

Full Length Research Paper

Rapid determination of cinnamic acid and harpagoside in a traditional Chinese medicine of *Scrophularia ningpoensis* by microwave-assisted extraction followed by high performance liquid chromatography (HPLC)

Taomin Huang*, Nianzu Chen, Yonghua Lai, Donglei Wang, Jinchao Yan and Jifeng Gu

Department of Pharmacy, Eye and ENT Hospital, Fudan University, Fenyang Road, No. 83, Shanghai 200031, China.

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Cinnamic acid and harpagoside are important active components present in *Scrophularia ningpoensis*, which has been used in the treatment of several diseases such as pharyngalgia. In the present study, a novel method based on high performance liquid chromatography (HPLC) following microwave-assisted extraction (MAE) was developed for the determination of cinnamic acid and harpagoside in a traditional Chinese medicine (TCM) of *S. ningpoensis*. Effective chromatographic separation was achieved on a C18 column at the detection wavelength of 278 nm. Microwave power of 400 W and irradiation time of 4 min were found to be the optimum conditions for the MAE process. In addition, to demonstrate the feasibility of the MAE-HPLC method, the conventional ultrasound extraction (USE) method was used for the analysis of cinnamic acid and harpagoside in the TCM. The results indicated that MAE-HPLC is a simple, rapid, efficient, and low cost method for the determination of cinnamic acid and harpagoside in TCM as well as a potential tool for the assessment of TCM quality.

Key words: *Scrophularia ningpoensis*, Cinnamic acid, harpagoside, microwave-assisted extraction, liquid chromatography, traditional Chinese medicine.

INTRODUCTION

Scrophularia ningpoensis Hemsl (Xuanshen in Chinese), recorded in the Pharmacopoeia of China (TSPCPR, 2005), is one of the most widely used TCMs. It has been commonly used in clinical practice for the treatment of several diseases, such as pharyngalgia, rheumatism, arthritis, tussis, constipation, and conjunctival congestion (Colas et al., 2006; Baghdikian et al., 1997; Dai et al., 2004). The main bioactive components of *S. ningpoensis* are cinnamic acid and harpagoside (Figure 1). Harpagoside is known to have anti-inflammatory, analgesic, antispasmodic, anti-anoxia, anti-arrhythmias, positive inotropic effects, and so on (Lanthers et al., 1992;

Andersen et al., 2004; Fiebich et al., 20010) and cinnamic acid is known to have antifungal, anti-oxidative activities (Lee et al., 2004; Neogi et al., 2003) and is capable of inducing differentiation of tumor cells.

In China, *S. ningpoensis* is cultured in several areas, such as in the Zhejiang, Henan, and Shandong provinces. It has been identified that various natural conditions including sun, oil, and climate lead to discrepancy in the TCM quality. It is possible to evaluate the TCM quality by the determination of cinnamic acid and harpagoside in the TCM. Thus, cinnamic acid and harpagoside play a key role in the course of disease treatment. This has resulted in the development of a simple, rapid, low-cost method for the determination of cinnamic acid and harpagoside in the TCM samples.

So far, several analytical methods have been reported to quantify some of the above-mentioned ingredients,

*Corresponding author. E-mail: tmhuang@fdeent.org. Tel/Fax: +86-21-64373955.

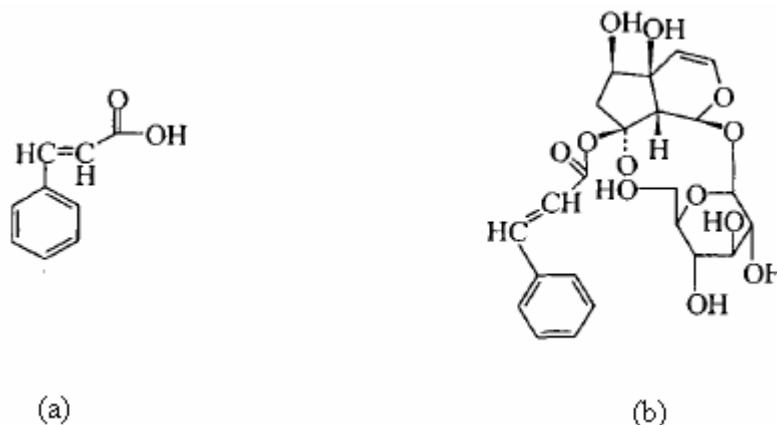


Figure 1. The structures of (a) cinnamic acid and (b) harpagoside.

