Journal of Chromatography A, 1218 (2011) 2827–2834

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Application of accelerated solvent extraction coupled with high-performance counter-current chromatography to extraction and online isolation of chemical constituents from *Hypericum perforatum* L.

Yuchi Zhang[a], Chunming Liu[a,*], Min Yu[b], Zhengkun Zhang[c], Yanjuan Qi[a], Jing Wang[a], Guimei Wu[a], Sainan Li[a], Jing Yu[a], Yang Hu[a]

[a] *Central Laboratory, Changchun Normal University, Changchun 130032, China*
[b] *Department of Nephrology, Affiliated Hospital, Academy of Military Medical Sciences, Beijing 100071, China*
[c] *Institute of Plant Protection, Jilin Academy of Agricultural Sciences, Gongzhuling 136100, China*

ARTICLE INFO

Article history:
Received 1 January 2011
Received in revised form 3 March 2011
Accepted 4 March 2011
Available online 12 March 2011

Keywords:
High-performance counter-current chromatography
Accelerate solvent extraction
Hypericum perforatum L.
Extraction and isolation

ABSTRACT

Accelerated solvent extraction (ASE) coupled with high-performance counter-current chromatography (HPCCC) was successfully used for the extraction and online isolation of five chemical constituents from the plant *Hypericum perforatum* L. The upper phase of the solvent system of ethyl acetate–methanol–water (5:2:5, v:v:v) was used as both the ASE solvent and the HPCCC stationary phase. Two hydrophobic compounds including 28.4 mg of hyperforin with a HPLC purity of 97.28% and 32.7 mg of adhyperforin with a HPLC purity of 97.81% were isolated. The lower phase of ethyl acetate–methanol–*n*-butanol–water (5:2:2.5:12, v:v:v:v) was used as both the ASE solvent and CCC stationary phase. Three hydrophilic compounds of 12.7 mg of 3,4,5-*O*-tricaffeoylquinic acid with a HPLC purity of 98.82%, 15.2 mg of 1,3,5-*O*-tricaffeoylquinic acid with a HPLC purity of 99.46% and 42.5 mg of 3-*O*-caffeoylquinic acid with a HPLC purity of 96.90%, were obtained in a one-step extraction–separation process with less than 3 h from 10.02 g of raw material of *H. perforatum*. The targeted compounds isolated, collected and purified by HPCCC were analyzed by high performance liquid chromatography (HPLC), the chemical structures of all five compounds above mentioned were identified by UV, MS and NMR.

1. Introduction

Hypericum perforatum L., a typical traditional Chinese medicine, is used all over the world for the treatment of depression [1,2]. Additionally, it has been reported that the extracts of *H. perforatum* may have an inhibitory effect [3,4], on human bladder cancer cells [5], and act as an antioxidant [6] and anticonvulsant [7]. The extracts of *H. perforatum* contain many constituents with documented biological activity such as quinic acids [8], phloroglucinols [9] and a broad range of flavonoids [8–10]. Exposure of the extracts of *H. perforatum* to light may lead to the degradation of phloroglucinols, which are extremely sensitive to oxidation and unstable in solution on exposure to air [11,12], therefore phloroglucinols are difficult to separate and isolate by conventional method. In this case, a method combining an extraction system with an isolation system online to avoid the exposure of the extracts to air and light is urgently needed.

Quinic acids have previously been shown to possess a multitude of pharmacological activities [13–15]. Tricaffeoylquinic acids have strong antihyperglycemic [16] and antimutagenic effects. However, the supplies of tricaffeoylquinic acids have been limited due to their very low content in natural plants and difficulties in isolating their pure compounds from natural sources. More efficient extraction and separation methods to provide bioactive components with high sample recovery are also needed.

Accelerated solvent extraction (ASE) has several advantages over traditional solvent extraction methods, including shorter extraction time, lower solvent consumption, higher extraction yields, high reproducibility [17] and less extraction discrimination [18]. Many applications of ASE have been reported in food and pharmaceutical field [19–22]. Compared to conventional liquid–solid separation methods, counter-current chromatography (CCC) has the advantage of sample recovery as no solid phase is employed, thus preventing the irreversible adsorption of analytes and allowing for a theoretical recovery rate of 100% [23–27]. Counter current chromatography has been extensively used for the separation and purification of natural products and other researches [26–30].

This paper is the first to report the combination of ASE and HPCCC (ASE–HPCCC) online, and the two instrumental setups of ASE–CCC successfully applied in this experiment. As a consequence of using the two instrumental setups of online extraction–isolation

* Corresponding author. Tel.: +86 431 8616 8777; fax: +86 431 8616 8777.
E-mail address: chunmingliu2000@yahoo.com.cn (C. Liu).

0021-9673/$ – see front matter.
doi:10.1016/j.chroma.2011.03.007

Table 1
Description of ASE extractions.

No.	Extraction solvent	For hydrophobic compounds[a] (upper phase as extraction solvent)			For hydrophilic compounds[b] (lower phase as extraction solvent)		
		Temperature (°C)	Pressure (psi)	Content dry weight[c] (mg/g)	Temperature (°C)	Pressure (psi)	Content dry weight[c] (mg/g)
1	HEX–EtOAc–MeOH–water (1:5:1:5, v:v:v:v)	60	600	0.26 ± 30.01	100	900	–[d]
2	HEX–EtOAc–MeOH–water (1:4:1:5, v:v:v:v)	90	800	0.25 ± 0.01	120	1200	–
3	HEX–EtOAc–MeOH–water (0.5:3:1:5, v:v:v:v)	130	1000	0.62 ± 0.02	140	1500	–
4	HEX–EtOAc–MeOH–water (0.5:2.5:1:5.5, v:v:v:v)	150	1200	0.53 ± 0.02	160	1800	–
5	EtOAc–MeOH–water (4:1:7, v:v:v)	80	600	7.33 ± 0.12	100	900	8.31 ± 0.16
6	EtOAc–MeOH–water (4:2:5, v:v:v)	100	800	7.44 ± 0.14	120	1200	8.42 ± 0.16
7	EtOAc–MeOH–water (5:2:5, v:v:v)	130	1000	7.65 ± 0.14	140	1500	8.45 ± 0.15
8	EtOAc–MeOH–water (6:2:4, v:v:v)	150	1200	7.45 ± 0.16	160	1800	8.58 ± 0.14
9	EtOAc–MeOH–BuOH–water (5:2:5:15, v:v:v:v)	80	600	7.10 ± 0.17	100	900	8.31 ± 0.15
10	EtOAc–MeOH–BuOH–water (5:2:2.5:12, v:v:v:v)	100	800	7.20 ± 0.16	140	1200	8.57 ± 0.18
11	EtOAc–MeOH–BuOH–water (6:2:1:10, v:v:v:v)	130	1000	7.30 ± 0.16	160	1500	8.53 ± 0.17
12	EtOAc–MeOH–BuOH–water (4:3:2:8, v:v:v:v)	150	1200	7.21 ± 0.17	180	1800	8.60 ± 20.13
13	EtOAc–BuOH–water (5:1:8, v:v:v)	60	600	7.79 ± 0.21	120	900	4.35 ± 0.10
14	EtOAc–BuOH–water (5:2:5, v:v:v)	90	800	7.91 ± 0.21	140	1200	5.06 ± 0.09
15	EtOAc–BuOH–water (3:1:2, v:v:v)	130	1000	7.89 ± 0.21	160	1500	5.09 ± 0.09

