the tripterine can be extracted exhaustively after three times of extraction, about 97.15% of tripterine can be extracted in the single extraction process. As increasing extraction times implies long analysis time and single extraction can satisfy the demands of the analysis, a single extraction is preferred in the extraction of tripterine.

The effect of the ratio of n-butanol to the material on the yield of tripterine was also studied. The results show that the changes of yield of tripterine are not obvious when the ratio ranges from 0.6 to 1.0 mL/g material. As the yield of tripterine was stable when the ratio was larger than 0.8 mL/g material, the ratio of 1.0 mL/g material was preferred.

In order to further shorten the extraction time, material quantity of 1 g, a cartridge whose capacity was 10 mL with 10.7 mm inner diameter and 140 mm length were used instead of 8 g material and a cartridge of 32 mL. In this way, the static and the dynamic extraction time were both shortened from 30 min to 15 min; about 90.5% of tripterine in the material can be extracted, which can also satisfy the demands of analysis.

According to the above investigations, the optimized conditions were finally set as follows: the cartridge with a capacity of 10 mL was used; n-butanol was selected as cosolvent and the ratio of n-butanol to the material was 1.0 mL/g material; the extraction temperature and pressure were 40°C and 35 MPa, respectively; the static and dynamic extraction time were both 15 min, and a single extraction was enough, as about 90.5% of tripterine in the material could be extracted this way.

Validation of the SFE Method

In order to validate the new SFE pretreatment method combined with HPLC for determination of tripterine in *Tripterygium wilfordii* Hook. F, the linearity, precision, stability, recovery, and the limit of detection and quantification were determined under the optimized conditions mentioned above.

Linearity and Precision

A linear relationship was found between tripterine peak areas and tripterine concentrations within the range of 7.36–184 μg/mL. The regression equation for calibration curve was $Y = 6245.2X - 34698$, $r^2 = 0.9944$ ($n = 7$). To evaluate the method precision, five independent samples of the root powder were extracted by SFE. The extracts were analyzed with HPLC. The RSD was less than 5%. To evaluate the chromatographic precision, three standard

Table 1. Absolute recoveries of SFE for determining the content of tripterine in *T. wilfordii* at low content level (n = 3)

Tripterine in the material (μg)	Adding amount (μg)	Detecting amount (μg)	Absolute recovery (%)	RSD (%)
196	285	470	96.1	3.2
196	190	389	101.6	2.9
196	95	294	103.2	5.4

sample solutions of different concentrations were injected five times and the RSD values of precision for continuous sample mountings were less than 2.41%.

Stability

The *T. wilfordii* root extract solution derived from SFE was injected at least six times on a single day (intra-day) and on different days (inter-day); the RSDs are 0.46% and 2.36% respectively.

Recovery Studies

The content of tripterine in the roots of *T. wilfordii* varies from 0.1 mg/g to 4 mg/g, depending on the parts of the roots and planting areas. So, the recovery was tested for SFE at both low and high content levels. The recovery at low content level was carried out using the standard addition procedure. Different amounts of tripterine standard sample were spiked into the powered roots whose content of tripterine had been determined. The percentage of recovery was calculated by comparing the detected amount with the added amount. The recoveries at three levels were examined at least three times. The average recoveries of tripterine ranged from 96.1 to 103.2% (Table 1). The recovery experiments at high content level were carried out with the whole roots powder of *T. wilfordii*, whose content of tripterine was much higher. The recovery was calculated based on extraction efficiency of three times extractions with SFE. The results (Table 2) show that the efficiency of

Table 2. Recoveries of SFE and soxhlet extraction for determining the content of tripterine in *T. wilfordii* at high content level (n = 3)

Methods (%)	Soxhlet exrtaction	SFE	Three times extraction with SFE
Yield of tripterine	0.3153	0.3781	0.4176
Recovery	75.50	90.50	100
RSD	3.67	3.23	4.06

SFE is higher than soxhlet extraction. The recovery of 90.5% can also meet the requirements of analysis.

Limit of Detection and Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the lowest concentration giving a response of three times and ten times the average of the baseline noise. LOD and LOQ for tripterine were 2.2 μg/mL and 7.36 μg/mL.

Comparison of SFE with the Soxhlet Extraction Method

The comparison of the extraction methods (Table 3) shows that the soxhlet extraction is time-consuming and uses large volumes of organic solvents. The use of large volumes of solvents implies additional costs, due to the fee associated with the purchasing and disposal of toxic solvents, and the environmental hazard. The SFE method proved to be a clean and simple extraction method for its low consumption of organic solvents, reduced operation procedures, and short extraction time. Moreover, the efficiency of SFE for tripterine is higher than that of the soxhlet extraction.

Figure 5 shows the chromatogram of the roots extract derived from SFE and soxhlet extraction. For the soxhlet extraction, many impurities were extracted, so cleaning up over silica gel column chromatography is often included to remove the impurities and yield a better baseline. For the extract obtained from SFE, the cleanup procedure can be omitted; the impurities are less.