such as micellar electrokinetic capillary chromatography (Lu et al., 2004; Huang et al., 1997a,b) capillary electrophoresis (Li et al., 1999) chemiluminescence detection (Han et al., 1999) high-performance liquid chromatography with ultraviolet detection (HPLC–UV), relative fluorescence (HPLC–RF), electrochemical detection (HPLC–ECD) (Dai et al., 2004; Song et al., 2002; Sladkovsky et al., 2001; Okamura et al., 1999; Guenther et al., 2005; Diaz et al., 1998; Guillerault et al., 1994) differential-pulse polarography (Ferreira et al., 1996) chemometrics (Riahi et al., 2009).

In the above-mentioned techniques, it was necessary to isolate and extract cinnamic acid and harpagoside from the TCM prior to the analyses. This could be done using various extraction techniques, such as soxhlet extraction, steam distillation (SD), hydro-distillation, and solvent extraction (Deng et al., 2006a, b; Dong et al., 2006; Yu et al., 2004; Li et al., 2004). However, the extraction methods could yield low extraction efficiency and result in the presence of toxic solvent residue in the extract. Moreover, these extraction procedures are time-consuming. Owing to such shortcomings, a new technique has been developed that typically uses less solvent, time, and energy such as supercritical fluids, microwave, and infrared light extraction.

Of late, there has been widespread interest in the application of microwave heating to the analysis of active compounds in plant herbs (Bilia et al., 2002; Deng et al., 2006c, 2007). The main advantage of microwave-assisted extraction (MAE) is the reduction of organic solvent as well as extraction time. Thus, MAE could be used for the determination of cinnamic acid and harpagoside in the TCM. HPLC–UV is a widespread method in laboratories (Okamura et al., 1999). Therefore, to develop a MAE method based on HPLC–UV is very useful and promising.

In this study, for the first time, MAE followed by high performance liquid chromatography was developed for the quantitative analysis of cinnamic acid and

harpagoside in the *S. ningpoensis*. The extraction conditions were optimized and the method validations were also performed.

MATERIALS AND METHODS

S. ningpoensis sample and chemicals

Dried roots of *S. ningpoensis* Hemsl were obtained from the eye and ENT Hospital of Fudan University in Shanghai, China. The TCM samples were ground into fine powder. Cinnamic acid (analytical grade, lot No.111730-200604) (Figure 1a) and harpagoside (analytical grade, lot No.110786-200503) (Figure 1b) were both purchased from the National Institute for the Control of Pharmaceuticals and Biological products in Beijing, China. Methanol (HPLC grade) was purchased from Merck (New Jersey, USA), and acetic acid was obtained from the revitalization of Chinese chemical plant in Jiangsu, China. Ethylparaben (analytical grade, lot No. 20080701) was obtained from the Taishan Xinning Pharmaceutical Co. Ltd. in Guangzhou, China, as it was not detected in the samples of *S. ningpoensis* Hemsl, and was used as the Internal Standard (IS) in the study. Double distilled water was obtained from a Millipore (USA) Direct-Q water-purification system.

Microwave-assisted extraction procedure

The MAE apparatus used in this study is illustrated in Figure 2. The microwave oven, with a maximum delivered power of 700 W, was purchased from Haier Company in Qingdao, China. Here it should be mentioned that since a hole in the casing of the microwave unit might cause energy to leak, the analyst must take precautions when using the microwave oven (Deng et al., 2006a, b).

