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# On-line purity monitoring in high-speed counter-current chromatography: Application of HSCCC-HPLC-DAD for the preparation of 5-HMF, neomangiferin and mangiferin from *Anemarrhena asphodeloides* Bunge

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**Abstract**

An efficient on-line purity monitoring strategy based on on-line coupling of high-speed counter-current chromatography (HSCCC) with high-performance liquid chromatography-diode array detection (HPLC-DAD) was successfully applied for the first time to the isolation and purification of 5-hydroxymethyl-furancarboxaldehyde (5-HMF), mangiferin and neomangiferin from the Chinese medicinal plant *Anemarrhena asphodeloides* Bunge, a plant used in the traditional Chinese medicine. The introduction of on-line purity monitoring in HSCCC has greatly improved the efficiency of this technique by overcoming the drawbacks of post-purification sample handling in HSCCC isolation. The effluent from the outlet of HSCCC was splitted into two parts, and one was collected, while the other was introduced directly through a switch valve into a HPLC-DAD system for purity monitoring. Using this method the desired fractions with high purities could be collected. From 600 mg partially purified extract, 165.6 mg neomangiferin and 292.8 mg mangiferin with purities of 98.9 and 99.5%, respectively, were obtained with a two-phase solvent system composed of *n*-butanol–water (1:1, v/v) by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.2 ml $min^{-1}$ after 210 min. A 17.1 mg 5-HMF with purity of 96.6% was also isolated for the first time.
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*Keywords:* *Anemarrhena asphodeloides* Bunge; 5-HMF; Neomangiferin; Mangiferin; On-line purity monitoring; Counter-current chromatography

## 1. Introduction

The purity of compounds is a critical component in the whole process of obtaining the desired biological active material needed for screening and for subsequent investigation of structure activity relationships. With resources expended collecting and tracking each fraction, it usually is more efficient to collect only the fractions with the desired compounds. Therefore, great effort in developing and integrating on-line purity monitoring is one of the major challenging objectives in modern high-throughput purification.

*Anemarrhena asphodeloides* Bunge is one of the most widely used medicinal plants officially listed in the Chinese Pharmacopoeia, which is often used to remove heat and quench fire, promote the production of body fluid and relieve dryness syndrome [1]. Many compounds have been separated from it, including steroidal saponins, flavones, lignans, anemarans and xanthones, and their bioactive effects have been widely reported. Mangiferin, main bioactive xanthone of *A. asphodeloides* Bunge, possesses radioprotection [2], anti-inflammatory [3], antidiabetic [4], antioxidant [5] and antitumor [6] effects. Neomangiferin, another xanthone compound, is also antidiabetic [7,8]. Further studies on pharmacological and clinical effects of mangiferin and neomangiferin necessitate the development of an efficient preparative separation method of these drugs. Such a method will also facilitate quality control and improvement of the quality of existing *A. asphodeloides* Bunge products.

At present, mangiferin and neomangiferin are commercially purified from *A. asphodeloides* Bunge by several steps such as crystallization and chromatography. All these conventional

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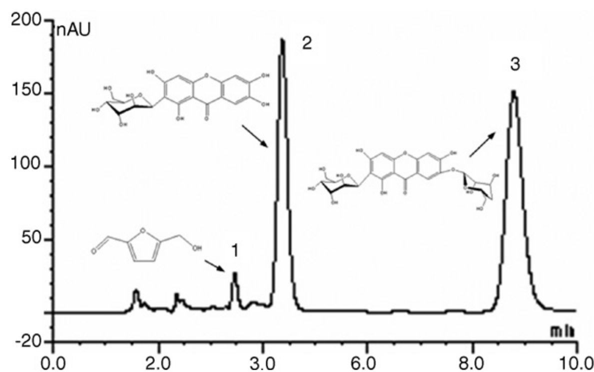