[a] Amounts of hyperforin plus adhyperforin.
[b] Amounts of 3,4,5-*O*-tricaffeoylquinic acid plus 1,3,5-*O*-tricaffeoylquinic acid and 3-*O*-caffeoylquinic acid.
[c] Data are expressed as mean ± SD. For each sample $n = 3$.
[d] Not determined.

process, five compounds, including hyperforin, adhyperforin, 3,4,5-*O*-tricaffeoylquinic acid, 1,3,5-*O*-tricaffeoylquinic acid and 3-*O*-caffeoylquinic acid were separated and purified from *H. perforatum*.

2. Experimental

2.1. Reagents and materials

Ethyl acetate, *n*-hexane, *n*-butanol, methanol and ethanol used were of analytical grade (Beijing Chemicals, Beijing, China). Water was purified on a Milli-Q water purification system (Millipore, Boston, USA). Acetonitrile and acetic acid were of HPLC grade (Fisher Scientific, Pittsburg, PA, USA). *H. perforatum* L. was harvested from Qiannan autonomous region of Guizhou province (Qiannan, China) and identified by Yuchi Zhang (Changchun Normal University, Changchun, China).

2.2. Apparatus

Accelerated Solvent Extraction 150 System (Dionex, Sunnyvale, CA, USA) with 100 ml stainless steel ASE vessels was used for the pressurized liquid extraction. High-performance countercurrent chromatography was performed on a DE Spectrum HPCCC (Dynamic Extractions, Slough, UK). The multilayer coil separation column was prepared by winding a 28 m × 2.6 mm I.D. PTFE tube directly onto one of the holders forming multiple coiled layers to give a total capacity of 125 ml. The β-value varied from 0.33 at the internal terminal to 0.58 at the external terminal ($R = 8$ cm, $\beta = r/R$, where r is the distance from the coil to the holder shaft and R is the revolution radius or the distance between the holder axis and the central axis of the centrifuge). The rotation speed was adjusted in a range of from 0 to 1600 rpm and 1400 rpm was used in the present study. The HPCCC system was equipped with a solvent delivery module of BT 8100 (Biotronic, Maintal, Germany), an integrator of D-2500 (Merck Hitachi, Darmstadt, Germany), and an injection valve with a sample loop of 10 ml. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Finnigan LCQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, USA). High-performance liquid chromatography (HPLC) was carried out on a Waters 2695 coupled with a Waters 2998 Diode array detector (DAD) (Milford, USA). Nuclear magnetic resonance spectra were recorded on a Bruker AV 500 spectrometer (Bruker BioSpin, Rheinstetten, Germany).

2.3. Accelerated solvent extraction

An ASE 150 System with 100 ml stainless steel ASE vessels was used for the pressurized liquid extraction. About 10.02 g of *H. perforatum* powder was mixed homogeneously with the same weight of diatomaceous earth and placed into the extraction cell. The extraction cells were placed into the ASE system and the extraction conditions and process were as follows: firstly, static time of 5 min, followed by a flush elution with 60% volume, and followed by the nitrogen purge of 60 s, and extract one time [31]. The extraction pressure and the extraction temperature were optimized in the subsequent experiments (provided in Table 1).

2.4. Selection of the two-phase solvent systems of HPCCC and ASE extraction solvent

In view of the upper phase and lower phase of the two-phase solvent system of HPCCC used as ASE extraction solvent, we investigated the extraction solvent and isolation solvent system simultaneously. A series of solvent systems was selected as the ASE solvent and HPCCC separation solvent systems with the extraction conditions summarized in Table 1. First, the upper phase of the solvent system was used as the ASE solvent for extracting the hydrophobic compounds, and after extraction, 3 ml of ASE solution was added into a test tube, and then the same volume of the

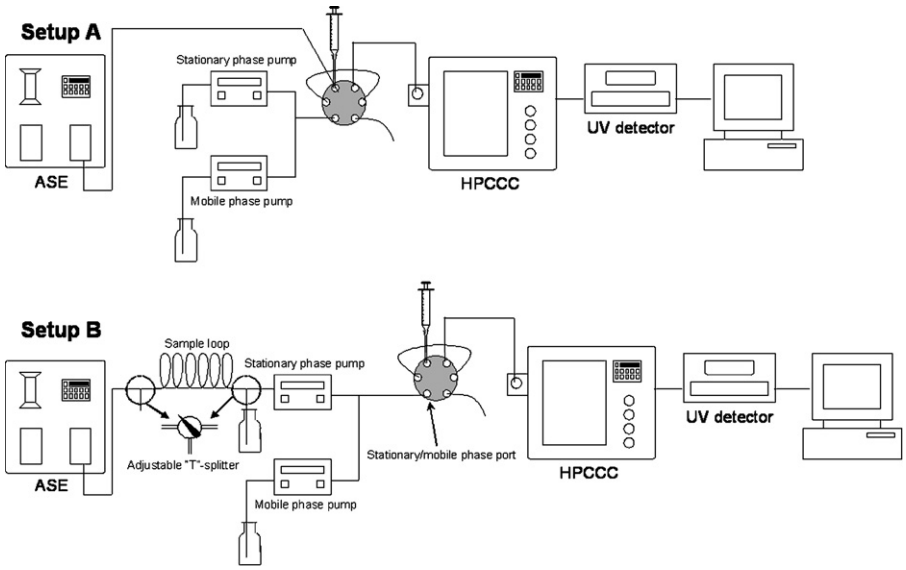


Fig. 1. The diagram presents two setups (A and B) enabling hyphenation of ASE and HPCCC. The first instrumental setup of ASE–HPCCC: the powder of raw plant material was mixed with diatomaceous earth, and then extracted by ASE. After extraction by the solvent of upper phase and/or lower phase we chose, the extracted solution was injected into the HPCCC system with the aid of pressure from the ASE *via* the sample injection port of the HPCCC. The second instrumental setup of ASE–HPCCC was created by turning the first T-splitter in order to make the ASE system and the sample loop interlinked, simultaneously the second T-splitter was turned to make the sample loop and stationary loop interlinked. The ASE solution was pumped into a sample loop; then the first T-splitter was turned to make the sample loop interlinked with air while the second T-splitter was turned to make the sample loop and stationary phase pump interlinked. The stationary pump was then turned on to pump the extracted solution into the HPCCC *via* the stationary/mobile phase port.

corresponding lower phase was added to it. Having been shaken vigorously for 10 min, the mixture was separated by centrifugation for 3 min. Then, an aliquot of each phase (0.5 ml) was delivered into a test tube separately, each was diluted with an equal volume (1 ml) of methanol and analyzed by HPLC. The *K* value was expressed as the peak area of the target compound in the upper phase divided by that in the lower phase (*K*, for hydrophobic compounds). Second, the lower phase of the solvent system above mentioned was used as the ASE solvent for extracting the hydrophilic compounds, and then 3 ml of the extracted solution was added into a test tube, and then the same volume of the corresponding upper phase was added to it. With the same operation, the *K* value was expressed as the peak area of the target compound in the lower phase divided by that in the upper phase (for hydrophilic compounds). The *K* value is the ratio of the contents of the solute distributed between the two mutually equilibrated solvent phases. Usually the composition of the two-phase solvent system is selected according to the partition coefficient of the targeted compound of the crude sample. The *K* value was calculated based on the HPLC peak areas obtained prior (A_1) and after equilibration (A_2) using the equation: $K = (A_1 - A_2)/A_2$.