The *S. ningpoensis* samples were desiccated and ground into powder. Subsequently, 1.25 g of *S. ningpoensis* was put into a 100 ml round-bottom flask containing 50 ml of distilled solvent spiked with 30 µg/ml of IS. The flask was then placed in the MO-2270M1 model microwave oven. The flask with the sample was heated by the microwave at a power of 200–700 W for 1–6 min. Simultaneously, a condenser with a continuous flow of cooling water was used to condense the solvent vapor in order to allow the solvent to repeatedly take part in the extraction process. After the microwave irradiation was complete, the flask was cooled in the air. Subsequent to the cooling, the solvent was centrifuged at 1200 x g

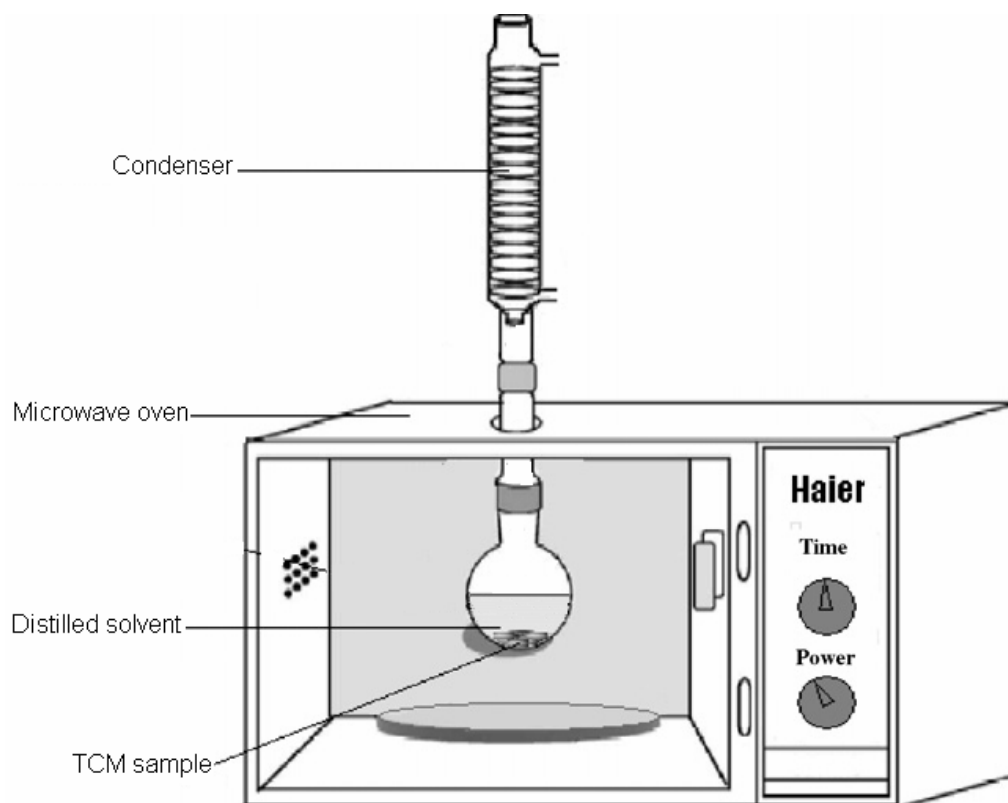


Figure 2. The apparatus of MAE extraction.

for 10 min and the clear supernatant solution (20 μ l) was analyzed by HPLC.

Ultrasonic extraction procedure

An ultrasonicator model B5500s-MTH (Branson, Shanghai, China) was used in the USE procedure. For this procedure, 1.25 g of *S. ningpoensis* was put into a 50 ml volumetric flask containing 50 ml of distilled solvent spiked with 30 μ g/ml of IS. The flask with sample completely immersed in the solvent was sonicated with continuous power for 30 min. After the USE procedure was complete, the flask was cooled in the air. Subsequent to the cooling, the solvent was centrifuged at 1200 \times g for 10 min and the clear supernatant solution (20 μ l) was analyzed by HPLC.

HPLC analysis

Chromatographic analysis was performed using an Agilent (Palo Alto, CA, USA) 1100 LC system equipped with a G1311A binary pump, a G1322A vacuum degasser, a G1316A thermostatted column compartment, a G1314A variable wavelength UV-visible detector, and an HP 1100 series manual injector with 20 μ l fixed loop. The detector was operated at 278 nm and the peak areas were automatically integrated by use of the Hewlett-Packard ChemStation software program (Rev. A. 10. 02 [1757]).