Fig. 1. HPLC chromatogram of partially purified extract from *Anemarrhena asphodeloides* Bunge after cleaning-up by D101 macroporous resin. Column: reversed-phase Diamonsil$^{TM}$ $C_{18}$ (4.6 mm × 200 mm i.d., 5 μm); mobile phase: methanol–water–acetic acid (34:66:1, v/v/v); flow-rate: 1.0 ml min$^{-1}$ and UV wavelength: 254 nm; 1: 5-HMF, 2: neomangiferin and 3: mangiferin.

methods are tedious, time consuming, thus, are not suitable for large-scale isolation. High-speed counter-current chromatography (HSCCC), a support-free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support [9], and has been widely used in preparative separation of natural products [10–12]. The preparative separation and purification of mangiferin and neomangiferin from *Rhizoma Anemarrhenae* by UV-triggered HSCCC has been reported recently [13]. Since collecting fractions based on UV response generally results in a large number of samples with few exceptions, the fractions collected on the preparative HSCCC system were analysed by high-performance liquid chromatography (HPLC) following purification to determine if the material meets the purity standards. However, the need for manual intervention and off-line sample processing delay the acquisition of the post-purification sample data, and, ultimately, the delivery of compounds for additional testing.

In this study, an effective on-line purity monitoring preparative system has been successfully applied for the first time to the isolation and purification of mangiferin and neomangiferin from *A. asphodeloides* Bunge. It involves on-line coupling of HSCCC with high-performance liquid chromatography-diode array detection (HSCCC-HPLC-DAD). On-line purity monitoring can analyse representative aliquot from each fraction automatically, thus, can remove those additional steps and decrease instrument idle time. At the same time, 5-hydroxymethyl-2-furancarboxaldehyde (5-HMF), which possesses uterotonic activity [14], anti-platelet aggregation activity [15] and anti-myocardial ischemia activity [16], was also isolated. To our best knowledge this is the first report of isolating 5-HMF from *A. asphodeloides* Bunge. The chemical structures of 5-HMF, mangiferin and neomangiferin are shown in Fig. 1.

## 2. Experimental

### 2.1. HSCCC-HPLC-DAD apparatus

The automated purification system contained a standard Prep HSCCC system and an on-line HPLC purity monitoring system. The HSCCC system used for this work consisted of the following commercial components: a model TBE–300A high-speed counter-current chromatography (Shenzhen, Tauto Biotech, China) with three polytetrafluoroethylene (PTFE) preparative coils (diameter of tube, 2.6 mm; total volume, 300 ml), a 20 ml sample loop, a model S constant flow pump, a UV–vis detector operating at 254 nm, a model N2010 workstation (Zhejiang University, Hangzhou, China), and a 701D fraction collector (Varian) which was used to control fraction collection. The revolution radius or the distance between the holder axis and central axis of the centrifuge ($R$) was 5 cm, and the $\beta$ value varied from 0.5 at internal terminal to 0.8 at the external terminal ($\beta = r/R$, where $r$ is the distance from the coil to the holder shaft). The temperature was adjusted by HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China). On-line purity analysis was performed with a six-port, two-position valve including a 10 μl sample loop (C2-2006, Unimicro Technologies, Shanghai, China), a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA) including P680 pump, thermostatted column compartment and PDA-100 photodiode array detector. Chromeleon software (Version 6.50) was used for evaluation and quantification. The details of the schematic diagram of the hyphenated HSCCC-HPLC-DAD system have been described in our previous work [17].

### 2.2. Reagents

D101 macroporous resin (Tianjin Agricultural Chemical Co. Ltd., Tianjin, China), a kind of milkwhite spherical granule, shows stronger intension, larger adsorption capability and more easy activation than some other sorbents.

All organic solvents used for HSCCC were of analytical grade and purchased from WuLian Chemical Factory, Shanghai, China. Methanol used for HPLC was of chromatographic grade (Merck, Germany). Reverse osmosis Milli-Q water (18 M Ω) (Millipore, USA) was used for all solutions and dilutions.