Determination of Tripterine in *T. wilfordii* with SFE

Tripterine in the materials listed in above were measured with the method described here. The results in Fig. 6 show that the content of tripterine is 0.0311% in the roots without bark while the content of tripterine is

Table 3. Comparison of SFE with soxhlet extraction method

	Consumption of solvents				
Extraction method	Methanol (mL)	Chloroform (mL)	n-butanol (mL)	Material (g)	Extraction time (h)
Soxhlet extraction	200	150	None	8.0	5.0
SFE	None	None	1.0	1.0	0.5

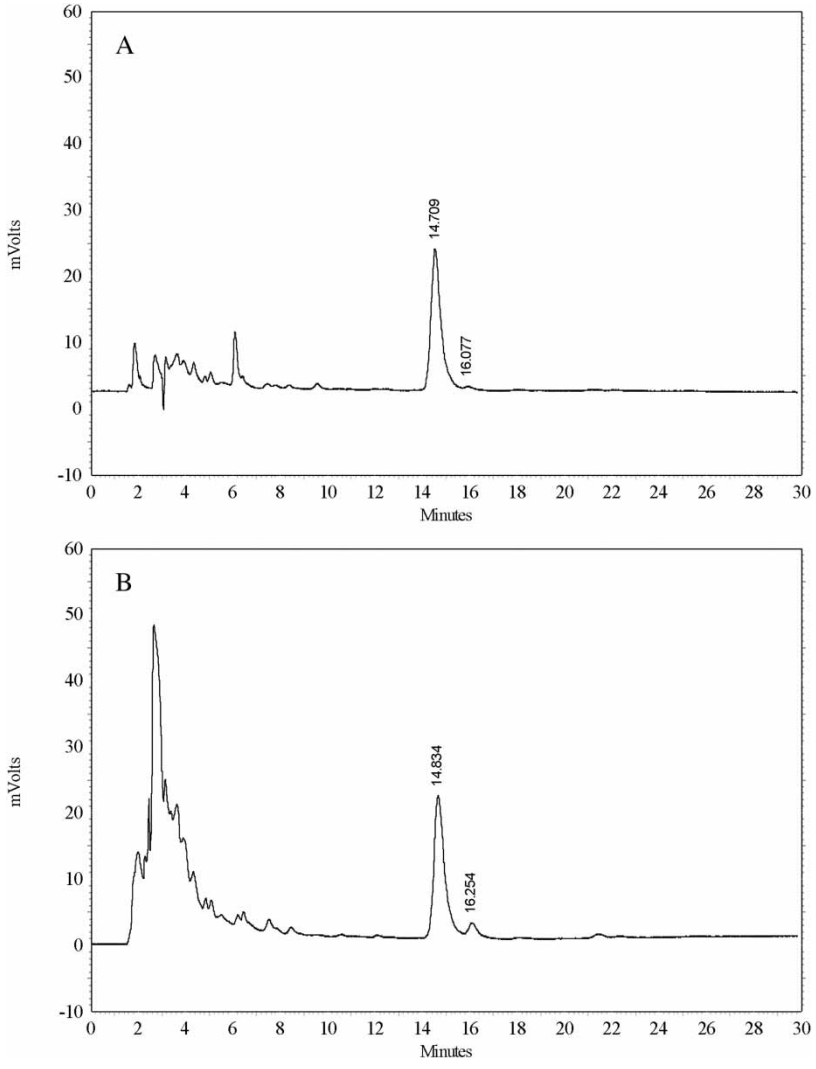


Figure 5. HPLC chromatogram of roots extract derived from SFE (A) and roots extract derived from soxhlet extraction (B).

0.3781% in the whole roots for the *T. wilfordii* growing in Hunan province. So, it can be concluded that tripterine mainly exists in the roots' bark. For the roots without bark from different planting areas, the content of tripterine is also different. The content of tripterine is the highest in the material from Guangxi province and the content of tripterine in the material from Hunan province takes second place.

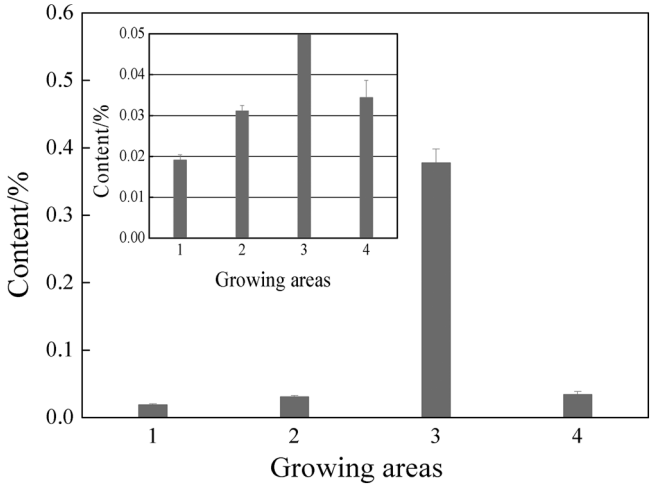


Figure 6. Determination of tripterine in *T. wilfordii* with SFE.

CONCLUSIONS

A new pretreatment method of SFE for determination of tripterine in *Tripterygium wilfordii* Hook. F was found and the optimized conditions were finally determined, where n-butanol was selected as cosolvent and the ratio of n-butanol to the material was 1.0 mL/g material. The extraction temperature and pressure were 40°C and 35 MPa, respectively. The static and dynamic extraction time were both 15 min. The validation of the method by determination of the linearity and precision, stability, recovery, and limits of detection and quantification indicated good reliability of the SFE method. Recovery of tripterine for SFE varied from 90.5 to 103.2% for samples with different content levels. Comparison of SFE with a soxhlet extraction method shows that SFE is a preferred simple, rapid, low-cost, clean and effective pretreatment method for the determination of tripterine in the roots of *T. wilfordii*. The selectivity of SFE for tripterine is high and the extract can be analyzed without further cleanup procedures.

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