2.5. ASE coupled with HPCCC online separation procedure

Two instrumental setups of ASE coupled with HPCCC online were successfully used in this experiment. In the first instrumental setup of ASE–HPCCC (see Fig. 1), the powder of raw plant material was mixed with diatomaceous earth, and then extracted by ASE. After extraction by the solvent of upper phase and/or lower phase we chose, the extracted solution was injected into the HPCCC system with the aid of pressure from the ASE *via* the sample injection port of the HPCCC. The second instrumental setup of ASE–HPCCC was created by turning the first T-splitter in order to make the ASE system and the sample loop interlinked, and simultaneously the second T-splitter was turned to make the sample loop and stationary phase loop interlinked. The ASE solution was pumped into a sample loop; then the first T-splitter was turned to make the sample loop interlinked with air while the second T-splitter was turned to make the sample loop and stationary phase pump interlinked. The stationary pump was then turned on to pump the extraction solution into the HPCCC *via* the stationary/mobile phase port (Fig. 1). Except those of the ASE solvent, other extraction parameters were described in Section 2.3. Before HPCCC separation was performed, the multilayered coiled column was first entirely filled with the upper phase (hydrophobic compounds) or lower phase (for hydrophilic compounds) as the stationary phase. Then the apparatus was rotated at 1400 rpm. In conjunction the mobile phase was pumped into the column at a flow-rate of 1.5 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the ASE solution was injected into the HPCCC system *via* the sample injection port or stationary/mobile phase port. The effluent of the column was continuously monitored with a UV detector at 254 nm. The temperature of the apparatus was set at 30 °C. Peak fractions were collected according to the elution profile and evaporated *via* a rotary evaporator. The residue was stored in a refrigerator for HPLC, MS and NMR analyses. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed.

2.6. HPLC analysis for K value and purity examination

The ASE extract and each HPCCC peak fraction were analyzed by using HPLC. The analysis was accomplished with an Agilent ODS C18 column (250 mm × 4.6 mm I.D., 5 μm) at 30 °C. Acetonitrile (Phase A) and 0.5% acetic acid in water (Phase B) were used as the mobile phase in gradient elution mode as follows: 0–60 min, 40–80% (Phase A, for upper-phase extract); 0–30 min, 13–30% (Phase B, for lower-phase extract). The flow-rate of the mobile phase was 1.2 ml/min. The effluents were monitored at 254 nm by a photodiode array detector. Identification of the HPCCC peak fractions was performed by MS and NMR techniques.

2.7. MS and NMR analyses

The ESI-MS conditions were optimized for a 3-*O*-caffeoylquinic acid detection prior to sample analyses, which was performed in

Table 2
Partition coefficient (K) values of hyperforin and adhyperforin in various two-phase solvent systems.

No.	Solvent system	K_1 [a]	K_2
1	Ethyl acetate–methanol–water (4:1:7, v:v:v)	0.62	0.71
2	Ethyl acetate–methanol–water (4:2:5, v:v:v)	0.85	0.91
3	Ethyl acetate–methanol–water (5:2:5, v:v:v)	1.60	1.94
4	Ethyl acetate–methanol–water (6:2:4, v:v:v)	2.25	3.06
5	Ethyl acetate–methanol–*n*-butanol–water (5:2:5:15, v:v:v:v)	1.73	2.17
6	Ethyl acetate–methanol–*n*-butanol–water (5:2:2.5:12, v:v:v:v)	1.92	2.39
7	Ethyl acetate–methanol–*n*-butanol–water (6:2:1:10, v:v:v:v)	2.19	2.74
8	Ethyl acetate–methanol–*n*-butanol–water (4:3:2:8, v:v:v:v)	6.12	6.68
9	Ethyl acetate–*n*-butanol–water (5:1:8, v:v:v)	16.26	19.24
10	Ethyl acetate–*n*-butanol–water (5:2:5, v:v:v)	15.95	19.02
11	Ethyl acetate–*n*-butanol–water (3:1:2, v:v:v)	18.32	22.94

[a] K_1: hyperforin; K_2: adhyperforin.

order to achieve the maximum sensitivity. As a result, the optimized MS conditions were obtained as follows: sheath gas flow rate: 60 bar; auxiliary gas flow rate: 10 bar; the electrospray voltage of the ion source: 5 kV; capillary voltage: 10 V; capillary temperature: 150 °C. The full scan of ions ranged from 100 to 2000 mass to charge ratios (m/z) units and conducted in the negative ion mode. MS/MS experiments were performed to obtain the detailed structural information of the compounds. NMR spectra were recorded at 25 °C on a Bruker AV 500 operated at a ^{1}H frequency of 500.13 MHz (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts (δ) were expressed in ppm and coupling constants (J) were reported in Hz. Samples were dissolved in deuterated methanol (CD_3OD).

3. Results and discussion

3.1. Selection of ASE extraction solvent

Several amounts of hydrophobic compounds were obtained by using different extraction solvents (Table 1). No matter which ASE solvent was used, a good repeatability was obtained. The calculated relative standard deviation (RSD, %: standard deviation divided by the mean and multiplied by 100) did not exceed 5.0%. According to the yield (the contents of hyperforin plus adhyperforin obtained from 1 g of crude plant material) data, if the upper phases of the solvent systems of *n*-hexane–ethyl acetate–methanol–water were used as the extraction solvent, the contents of hyperforin and adhyperforin were very low (0.25–0.62 mg/g). If the upper phases of ethyl acetate–methanol–water, ethyl acetate–methanol–*n*-butanol–water and ethyl acetate–*n*-butanol–water at different volume ratios were used as the extraction solvent, the yields of hyperforin and adhyperforin extracted with different solvent systems were not significantly different (7.10–7.91 mg/g). Therefore, the solvent systems of ethyl acetate–methanol–water at the volume ratios of 4:1:7, 4:2:5, 5:2:5 and 6:2:4 (v:v:v), the solvent systems of ethyl acetate–methanol–*n*-butanol–water at the volume ratios of 5:2:5:15, 5:2:2.5:12, 6:2:1:10 and 4:3:2:8 (v:v:v:v) and the solvent systems of ethyl acetate–*n*-butanol–water at the volume ratios of 5:1:8, 5:2:5 and 3:1:2 (v:v:v) to considered to meet the extraction requirements, and were selected for K evaluation.