The analytes were separated on a Diamonsil C18 analytical column (200 mm \times 4.6 mm i.d., 5 μ m particle size). The mobile phase consisted of methanol-1% acetic acid aqueous solution (55:45, V/V) at a flow-rate of 1 ml/min. All analyses were performed

under isothermal conditions at 25°C. The peak area measurements were used for the quantification.

Preparation of calibration solution

Stock solutions (500 μ g/ml) of cinnamic acid and harpagoside were prepared by dissolving cinnamic acid and harpagoside into methanol. The stock solution of IS (300 μ g/ml) was prepared with water. All the stock solutions were stored in a refrigerator at 4°C. For quantitative analysis of cinnamic acid and harpagoside in *S. ningpoensis*, working standard solutions with the concentrations of 5, 10, 25, 50, 75, and 100 μ g/ml were prepared by diluting stock solutions of cinnamic acid and harpagoside with solvent of ethanol: water (50:50 V/V) spiked with 30 μ g/ml of IS. The calibration samples were then aliquoted and stored at 4°C.

Optimization of MAE parameters

In the proposed method, cinnamic acid and harpagoside were isolated from *S. ningpoensis* using MAE. The experimental parameters of MAE (extraction solvent, microwave power, and irradiation time) were examined. The analysis of the average peak area of cinnamic acid and harpagoside was replicated three times in order to determine the optimal parameters.

Validation of the HPLC method

To assess the limits of detection (LOD) and quantification (LOQ),

Table 1. The extraction efficiencies of cinnamic acid and harpagoside in different extraction solvent.

Solvent	Cinnamic acid (%)	Harpagoside (%)
Water	0.029	0.119
95% ethanol	0.032	0.128
75% ethanol	0.038	0.145
50% ethanol	0.035	0.141
30% ethanol	0.032	0.118
95% methanol	0.028	0.115
75% methanol	0.039	0.145
50% methanol	0.034	0.142
30% methanol	0.031	0.125

the dilute solution of the reference compounds was further diluted to a series of concentrations with the solvent of ethanol: water (50:50). The LOD and LOQ were determined as signal-to-noise (S/N) ratios of 3 and 10, respectively.

The intra- and inter-day precisions were determined by analyzing the calibration samples during a single day and on three consecutive days, respectively. The relative standard deviation (RSD, %) was calculated on the basis of the peak area obtained.

To confirm the reproducibility, six different working solutions prepared from the *S. ningpoensis* sample were analyzed. The RSD value was considered as a measure of precision and reproducibility. The recovery test was used for evaluating the accuracy of this method. Accurate amounts of reference compounds were added to a *S. ningpoensis* sample, and then extracted and analyzed by the proposed method. The recoveries were estimated using the following formula: recovery (%) = (amount found – original amount) / amount added x 100%. Sextuple measurements were performed by MAE-HPLC.

RESULT AND DISCUSSION

Optimization of MAE parameters

The *S. ningpoensis* sample was treated using the MAE method described above. The experiment parameters of MAE (extraction solvent, microwave power, and irradiation time) were examined (Deng et al., 2006 a, b). The extraction efficiencies of cinnamic acid and harpagoside in the sample were investigated, and the analytical results obtained by HPLC measurement under different conditions were compared in order to obtain the optimum extraction conditions.

Selection of extraction solvent

The extraction solvent of MAE was first considered. MAE of the TCM sample was performed at 200 W for 6 min. The effects of the extraction solvent on water, 95% ethanol, 75% ethanol, 50% ethanol, 30% ethanol, 95% methanol, 75% methanol, 50% methanol, and 30% methanol analysis in *S. ningpoensis* are shown in Table 1. The results obtained by the HPLC measurement were compared, and the solvent that offered the best extraction

efficiencies was selected. The observed concentrations in Table 1 are mean values of triplicate measurements. It can be seen that although 75% methanol provided the best extraction efficiencies, 75% ethanol was used as the extraction solvent owing to its less toxic nature. The reason for the difference in the extraction efficiencies of the tested solvents is, however, not clear.