The *A. asphodeloides* Bunge was purchased from a local drug store and identified by Professor Luping Qin (Department of Pharmacognosy, College of Pharmacy, the Second Military Medical University, Shanghai, China).

### 2.3. Preparation of the partially purified extract

About 300 g of dried *A. asphodeloides* Bunge was chopped and extracted three times by reflux with 1500 ml volume of 70% ethanol in a haven for 2 h. After filtration, the extract was combined and evaporated to dryness by rotary vaporization at 90 °C under reduced pressure and re-dissolved in water. The water soluble extract was then chromatographed on a glass column (4.0 cm × 60 cm, containing 350 g D101 macroporous resin) by eluting stepwise with water and 30% ethanol. Water was first used to remove some un-target chemicals, which have no or little retention on D101 macroporous resin, 30% ethanol was then used to yield target sample and 95% ethanol was used to activate the resin for another use. A 7.2 g of dried material was obtained as partially purified extract, which may be

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responsible for the antidiabetic activities of this drug. Then this partially purified extract of *A. asphodeloides* Bunge was subjected to HSCCC for isolation and purification of mangiferin and neomangiferin.

The sample solutions were prepared by dissolving the partially purified extract in 10 ml lower phase of the solvent system for isolation and purification.

### 2.4. HSCCC separation procedure

For the present study, a two-phase solvent system composed of *n*-butanol–water (1:1, v/v) was prepared. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two-phases were separated shortly before use. In HSCCC separation, the coil column was first entirely filled with the upper phase of the solvent system, and then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow-rate of 1.0 ml $min^{-1}$. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 10 ml sample solution containing 600 mg of the partially purified extract was injected through the injection valve. The flow-rate of the mobile phase was increased to 2.2 ml $min^{-1}$ after 210 min, and the effluent of the column was continuously monitored with the UV detector at 254 nm. A total of three peaks were obtained.

### 2.5. HPLC-DAD purity analysis of CCC peak fractions

When the HSCCC elution times reach 74, 84, 94, 104, 114, 124, 134, 183, 193, 203, 283, 293, 303, 313, 323 and 333 min, the valve was switched and the factor collector began to collect fractions. In the meantime, the representative aliquot sample was sent to the analytical unit and the HPLC-DAD began to perform a purity analysis run. After 1 min the valve was triggered back for the next analytical run.

The column used was a reversed-phase Diamonsil™ $C_{18}$ (4.6 mm × 200 mm i.d., 5 μm) (Dikma Technologies Company, China) with a pre-column equipped with the same stationary phase, the mobile phase was methanol–water–acetic acid (34:66:1, v/v/v). The solvent flow-rate was 1.0 ml $min^{-1}$ and the column temperature was set at 25 °C. The injected volume was 10 μl. The DAD recorded UV spectra in the range from 190 to 800 nm, and the HPLC chromatogram was monitored at 254 nm.

### 2.6. Comparison of on-line selective collection with off-line purification of full fractions by semi-preparative HPLC

In order to determine if the aliquot collected using the on-line purity monitoring system was representative of the collected fraction, the purity and the recovery of the aliquot selectively collected from peak I during purification were determined and the results were compared with those obtained following further purification by semi-preparative HPLC after off-line evaporation and re-dissolution of the full fractions of peak I.

The semi-preparative HPLC equipment consisted of a 515 pump and a 2487 detector (Waters). A model N2000 recorder for evaluation and quantification and a sample injector with a 750 μl loop. The column used was a reversed-phase YWG $C_{18}$ (10.0 mm × 200 mm i.d., 10 μm) (Elite, Dalian, China) with a pre-column equipped with the same stationary phase.