The amounts of hydrophilic compounds extracted by different extraction solvents were also obtained with the result shown in Table 1. The solvent systems of ethyl acetate–*n*-butanol–water should not be taken into account, since the yield of 3,4,5-*O*-tricaffeoylquinic acid plus 1,3,5-*O*-tricaffeoylquinic acid and 3-*O*-caffeoylquinic acid were very low (38.16–42.45 mg/10 g). Therefore, the solvent systems of ethyl acetate–methanol–water at the volume ratios of 4:1:7, 4:2:5, 5:2:5 and 6:2:4 (v:v:v), the solvent systems of ethyl acetate–methanol–*n*-butanol–water at the volume ratios of 5:2:5:15, 5:2:2.5:12, 6:2:1:10 and 4:3:2:8 (v:v:v:v) were considered to meet the extraction requirements, and were also selected for K evaluation.

The temperature and pressure did not significantly influence the ASE (Table 1, extraction yields by the upper phase of ethyl acetate–methanol–water at the volume ratios of 4:1:7, 4:2:5, 5:2:5 and 6:2:4 were 7.33 mg/g, 7.44 mg/g, 7.65 mg/g and 7.45 mg/g respectively, RSD% value of the extraction yield by different extraction solvents was 1.78%, the extraction yields by the lower phase of ethyl acetate–methanol–*n*-butanol–water at volume ratios of 5:2:5:15, 5:2:2.5:12, 6:2:1:10 and 4:3:2:8 were 8.31 mg/g, 8.58 mg/g, 8.53 mg/g and 8.60 mg/g respectively, RSD% value of the extraction yield by different extraction solvents was 1.57%, did not exceed 2.0%). Then we selected optimized conditions which provide the high yields. The ASE conditions we optimized were: at a temperature of 130 °C, a pressure of 1000 psi, one cycle and a static time of 5 min.

3.2. Optimization of ASE–HPCCC conditions and identification of HPCCC fractions

3.2.1. Upper phase (organic phase) as the ASE solvent for hydrophobic compounds

Successful separation by HPCCC largely depends upon the selection of a suitable two-phase solvent system. According to the description in Section 3.1, the solvent systems we investigated were composed of ethyl acetate–methanol–water, ethyl acetate–*n*-butanol–methanol–water and ethyl acetate–*n*-butanol–water at different volume ratios. The partition coefficient values (K) of hyperforin and adhyperforin in different solvent systems are shown in Table 2. The results indicate that the solvent systems composed of ethyl acetate–*n*-butanol–water at the different volume ratios had large K values, hyperforin and adhyperforin could not be eluted. The appropriate K values can be obtained by the solvent systems of ethyl acetate–methanol–*n*-butanol–water and ethyl acetate–methanol–water at different volume ratios. The solvent systems of ethyl acetate–*n*-butanol–water at different volume ratios have a poor reservation in the column of HPCCC. If the solvent systems of ethyl acetate–methanol–water at the volume ratios of 4:1:7 and 4:2:5 were used as two-phase solvent systems, the two compounds could not be separated. For the purpose of shortening the lamination time and enhancing the reservation of the stationary phase in the column, the two-phase solvent system composed of ethyl acetate–methanol–water (5:2:5, v:v:v) was selected to isolate and purify the hydrophobic compounds.

ASE coupled with HPCCC online is a combination technique, which needs to meet both the requirements in order to make the experiment successful. The extraction system combined with a separation system requires an extraction solution transmission device and a power device. In constructing the first instrumental setup

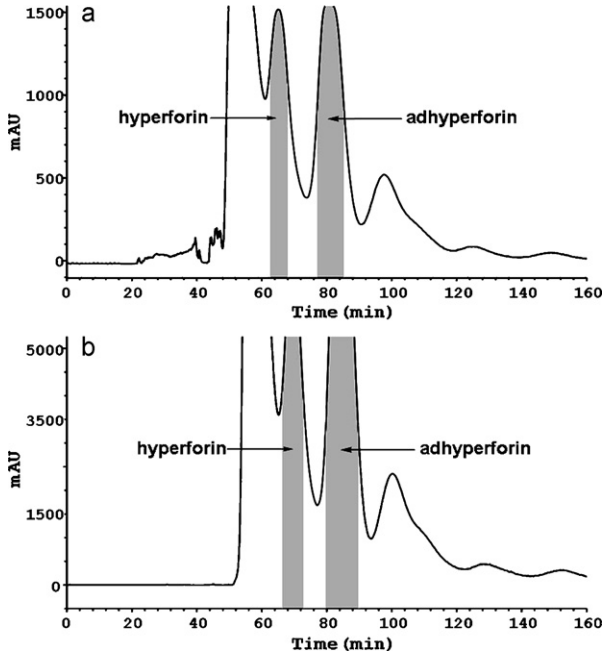


Fig. 2. ASE–HPCCC chromatograms of hyperforin and adhyperforin extracts from raw plant *H. perforatum*. By the first combination mode of ASE and HPCCC (a) and the second combination mode of ASE and HPCCC (b). *ASE conditions*: extraction solvent: the upper phase of the solvent system of ethyl acetate–methanol–water at a volume ratio of 4:2:5; extract temperature: 80 °C; extract pressure: 800 psi; static time: 5 min; nitrogen purge time of 60 s; flush volume of 60%. *HPCCC conditions*: column volume: 125 ml; rotation speed: 1400 rpm; solvent system: ethyl acetate–methanol–water (4:2:5, v:v:v); mobile phase: lower phase; flow-rate: 1.5 ml/min.