Microwave extraction time and power

The effects of microwave extraction time and microwave power on the analytical results are shown in Figure 3. 75% ethanol was used as the extraction solvent for MAE, and the analytical results obtained by HPLC measurement were compared based on the different values of microwave extraction time and microwave power. It was found that the best extraction efficiencies were obtained for the microwave power of 400 W and the extraction time of 4 min. However, the peak areas reached a relative maximum at the extraction time of 4 min when the microwave power was 400 and 700 W.

A subsequent decrease was observed for a longer exposure time, which was possibly owing to the degradation of the compounds as a result of the higher temperature. Meanwhile, it was observed that response signals generally decreased with an increase in the microwave power at the same irradiation time. This behavior could also be attributed to the same reason of degradation of the analytes. As seen in Figure 3, the best extraction efficiencies were obtained at the microwave power of 400 W and the extraction time of 4 min. Thus, these MAE conditions were used for conducting the study.

Optimization of the chromatographic conditions

The HPLC parameters were optimized by investigating the influence of the mobile phase and detection wavelength on the resolution and sensitivity. The initial separation of the *S. ningpoensis* extracts was carried out

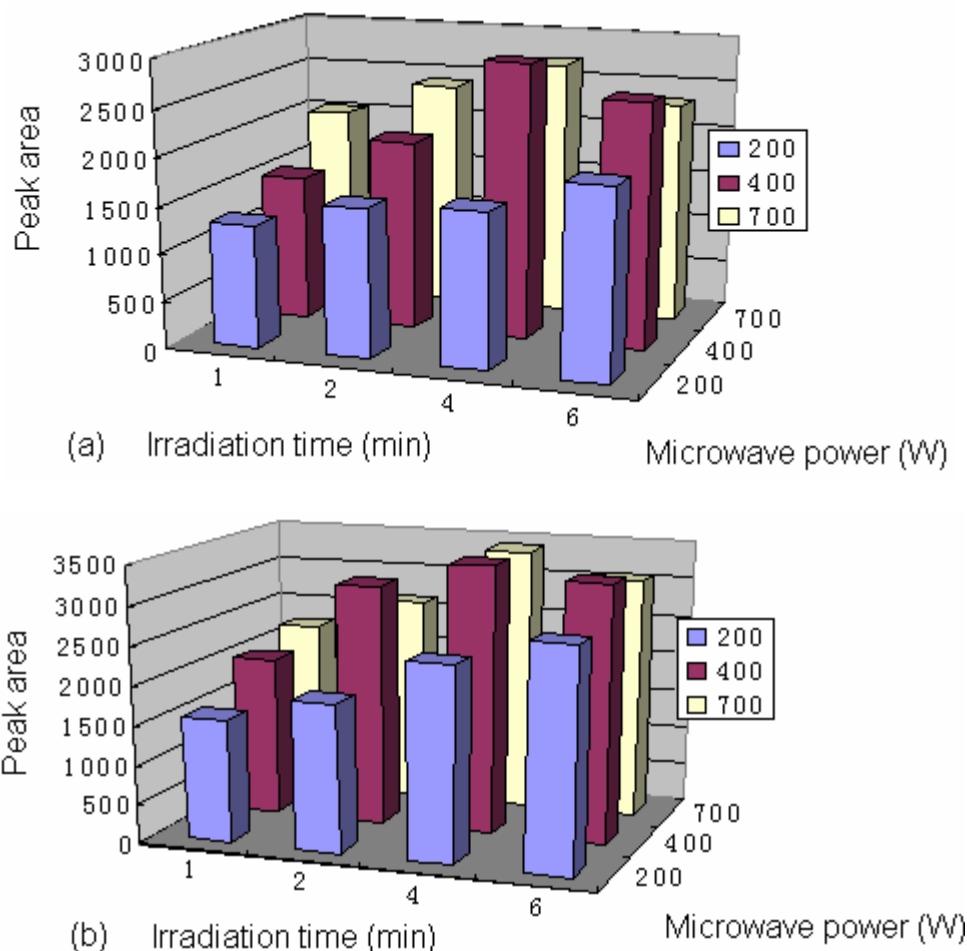


Figure 3. The effects of microwave powder and irradiation time on the extraction efficiencies of (a) cinnamic acid and (b) harpagoside in *S. ningpoensis*.