## 3. Results and discussion

### 3.1. Optimization of HPLC conditions

In order to reduce the run time of the HPLC analysis, and thereby increase the number of runs per unit volume from HSCCC, the HPLC conditions were optimized. In the present study, organic aqueous-based mobile phases were tested on a reversed-phase $C_{18}$ column, including methanol–water and acetonitrile–water in combination with acetic acid, phosphate buffer and phosphoric acid. The flow-rate of the mobile phase and the temperature of the column, which might affect the separation, were also tested. It was found that an excellent separation was achieved by the following separation conditions: the mobile phase composed of methanol–water–acetic acid (34:66:1, v/v/v) was isocratically eluted at a flow-rate of 1.0 ml $min^{-1}$, and UV detection was set at 254 nm. No complex gradient of mobile phase and no buffer were necessary. The HPLC chromatogram of the partially purified extract is shown in Fig. 1; it contained several compounds including 5-HMF, neomangiferin and mangiferin (peaks I, II and III).

### 3.2. Optimizing HSCCC operation

In HSCCC, a suitable two-phase solvent system was critical for a successful isolation and separation. 5-HMF, mangiferin and neomangiferin are not soluble in non-polar solvent, but have some solubility in ethyl acetate, *n*-butanol, methanol and chloroform. On the basis of these properties, several solvent

Table 1
The partition coefficient of 5-HMF, mangiferin and neomangiferin in different solvent systems

| Solvent system | Neomangiferin | 5-HMF | Mangiferin |
|---|---|---|---|
| Chloroform–methanol–water (4:3:2) | 11.39 | 1.80 | 8.16 |
| Ethyl acetate–*n*-butanol–water (2:1:3) | 0.11 | 1.48 | 2.89 |
| Ethyl acetate–*n*-butanol–water (2:3:5) | 0.43 | 1.46 | 5.21 |
| *n*-Butanol–water (1:1) | 0.52 | 1.11 | 3.75 |
| *n*-Butanol–acetic acid–water (4:1:5) | 0.56 | 0.87 | 2.03 |

Experimental protocol: about 4 ml of each phase of pre-equilibrated two-phase solvent system was mixed with 2 mg of the partially purified extract in a 10 ml test tube. After shaking vigorously for 5 min to equilibrate the sample thoroughly with the two-phases, the two-phase was separated and evaporated to dryness under reduced pressure. The residue was diluted with 30% $CH_3OH$ and analysed by HPLC. The partition coefficient (*K*) value was expressed as the peak area of target components in the upper phase divided by that in the lower phase.

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systems including chloroform–methanol–water (4:3:2, v/v/v), *n*-butanol–water (1:1, v/v), *n*-butanol–acetic acid–water (4:1:5, v/v/v) and ethyl acetate–*n*-butanol–water at different volume ratios (2:1:3, 2:3:5, v/v/v) were tested. After trying five kinds solvent systems (Table 1), the one composed of *n*-butanol–water (1:1, v/v) was found to be satisfactory for the separation of 5-HMF, mangiferin and neomangiferin from the partially purified extract.

Although the selection of the two-phase system is critical, the flow-rate of the mobile phase may also affect the separation. If only one component needs to be separated from the others, the standard HSCCC method, which uses a constant flow-rate of the mobile phase, could be used. In order to separate more different compounds, stepwise elution or stepwise increasing the flow-rate of the mobile phase might be applied [11]. Although 5-HMF was purified and separated at the flow-rate of 1.0 ml $min^{-1}$, mangiferin was retained in the column for a long time (>8 h) and more mobile phase was required. While the flow-rate of the mobile phase was increased to 2.2 ml $min^{-1}$, mangiferin could be separated from other compounds, but 5-HMF would be eluted together with other compounds. Finally, the method with stepwise increasing the flow-rate of the mobile phase was attempted with this two-phase solvent system. That is, the flow-rate of the mobile phase was kept at 1.0 ml $min^{-1}$ before 3.5 h, and then increased to 2.2 ml $min^{-1}$ after 210 min. Under the above optimized separation conditions, the isolation of the target compounds was achieved with good resolution and the retention of the stationary phase remained constant (30%), though the hydrodynamic equilibrium was initially established at a flow-rate 1.0 ml $min^{-1}$, which was then increased to 2.2 ml $min^{-1}$ after 210 min.

