# A High-Speed Counter-Current Chromatography–HPLC–DAD Method for Preparative Isolation and Purification of Two Polymethoxylated Flavones From *Taraxacum mongolicum*

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## Abstract

**After an initial clean-up step on silica gel, a preparative high-speed counter-current chromatography coupled with on-line high-performance liquid chromatography–diode array detection method (HSCCC–HPLC–DAD) was successfully developed for the isolation and determination two polymethoxylated flavones, 3′,4′,7-trimethoxyquercetin and artemetin, from the aerial part of *Taraxacum mongolicum*. The HSCCC separation was performed with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (6:5:6:5, v/v/v/v) at a flow rate of 1.5 mL/min and at 800 rpm. The on-line purity monitoring of a representative aliquot from each HSCCC fraction was operated automatically. Using this method, fractions with high purity were collected. The HSCCC purification step was done in 5 h, and afforded 84.2 mg of 3′,4′,7-trimethoxyquercetin and 52.3 mg of artemetin, with purity over 98% from 200 mg of the enriched extracts of *T. mongolicum*. The structures were identified by electorspray ionization mass spectrometry and $^1$H NMR experiments. To our best knowledge, 3′,4′,7-trimethoxyquercetin was obtained from the plant of genus *Taraxacum* for the first time by our group. This hyphenated method could be used for the preparation of bioactive compounds with higher purity from natural products.**

## Introduction

The genus *Taraxacum* is a member of the family Asteraceae, which is widely distributed in the warmer temperature zones of the Northern Hemisphere. This perennial weed has been known since ancient times for its curative properties and has been utilized for the treatment of various ailments such as dyspepsia, heartburn, spleen and liver complaints, hepatitis, and anorexia (1,2). In traditional Chinese medicine (TCM), which sometimes combines herbs, it has been used in preparations to enhance immune response to upper respiratory tract infections, bronchitis, or pneumonia, and as a compress for its anti-mastopathy activity (3,4). *Taraxacum mongolicum* Hand.-Mazz. is a member of genus *Taraxacum*, which has been widely used in TCM for its remarkable curative effects, especially on mastopathy, faucitis, furuncles, pneumonia, appendicities, jaundice, gonorrhoea, tonsillitis, nephritis, etc. (5), and it is officially listed in the Pharmacopoeia of the PR China (6). Furthermore, fresh leaves of *T. mongolicum* have been used by local people as vegetable food in Northern China. Additionally, extracts are used as flavor components in various food products, including alcoholic beverages and soft drinks, frozen dairy desserts, candy, baked goods, gelatins, puddings, and cheese. However, a literature search yielded only a few articles reported for the isolation and identification of metabolites from *T. mongolicum* (7–11). Our preliminary experiments of *T. mongolicum* disclosed that the ethyl acetate extract of this plant was rich in flavonoids, which have been reported to act as antioxidants in various biological systems (12–14). In natural products, flavonoids are the most abundant, but the highly methoxylated flavonoids exhibit higher biological activity, even though they occur in much lower concentrations (15). However, pharmacological properties of the compounds from *T. mongolicum* were still a neglected field over the last years; medical plant therapy is mainly based on the empirical findings during hundreds and thousands of years (16). Therefore, isolation and purification of bioactive constituents from *T. mongolicum* in quantities is of great importance for the further study of their biological, pharmacological, and clinical effects, both in vitro and in vivo.

High-speed counter-current chromatography (HSCCC), first invented by Ito (17), is a support-free liquid–liquid chromatographic technique based on the partitioning of compounds between two immiscible liquid phases, which can eliminate irreversible adsorption of samples on solid support in conven-

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tional column chromatography (18). This method has been successfully applied to the separation and isolation of many natural products (19–23).

The purity of compounds is a critical element in the whole process of obtaining the desired biologically active material needed for screening and for subsequent formation of structure-activity relationships (24). Usually, each fraction after HSCCC purification was collected based on UV response, and then the purity was determined by high-performance liquid chromatography (HPLC) or thin-layer chromatography. Obviously, this method delayed the acquisition of the post-purification sample data. Reports by Zhou have concerned on-line purity monitoring the HSCCC fractions (22,23). The greatest benefit of the method is that, besides the preparative isolation of target compounds, the purity of the fractionations can be measured on-line, which can decrease the instrument idle time. However, reports concerning on-line hyphenated HSCCC–HPLC are scarce.