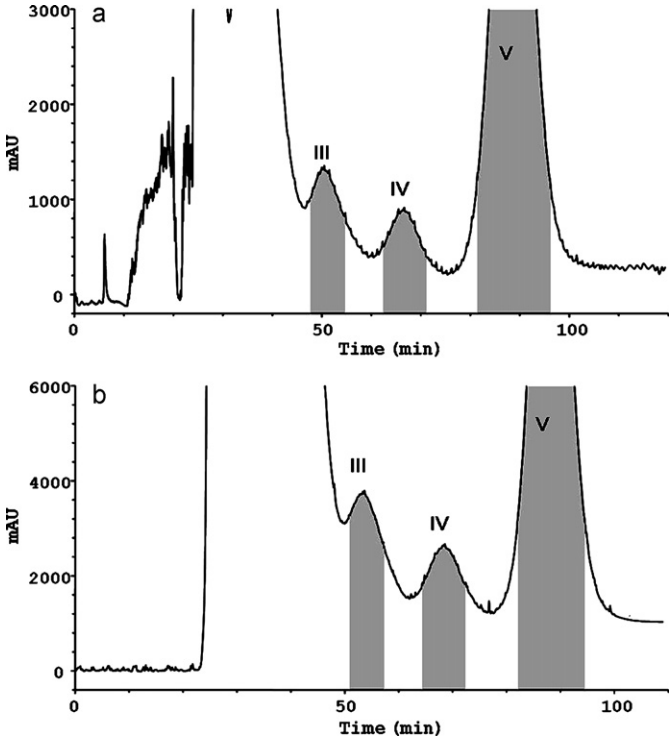


Fig. 4. ASE–HPCCC chromatograms of three caffeoylquinic acids extracted from raw plant material of *H. perforatum*. By the first instrumental setup of the combination of ASE and HPCCC (a) and the second instrumental setup of the combination of ASE and HPCCC (b). *ASE conditions*: extraction solvent: the lower phase of the solvent system of ethyl acetate–methanol–*n*-butanol–water at a volume ratio of 5:2:2.5:12; extract temperature: 150 °C; extract pressure: 1500 psi; static time: 5 min; nitrogen purge time of 60 s; flush volume of 60%. *HPCCC conditions*: column volume: 125 ml; rotation speed: 1400 rpm; solvent system: ethyl acetate–methanol–*n*-butanol–water (5:2:2.5:12, v:v:v:v); mobile phase: lower phase; flow-rate: 1.5 ml/min.

of ASE-HPCCC online (see Fig. 1 mode A), the ASE system and HPCCC system are connected directly by a short loop (100.0 cm, inside diameter of 0.2 mm). The ASE extract reached the HPCCC system under the pressure of ASE *via* the sample injection port of HPCCC. The HPCCC chromatogram is shown in Fig. 2. Peak fractions were collected according to the elution profile and evaporated under reduced pressure. The residual substance was dissolved in methanol for HPLC and MS analyses. The retention of the stationary phase was 87.2%. The advantages of this instrumental setup are convenience and efficiency with the retention of the stationary phase being relatively high. But the disadvantages of this instrumental setup are that the volume of sample solution is limited, and the maximum volume was just 5 ml since the sample solutions get into the HPCCC system *via* sample injection port. About 4.6 mg of hyperforin with a HPLC purity of 98.34% and 4.9 mg of adhyperforin with a HPLC purity of 98.61% were obtained.

The second instrumental setup of ASE–HPCCC online features the ASE and HPCCC systems connected by a sample loop (400.0 cm, inside diameter of 2.0 mm), a stationary phase pump and two T-splitters. The HPCCC chromatogram is shown in Fig. 2(b). Peak fractions were collected according to the elution profile and evap-

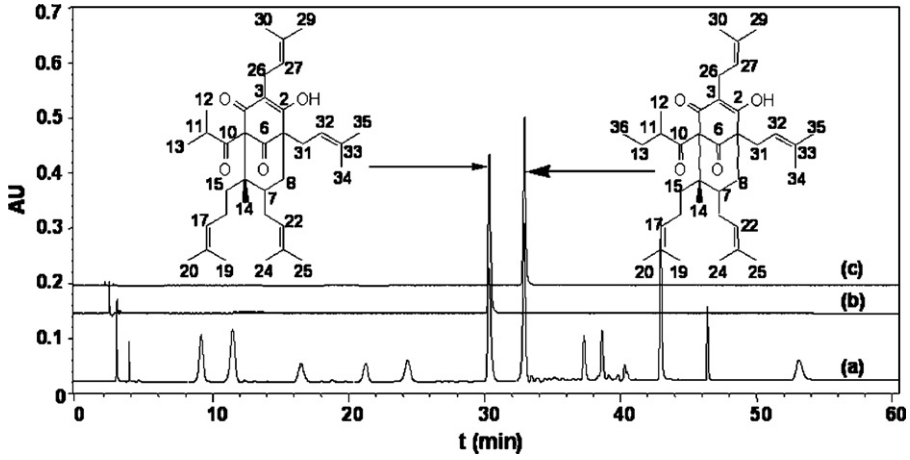


Fig. 3. HPLC chromatograms of two fractions eluted by HPCCC, of ASE extracts by the upper phase of solvent system of ethyl acetate–methanol–water at a volume ratio of 5:2:5 (v:v:v). (a) ASE extracts, (b) hyperforin, and (c) adhyperforin. An Agilent ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm) at 30 °C. Acetonitrile (Phase A) and 0.5% acetic acid in water (Phase B) were used as the mobile phase in gradient elution mode as follows: 0–60 min, 40–80% (Phase A). The flow-rate of the mobile phase was 1.2 ml/min.

Table 3
^{1}H and ^{13}C NMR data of hyperforin and adhyperforin.

	Hyperforin		Adhyperforin	
	H δ (multiplicity, J)	C δ	H δ (multiplicity, J)	C δ
1	–	210.0	–	211.4
2	–	184.2	–	184.3
3	–	122.3	–	118.6
4	–	182.2	–	188.1
5	–	61.7	–	60.76
6	–	82.2	–	82.7
7	1.71 m	43.6	1.75 m	42.2
8	1.83 (dd J = 13.4, 4.1 Hz, J = 13.4, 13.0 Hz)	40.4	1.88 (dd, J = 13.2, 4.1 Hz, J = 13.2, 13.1 Hz)	39.6
9	–	49.3	–	46.5
10	–	212.6	–	213.6
11	2.02 (m J = 6.5 Hz)	42.2	2.11 (m, J = 6.5 Hz)	41.4
12	0.94 (d J = 6.5 Hz)	21.2	0.96 (d, J = 6.5 Hz)	20.6
13	0.95 (d J = 6.5 Hz)	19.4	0.93 (d, J = 6.5 Hz)	21.1
14	0.84 s	15.6	0.86 s	13.2
15	1.67 m	38.7	1.73 m	36.6
16	1.97 m	28.2	1.92 m	25.3
17	4.89 m	123.7	4.92 m	125.6
18	–	133.5	–	136.4
19	1.58 s	25.7	1.52 s	24.2
20	1.48 s	17.5	1.47 s	16.8
21	1.83 m	25.8	1.98 m, 1.63 m	27.6
22	4.96 m	126.4	4.92 m	123.3
23	–	131.3	–	131.3
24	1.54 s	26.2	1.56 s	25.7
25	1.47 s	18.3	1.45 s	16.4
26	3.07 (dd, J = 14.8, 7.3 Hz, J = 14.8, 7.1 Hz)	22.5	3.02 (dd, J = 14.6, 7.2 Hz, J = 14.6, 7.1 Hz)	22.3
27	5.03 (t, J = 5.8 Hz)	124.2	5.06 (t, J = 6.8 Hz)	124.1
28	–	132.5	–	126.6
29	1.57 s	26.3	1.54 s	24.3
30	1.68 s	18.4	1.61 s	16.5
31	2.32 (dd, J = 14.5, 7.0 Hz, J = 14.5, 6.5 Hz)	30.3	2.33 (dd, J = 14.2, 6.9 Hz, J = 14.1, 6.4 Hz)	29.3
32	4.93 m	121.6	5.22 (t, J = 6.6 Hz)	123.2
33	–	134.3	–	135.4
34	1.57 s	26.1	4.76 s	125.4
35	1.58 s	18.6	1.62 s	12.3
35	–	–	1.57 s	16.4

orated under reduced pressure. The residue was dissolved in methanol for HPLC, MS and NMR analyses. The retention of the stationary phase was 68.6%. The advantages of this instrumental setup include a large volume of sample solution, suitable for preparative extraction and separation. The maximum volume in theory is the same as the volume of the HPCCC column. But the disadvantage of this instrumental setup is that the retention of the stationary phase is relatively low.