### 3.3. Purification of 5-HMF, mangiferin and neomangiferin with on-line purity monitoring by HSCCC-HPLC-DAD

A total of 17.1 mg 5-HMF and 292.8 mg mangiferin from 600 mg partially purified extract was obtained with a two-phase solvent system composed of *n*-butanol–water (1:1, v/v) by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.2 ml $min^{-1}$ after 210 min. On-line HPLC purity monitoring of each peak fraction of this HSCCC system revealed that the purity of 5-HMF and mangiferin corresponding to peaks II and III was over 96.6 and 99.5%, while the purity of peak I was not satisfactory before 114 min. Therefore, the collection had been performed selectively from the fifth point (114 min) according to the on-line purity detection, and 165.6 mg neomangiferin with the purity of 98.6% were obtained. Fig. 2 shows the HSCCC separation of 600 mg of the partially purified sample with on-line HPLC chromatograms at several representative points of the three peaks.

### 3.4. Comparison of on-line selective collection with off-line purification of full fractions by semi-preparative HPLC

With resources expended collecting and tracking each fraction, it is usually more efficient to collect only the fractions with the desired compounds. However, since the run time of HPLC analysis make on-line purity detection discontinuous, the selective collection may induce part of the fractions missing. In order to assess the efficiency of the on-line purity monitoring system, the off-line evaporated and re-dissolved full fractions of peak I were further purified by semi-preparative HPLC (see Fig. 3). Then a comparison was made between the results obtained