on a Diamonsil C18 column using a mixture of methanol and water as the mobile phase. However, the separation achieved was not beneficial. Thus, by considering the presence of acidic ingredients in the herbal extraction, a small amount of acetic acid was added to the mobile phase in order to suppress the ionization of these compounds. This helped in achieving the optimum mobile phase with an aqueous phase (containing 1% acetic acid).

Method validation

The representative chromatograms of standards with cinnamic acid and harpagoside (equivalent to 25 $\mu\text{g/ml}$) as well as the *S. ningpoensis* sample spiked with 30 $\mu\text{g/ml}$ IS are shown in Figure 4. It can be seen that cinnamic acid and harpagoside were well resolved with good symmetry with retention times of 10.3 and 12.7 min, respectively. As seen in Figure 4b, no significant direct interference was observed at the retention time of the

analyte.

In order to obtain the calibration curves, HPLC analysis of the calibration solutions was performed in the range of 5 to 100 $\mu\text{g/ml}$ at known concentrations (5, 10, 25, 50, 75, and 100 $\mu\text{g/ml}$) spiked with 30 $\mu\text{g/ml}$ of IS. For each solution, three replicated analyses were performed. The calibration curve for quantifying cinnamic acid was obtained as: $y=0.08589x-0.03736$, $r=0.9995$ (y : peak area ratio of cinnamic acid to IS; x : cinnamic acid concentration, $\mu\text{g/ml}$). Similarly, the calibration curve for quantifying harpagoside was obtained as: $y=0.02487x-0.00438$, $r=0.9996$ (y : peak area ratio of harpagoside to IS; x : harpagoside concentration, $\mu\text{g/ml}$).

The results of precision, reproducibility, and recovery tests are shown in Tables 2 and 3. It can be seen that the RSD values were below 3% and the recovery values were between 95 and 105%. The detection limit values for cinnamic acid and harpagoside were calculated on the basis of the S/N ratio of 3, and the values obtained were 0.02 and 0.60 $\mu\text{g/ml}$, respectively. These values are observed to be considerably lower than the respective

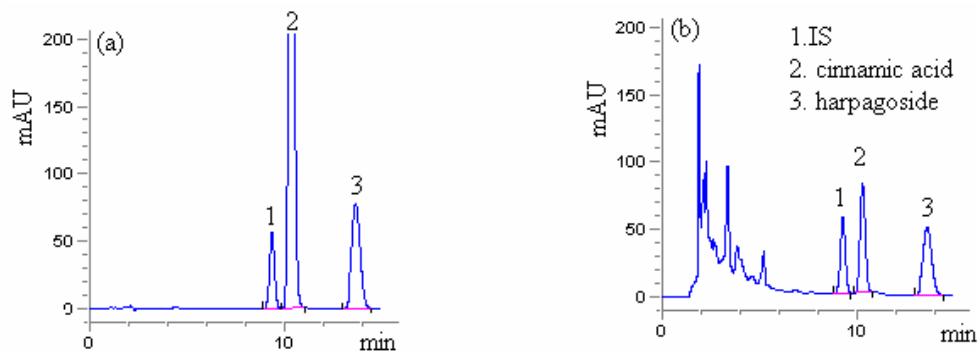


Figure 4. Representative chromatograms of standards with cinnamic acid and harpagoside (equivalent to 25 µg/ml) (a) and *S. ningpoensis* sample (b) spiked with 30 µg/ml IS by MAE.

Table 2. Precisions of cinnamic acid and harpagoside.