Two compounds, 28.4 mg of hyperforin with a purity of 97.28% and 32.7 mg of adhyperforin with a purity of 97.81%, were well extracted and isolated. The HPLC chromatograms are shown in Fig. 3. The structures were identified by UV, MS and NMR spectra.

HPCCC Peak 1: UV λ_{max} at 204 and 276 nm, ESI-MS (m/z): 535 $[M-H]^-$, 466 $[M\text{-}C_5H_9\text{-}H]^-$, 397 $[M\text{-}H\text{-}C_5H_9\text{-}C_5H_9]^-$, 383 $[M\text{-}H\text{-}C_5H_9\text{-}C_6H_{11}]^-$. HPCCC Peak 2: UV λ_{max} at 204 and 276 nm, ESI-MS (m/z): 549 $[M-H]^-$, 480 $[M\text{-}C_5H_9\text{-}H]^-$, 411 $[M\text{-}H\text{-}C_5H_9\text{-}C_5H_9]^-$, 397 $[M\text{-}H\text{-}C_5H_9\text{-}C_6H_{11}]^-$. The subsequent structural identification of the peak fractions collected by HPCCC were performed in comparison with the previous ^{1}H NMR and ^{13}C NMR data [8,32–34]. The NMR data are shown in Table 3.

3.2.2. Lower phase (aqueous phase) as the ASE solvent for hydrophilic compounds

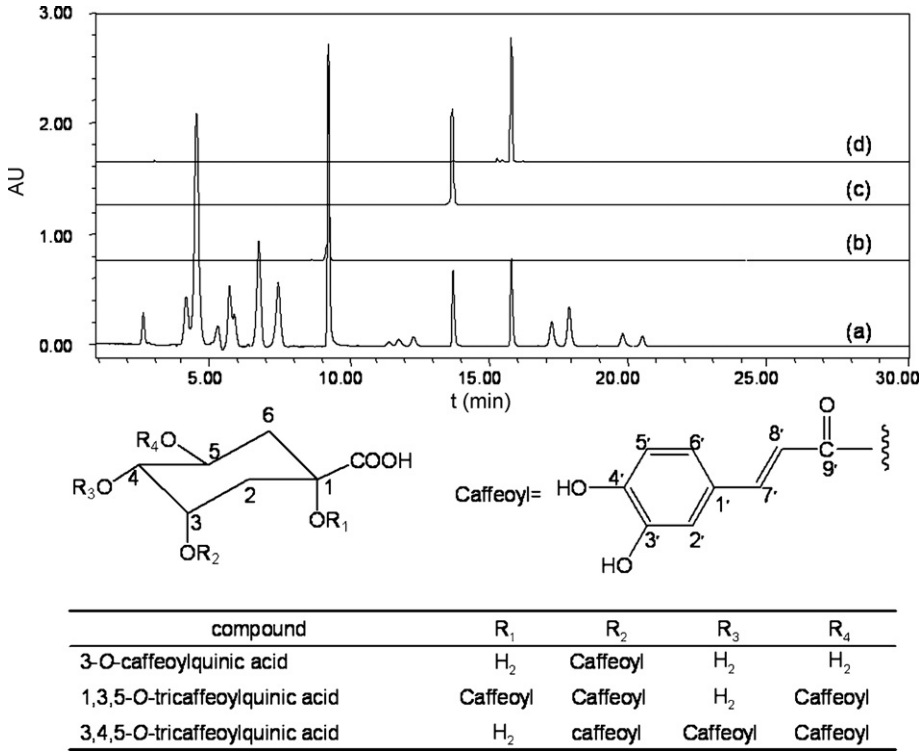
According to the K values of compounds 3–5 in different two-phase solvent systems presented in Table 4, the solvent system of ethyl acetate–methanol–water at a volume ratio of 6:2:4 and the solvent systems of ethyl acetate–methanol–*n*-butanol–water at volume ratios of 6:2:1:10 and 4:3:2:8 had relative large K values ($K > 1.0$), and the separation time will be long. When ethyl acetate–methanol–water at a volume ratio of 5:2:5:15 was used as the two-phase solvent system, the three compounds could not be separated and the purity of them became poor. When ethyl acetate–methanol–water systems at the volume ratios of 4:1:7, 4:2:5 and 5:2:5 were used, three compounds can be well separated, but the separation time was long and the HPCCC peak broadened seriously. Therefore, the solvent system of ethyl acetate–methanol–*n*-butanol–water (5:2:2.5:12, v:v:v:v) was chosen and practiced on by using HPCCC separation. The lower phase was selected as the stationary phase, the upper phase as the mobile phase, and the two instrumental setups of ASE–HPCCC were also carried out. Fig. 4(a) and (b) shows the HPCCC separation of the aqueous extracts by the two instrumental setups. Briefly, 1.0 mg of 3,4,5-*O*-tricaffeoylquinic acid with a HPLC purity of 97.12%, 1.2 mg of 1,3,5-*O*-tricaffeoylquinic acid with a HPLC purity of 97.84% and 3.7 mg of 3-*O*-caffeoylquinic acid with a HPLC purity of 98.05% were obtained by the first instrumental setup. By the second instrumental setup, 12.7 mg of 3,4,5-*O*-tricaffeoylquinic acid with a HPLC purity of 98.82%, 15.2 mg of 1,3,5-*O*-tricaffeoylquinic acid with a HPLC purity of 99.46% and 42.5 mg of 3-*O*-caffeoylquinic acid with a HPLC purity of 96.90% were obtained in a one-step extraction–separation process. The structure was identified by MS, ^{1}H NMR and ^{13}C NMR spectra (Fig. 5).

HPCCC Peak 3: ESI-MS (m/z): 677 $[M-H]^-$, 515 $[M\text{-}C_6H_{10}O_5(\text{Caffeoyl})]^-$, 353 $[M\text{-}2C_6H_{10}O_5(\text{Caffeoyl})]^-$, 191 $[M\text{-}3C_6H_{10}O_5(\text{Caffeoyl})]^-$. The NMR data are shown in Table 5. Compared with the reported data, the MS, ^{1}H NMR and ^{13}C NMR data are in agreement with those of 3,4,5-*O*-tricaffeoylquinic acid in the literatures [35,36].

Table 4
Partition coefficient (K) values of three caffeoylquinic acids in various two-phase solvent systems.