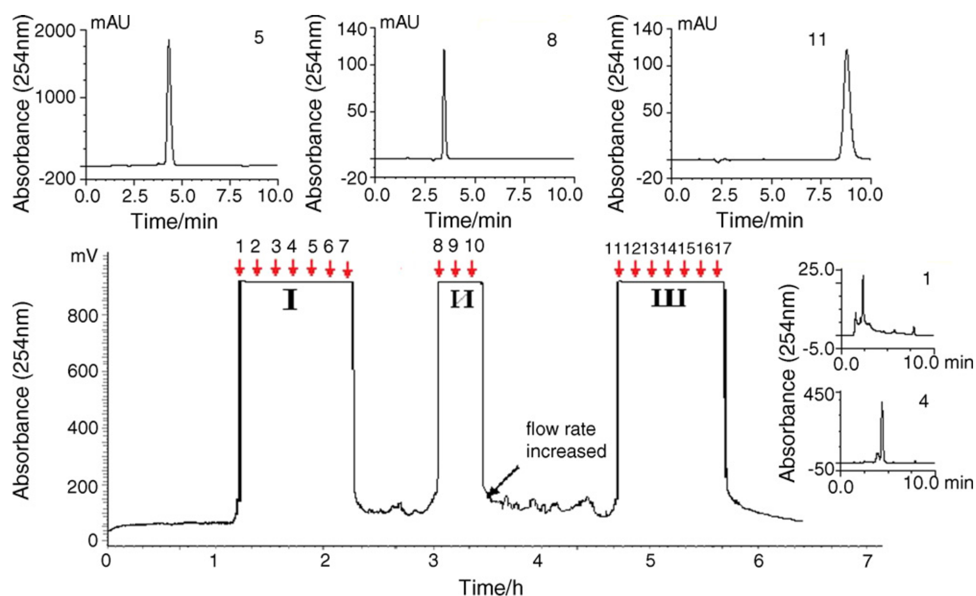


Fig. 2. HSCCC-HPLC-DAD chromatogram of the partially purified extract from *Anemarrhena asphodeloides* Bunge. Peak I: neomangiferin, peak II: 5-HMF and peak III: mangiferin. HSCCC conditions: solvent system: *n*-butanol–water (1:1, v/v); stationary phase: upper phase; mobile phase: lower phase; revolution speed: 800 rpm; separation temperature: 25 °C; flow-rate: 0–3.5 h, 1.0 ml $min^{-1}$ and 3.5–8 h, 2.2 ml $min^{-1}$; sample size: 600 mg; sample loop: 20 ml and detection wavelength: 254 nm. Retention of stationary phase: 30%. HPLC-DAD analysis conditions are the same as shown in Fig. 1. The sample time point: 1–74 min; 2–84 min; 3–94 min; 4–104 min; 5–114 min; 6–124 min; 7–134 min; 8–183 min; 9–193 min; 10–203 min; 11–283 min; 12–293 min; 13–303 min; 14–313 min; 15–323 min; 16–333 min and 17–343 min. The arrow at 3.5 h indicates that the flow-rate of the mobile phase was increased stepwise from 1.0 to 2.2 ml $min^{-1}$.

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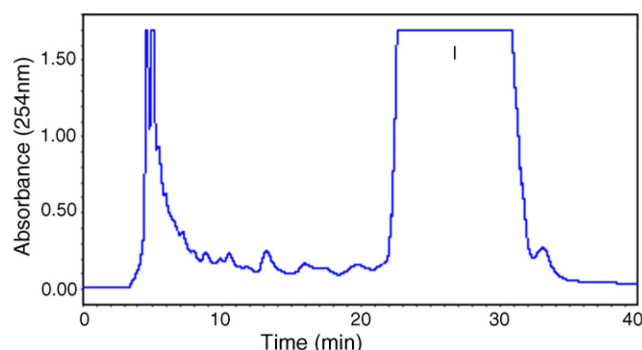


Fig. 3. Semi-preparative chromatogram of neomangiferin after separating by HSCCC. I = neomangiferin. Column: reversed-phase YWG $C_{18}$ (10.0 mm × 200 mm i.d., 10 μm); mobile phase: methanol–water (20:80, v/v); injection volume: 500 μl; flow-rate: 2.2 ml $min^{-1}$; UV wavelength: 254 nm.

using this on-line selective collection technique and the results obtained using traditional post-purification sample treatments. The two methods provided comparable relative purity and recovery determinations.

### 3.5. Origin of 5-HMF

To our best knowledge 5-HMF was isolated in this study for the first time from *A. asphodeloides* Bunge. 5-HMF possessing uterotonic activity, anti-platelet aggregation activity and anti-myocardial ischemia activity, has been separated from many plants [18–21]. Some of the plants contain 5-HMF itself, while others contain some constituents that can be transformed into 5-HMF under critical conditions such as high temperature. In our study, 5-HMF was extracted by reflux at 90 °C, thus it could be inferred that 5-HMF has probably been transformed from polysaccharides during the course of extraction.

## 4. Conclusion

An effective on-line purity monitoring preparative system, HSCCC-HPLC-DAD, has been successfully applied for the first time to the isolation and purification of 5-HMF, mangiferin and neomangiferin from *A. asphodeloides* Bunge with a two-phase solvent system composed of *n*-butanol–water (1:1, v/v). From 600 mg partially purified extract, 17.1 mg 5-HMF, 165.6 mg neomangiferin and 292.8 mg mangiferin with purities of 96.6, 98.9 and 99.5%, respectively, were obtained.

The results show that the purity results obtained from the on-line HSCCC-HPLC-DAD agree with those acquired when sampling from fractions that were evaporated and re-dissolved, one of the more traditional methods of post-separation fraction analysis. The present study indicates that the on-line purity monitoring has improved the efficiency of the overall purification process and HSCCC-HPLC-DAD is a very powerful technique for the preparative separation and purification of bioactive components from the plant materials.

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