Added (µg/ml)	Precision of cinnamic acid				Precision of harpagoside			
	Intra-day (n=3)		Inter-day (n=3)		Intra-day (n=3)		Inter-day (n=3)	
	Measured (µg/ml)	RSD (%)	Measured (µg/ml)	RSD (%)	Measured (µg/ml)	RSD (%)	Measured (µg/ml)	RSD (%)
10	9.85	0.87	9.89	1.12	9.92	1.17	10.12	1.35
50	49.34	1.13	49.57	1.21	48.97	1.15	50.37	1.44
100	101.35	0.89	101.23	1.05	98.83	0.98	99.54	1.28

Table 3. Reproducibilities and recoveries of cinnamic acid and harpagoside.

Analyte	Reproducibility (n=6)		Recovery (n=6)			
	Mean (mg/g)	RSD (%)	Added (mg)	Recorded (mg)	Recovery mean (%)	RSD
Cinnamic acid	0.35	0.97	0.352	0.336	96.0	2.72
Harpagoside	1.40	2.62	1.405	1.449	103.5	1.98

concentrations of cinnamic acid and harpagoside in *S. ningpoensis*. Thus, it can be concluded that the proposed method is sensitive enough to analyze cinnamic acid and harpagoside in *S. ningpoensis*. The stability values of cinnamic acid and harpagoside were determined by periodic analysis of the same sample (0, 4, 8, 12, 24 and 32 h), and the values obtained were 2.14 and 2.95%, respectively.

Determination of cinnamic acid and harpagoside in *S. ningpoensis* sample

The HPLC chromatogram of cinnamic acid and harpagoside in *S. ningpoensis* obtained by MAE at the optimal condition is shown in Figure 4b. The concentrations of cinnamic acid and harpagoside in *S. ningpoensis* were calculated in accordance to the calibration curves, and the analytical results are listed in

Table 4.

In addition, to demonstrate the reliability of the quantification performed by the proposed method, the conventional method of USE followed by HPLC was also used to quantitatively analyze cinnamic acid and harpagoside in the *S. ningpoensis* samples. The analytical results are listed in Table 4. It can be seen in Table 4 that the cinnamic acid and harpagoside concentrations in the *S. ningpoensis* obtained from the proposed method were higher than the respective concentrations obtained from the USE method with only 4 min.

Cinnamic acid and harpagoside are the main active components in *S. ningpoensis*, which play an important role in the course of disease treatment. It has been demonstrated that quality assessment can be performed based on the concentrations of active constituents in TCMs. Thus, it can be concluded that the proposed method has the potential for quality monitoring of the

Table 4. The concentrations of cinnamic acid and harpagoside in *S. ningpoensis* samples by MAE and USE.

Samples (Batch No.)	Cinnamic acid (mg/g)		Harpagoside concentration (mg/g)	
	MAE	USE	MAE	USE
1	0.38±0.008	0.32±0.002	1.45±0.005	1.26±0.006
2	0.37±0.005	0.33±0.003	1.43±0.024	1.25±0.014
3	0.38±0.004	0.31±0.005	1.46±0.017	1.22±0.011

TCM, *S. ningpoensis*.

Conclusion

In the present study, a novel method based on HPLC following MAE was developed for the determination of cinnamic acid and harpagoside in a Traditional Chinese Medicine (TCM) of *S. ningpoensis*. Effective chromatographic separation was achieved on a C18 column at the detection wavelength of 278 nm. Microwave power of 400 W and irradiation time of 4 min were found to be the optimum conditions for the MAE process. The cinnamic acid and harpagoside concentrations in the *S. ningpoensis* obtained from the proposed method were higher than the respective concentrations obtained from the USE method with only 4 min.

The MAE technique followed by HPLC measurement was successfully applied for the determination of cinnamic acid and harpagoside in the *S. ningpoensis*. The results obtained for precision and recovery serve for method validation. Owing to the relatively lower method detection limit, less extraction time, and high extraction efficiency, a simple technique with the MAE method (Deng et al., 2006 a, b) could be used for the determination of cinnamic acid and harpagoside in the TCM. The results indicated that the proposed method, MAE-HPLC, is feasible for analyzing the bioactive components in *S. ningpoensis*.

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