No.	Solvent system	K_1 [a]	K_2	K_3
1	Ethyl acetate–methanol–*n*-butanol–water (5:2:5:15, v:v:v:v)	0.48	1.28	1.35
2	Ethyl acetate–methanol–*n*-butanol–water (5:2:2.5:12, v:v:v:v)	0.84	1.77	1.89
3	Ethyl acetate–methanol–*n*-butanol–water (6:2:1:10, v:v:v:v)	1.92	3.34	3.59
4	Ethyl acetate–methanol–*n*-butanol–water (4:3:2:8, v:v:v:v)	2.24	3.96	4.27
5	Ethyl acetate–methanol–water (4:1:7, v:v:v)	0.89	2.52	2.71
6	Ethyl acetate–methanol–water (4:2:5, v:v:v)	0.75	1.86	2.06
7	Ethyl acetate–methanol–water (5:2:5, v:v:v)	1.15	2.03	2.18
8	Ethyl acetate–methanol–water (6:2:4, v:v:v)	1.65	2.74	2.95

[a] K_1: 3,4,5-*O*-tricaffeoylquinic acid; K_2: 1,3,5-*O*-tricaffeoylquinic acid; K_3: 3-*O*-caffeoylquinic acid.



compound	R_1	R_2	R_3	R_4
3-*O*-caffeoylquinic acid	H_2	Caffeoyl	H_2	H_2
1,3,5-*O*-tricaffeoylquinic acid	Caffeoyl	Caffeoyl	H_2	Caffeoyl
3,4,5-*O*-tricaffeoylquinic acid	H_2	caffeoyl	Caffeoyl	Caffeoyl

Fig. 5. HPLC chromatograms of three fractions, eluted by HPCCC, and that of ASE extracts by the lower phase of solvent system of ethyl acetate–methanol–*n*-butanol–water at a volume ratio of 5:2:2.5:12 (v:v:v:v). (a) ASE extract, (b) 3-*O*-caffeoylquinic acid, (c) 1,3,5-*O*-tricaffeoylquinic acid, and (d) 3,4,5-*O*-tricaffeoylquinic acid. An Agilent ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm) at 30 °C. Acetonitrile (Phase A) and 0.5% acetic acid in water (Phase B) were used as the mobile phase in gradient elution mode as follows: 0–30 min, 13–30% (Phase A). The flow-rate of the mobile phase was 1.2 ml/min.

Table 5
^{1}H and ^{13}C NMR data of 3,4,5-*O*-tricaffeoylquinic acid, 1,3,5-*O*-tricaffeoylquinic acid, and 3-*O*-caffeoylquinic acid.

	3,4,5-*O*-Tricaffeoylquinic acid		1,3,5-*O*-Tricaffeoylquinic acid		3-*O*-Caffeoylquinic acid	
	H δ (multiplicity, J)	C δ	H δ (multiplicity, J)	C δ	H δ (multiplicity, J)	C δ
1	–	74.4	–	80.8	–	76.5
2	2.20, 2.45 (1H each, d, J = 13.4 Hz)	39.2	2.41, 2.92 (1H each, dd, J = 3, 16 Hz, H-2)	32.9	1.80 m	39.2
3	5.83 m	67.4	5.46 m	72.9	5.21 m	70.6
4	5.35 (1H, dd, J = 9.9,3.4 Hz)	72.2	3.99 (1H, dd, J = 3, 8 Hz)	72.2	3.75 m	72.6
5	5.74 (1H, m, J = 3.2 Hz, H-5)	69.1	5.58 m	71.2	4.6 m	72.3
6	2.31, 2.38 (1H each, dd, J = 3, 3.5 Hz)	36.1	2.00, 2.67 (1H each, dd, J = 10, 13 Hz)	38.3	2.64 m	37.8
7	–	174.7	–	174.1	–	–
1′	–	166.2, 166.3, 166.3	–	127.5, 127.6, 127.8	–	125.3
2′	6.25, 6.25, 6.42 (3H, m, J = 15.9 Hz)		7.07 (3H, m, J = 2 Hz)		7.01 (1H, d, J = 1.8 Hz)	114.4
3′	7.59, 7.60, 7.68 (1H each, dd, J = 15.9 Hz)	124.3, 124.3, 124.4	–	–	–	144.2
4′	–	127.4, 127.4, 127.5	–	–	–	147.0
5′	6.96, 7.01, 7.11 (1H each, d, J = 2.0 Hz)	110.0, 110.1, 110.1	6.55, 6.65, 6.79 (1H each, d, J = 8 Hz)	115.3, 116.6, 116.6	6.63 (1H, d, J = 8.2 Hz)	115.5
6′	–	149.4, 149.5, 149.5	6.64, 6.78, 6.95 (1H each, dd, J = 2.8 Hz)	122.2, 123.0, 123.0	6.74 (1H, d, J = 8.1 Hz)	124.2
7′	–	151.5, 151.5,151.5	7.51, 7.55, 7.63 (1H each, J = 16 Hz)	147.4, 147.4, 147.9	7.49 (1H, d, J = 15.8 Hz)	145.9
8′	6.78, 6.83, 6.86 (1H each, d, J = 8.4 Hz)	111.3, 111.3,111.3	6.17, 6.22, 6.32 (1H each, d, J = 16 Hz)	115.0, 115.2, 115.3	–	115.9
9′	–	122.9, 122.9, 123.1	–	167.8, 168.7, 168.8	–	168.9

HPCCC Peak 4: ESI-MS (m/z): 677 $[M-H]^-$, 515 $[M-C_6H_{10}O_5(Caffeoyl)]^-$, 353 $[M-2C_6H_{10}O_5(Caffeoyl)]^-$, 191 $[M-3C_6H_{10}O_5(Caffeoyl)]^-$. The NMR data are shown in Table 5. The MS, ^1H NMR and ^{13}C NMR data were in agreement with those of 1,3,5-*O*-tricaffeoylquinic acid in the literatures [37,38].

HPCCC Peak 5: ESI-MS (m/z): 353 $[M-H]^-$, 191 $[M-C_6H_{10}O_5(Caffeoyl)]^-$. The NMR data are shown in Table 5. The MS, ^1H NMR and ^{13}C NMR data are in agreement with those of 3-*O*-caffeoylquinic acid (chlorogenic acid) in the literatures [39].

4. Conclusion

Two hydrophobic compounds of hyperforin and adhyperforin as well as three hydrophilic caffeoylquinic acids extracted from the nature plant *H. perforatum* were isolated by ASE coupled with HPCCC online. Under optimal ASE conditions, the contractions of hyperforin, adhyperforin, 3,4,5-*O*-tricaffeoylquinic acid, 1,3,5-*O*-tricaffeoylquinic acid and 3-*O*-caffeoylquinic acid from crude extract were 2.83 mg/g, 3.26 mg/g, 1.27 mg/g, 1.52 mg/g and 4.24 mg/g, respectively. At last, a purity of greater than 96% of the 5 compounds above mentioned was obtained by ASE–HSCCC with a two-phase solvent system composed of ethyl acetate–methanol–water at a volume ratio of 4:2:5 and that of ethyl acetate–methanol–*n*-butanol–water at an optimized volume ratio of 5:2:2.5:12. The combined use of the two instruments has been developed successfully in this paper. Two instrumental setups of ASE–HPCCC were successfully applied, the advantage of mode A is the configuration is relative uncomplicated, and the retention of the stationary phase is relative high, appropriate to the analytical ASE–HPCCC separation. The advantage of mode B is the amounts of compounds separated by ASE–HPCCC is large, appropriate to the preparative ASE–HPCCC separation. Furthermore, the main advantages of ASE–HPCCC are as follows. (1) Since ASE is a very effective extraction instrument, it can save a lot of extraction time as well as extraction solvent. (2) ASE–HPCCC is suitable for the extraction and isolation of the rare chemical constituents in natural plants. (3) Since ASE coupled HPCCC is an online extraction–separation technique, it can avoid the degradation and/or transformation of the unstable constituents under light and/or oxygen.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 30970299) and Major Project of Jilin Provincial Science and Technology Department (No. 20090936).

References

[1] A.J.P. Francis, Phytomedicine 12 (2005) 167.
[2] T. Mennini, M. Gobbi, Life Sci. 75 (2004) 1021.
[3] R. Capasso, F. Borrelli, F. Capasso, N. Mascolo, A.A. Izzo, Urology 64 (2004) 168.
[4] L. Luo, Q. Sun, Y.Y. Mao, Y.H. Lu, R.X. Tan, J. Ethnopharmacol. 93 (2004) 221.
[5] N.E. Stavropoulos, A. Kim, U.U. Nseyo, I. Tsimaris, T.D. Chung, T.A. Miller, M. Redlak, U.O. Nseyo, D. Skalkos, J. Photochem. Photobiol. B 84 (2006) 64.
[6] J. Benedi, R. Arroyo, C. Romero, S.M. Aragon, A.M. Villar, Life Sci. 75 (2004) 1263.
[7] H. Hosseinzadeh, G.R. Karimi, M. Rakhshanizadeh, J. Ethnopharmacol. 98 (2005) 207.
[8] B.A. Silva, J.O. Malva, A.C.P. Dias, Food Chem. 110 (2008) 611.
[9] B. Tirillini, A. Ricci, G. Pintore, M. Chessa, S. Sighinolfi, Fitoterapia 77 (2006) 164.
[10] F. Pellati, S. Benvenuti, M. Melegari, J. Chromatogr. A 1088 (2005) 205.
[11] H. Falk, Angew. Chem. Int. Ed. 385 (1999) 3116.
[12] A. Tolonen, A. Hohtola, J. Jalonen, Phytochem. Anal. 14 (2003) 306.
[13] Y. Li, P.P.H. But, V.E.C. Ooi, Antiviral Res. 68 (2005) 1.
[14] J.O. Ojwang, Y.H. Wang, P.R. Wyde, N.H. Fischer, W. Schuehly, J.R. Appleman, S. Hinds, C.D. Shimasaki, Antiviral Res. 68 (2005) 163.
[15] L. Gongora, R.M. Giner, S. Manez, M.C. Recio, G. Schinella, J.L. Rios, Life Sci. 71 (2002) 2995.
[16] T. Matsui, S. Ebuchi, T. Fujise, K.J.M. Abesundara, S. Doi, H. Yamada, K. Matsumoto, Biol. Pharm. Bull. 27 (2004) 1797.
[17] B. Kaufmann, P. Christen, Phytochem. Anal. 13 (2002) 105.
[18] J. Richter, I. Schellenberg, Anal. Bioanal. Chem. 387 (2007) 2207.
[19] Y. Li, T. Pang, Z.M. Guo, Y.Y. Li, X.L. Wang, J.H. Deng, K.J. Zhong, X. Lu, G.W. Xu, Talanta 81 (2010) 650.
[20] J.H. Chen, F.M. Wang, J. Liu, F.S.C. Lee, X.R. Wang, H.H. Yang, Anal. Chim. Acta 613 (2008) 184.
[21] H.X. Zhao, L.P. Wang, Y.M. Qiu, Z.Q. Zhou, X. Li, W.K. Zhong, J. Chromatogr. B 840 (2006) 139.
[22] S. Vichi, C. Santini, N. Natali, C. Riponi, E. Lopez-Tamames, S. Buxaderas, Food Chem. 102 (2007) 1260.
[23] B. Pinel, G. Audo, S. Mallet, M. Lavault, F.D.L. Poype, D. Seraphin, P. Richomme, J. Chromatogr. A 1151 (2007) 14.
[24] M. Gu, F. Ouyang, Z.G. Su, J. Chromatogr. A 1022 (2004) 139.
[25] Y. Yang, Y. Huang, D. Gu, A. Yili, G. Sabir, H.A. Aisa, Chromatographia 69 (2009) 963.
[26] Y. Wang, B. Liu, Phytochem. Anal. 18 (2007) 436.
[27] V.P. Silva, R.R. Oliveira, M.R. Figueiredo, Phytochem. Anal. 20 (2009) 77.
[28] P. Fan, A. Marston, A.E. Hay, K. Hostettmann, J. Sep. Sci. 32 (2009) 2979.
[29] A. Peng, H. Ye, X. Li, L. Chen, J. Sep. Sci. 32 (2009) 2967.
[30] S.G. Deng, Z.Y. Deng, Y.W. Fan, Y. Peng, J. Li, D.M. Xiong, R. Liu, J. Chromatogr. B 877 (2009) 2487.
[31] N. Bodin, H. Budzinski, K.L. Menach, N. Tapie, Anal. Chim. Acta 643 (2009) 54.
[32] E.C. Tatsis, S. Boeren, V. Exarchou, A.N. Troganis, J. Vervoort, I.P. Gerothanassis, Phytochemistry 68 (2007) 383.
[33] Y.M. Cui, C.Y.W. Ang, R.D. Beger, T.M. Heinze, L.H. Hu, J. Leakey, Drug Metab. Dispos. 32 (2004) 28.
[34] A. Charchoglyan, A. Abrahamyan, I. Fujii, Z. Boubakir, T.A.M. Gulder, T.M. Kutchan, H. Vardapetyan, G. Bringmann, Y. Ebizuka, L. Beerhues, Phytochemistry 68 (2007) 2670.
[35] B.N. Timmerman, J.J. Hoffmann, J. Nat. Prod. 46 (1983) 365.
[36] M.N. Clifford, W. Wu, J. Kirkpatrick, N. Kuhnert, J. Agric. Food Chem. 55 (2007) 929.
[37] I. Agata, S. Goto, T. Hatano, S. Nishibe, T. Okuda, Phytochemistry 33 (1993) 508.
[38] M.N. Clifford, S. Knight, N. Kuhnert, J. Agric. Food Chem. 53 (2005) 3821.
[39] N. Durust, S. Ozden, E. Umur, Y. Durust, M. Kucukislamoglu, Turk. J. Chem. 25 (2001) 93.