The present paper successively describes an on-line HSCCC–HPLC–diode array detection (DAD) method to prepare two polymethoxylated flavones, 3',4',7-trimethoxyquercetin and artemetin (Figure 1), with high purity from *T. mongolicum*. The best isolation conditions were optimized after investigation of the effects of two-phase solvent system, flow rate, and revolution speed. The on-line purity monitoring of a representative aliquot from each HSCCC fraction was operated automatically. The chemical structures of the two target compounds were elucidated by ESI–MS and $^1$H NMR. As far as we know, this is the first report to isolate and purify 3',4',7-trimethoxyquercetin from the plant of genus *Taraxacum*.

## Experimental

### HSCCC–HPLC–DAD instrumental set-up

The schematic diagram of the on-line hyphenated HSCCC–HPLC–DAD system was designed for the simultaneously preparative isolation and purity analysis (Figure 2) (22,23).

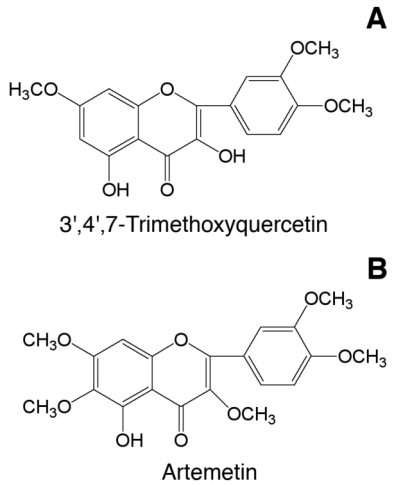


**Figure 1.** Chemical structures of 3',4',7-trimethoxyquercetin and artemetin.

Preparative HSCCC used for this work was carried out using a seal-free HSCCC instrument made by Prof. Qi-Zhen Du in the Institute of Food and Biological Engineering (Zhejiang Gongshang University, Hangzhou, China). The apparatus was equipped with one layer coil column made of convoluted polytetrafluoroethylene (PTFE) tubing with a 2.6 mm average i.d. and a 20-mL sample loop (total volume, 420 mL). The column revolves around its own axis at the angular velocity in the same direction. The revolution radius, or the distance between the holder axis and central axis of the centrifuge, was 8 cm, and the β value of the coils from the inner layer to the outer layer is 0.50–0.79. β = r/R, where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distant between the holder axis and central axis of the centrifuge. The revolution speed of the apparatus can be regulated with a speed controller in the range of 0–1000 rpm. The HSCCC system was also equipped with a model FMI constant-flow pump (Zhejiang Instrument Factory, Hangzhou, China), a PC300 variable wavelength detector operating at 254 nm, and a model SCJS-3000 workstation (Tianjin Scientific Instrument Ltd., Tianjin, China). On-line purity analysis was performed with a six-port, two-position valve including a 10-µL sample loop (Unimicro Technologies Co., Shanghai, China), a Shimadzu analytical HPLC system which consisted of two LC-8A pumps, a Prominence SPD-M20A diode array detector, and an LC Solution workstation (Shimadzu, Japan).

### Chemicals and reagents

All organic solvents used for preparation of crude extracts and HSCCC separation were of analytical grade and purchased from the Second Institute of Oceanography (Zhejiang, China). Acetronitrile used for HPLC was of chromatographic grade (Merk, Darmstadt, Germany). All aqueous solutions were prepared with pure water produced by Milli-Q water (18.2 MΩ) system (Millipore, Billerica, MD). Silica gel (200–300 mesh, Qingdao, China) was used for column chromatography.

The aerial part of *Taraxacum mongolicum* Hand.-Mazz. was purchased from Bozhou, Anhui province in January and identified by Prof. Liurong Chen (College of Pharmaceutical Sciences, Zhejiang University).

### Preparation of sample elution

The ground dried aerial parts of *T. mongolicum* (2.0 Kg) were extracted three times with 95% EtOH for 3 h at reflux temperature. Then the EtOH extract was concentrated under reduced pressure to give brown syrup (253 g), which was

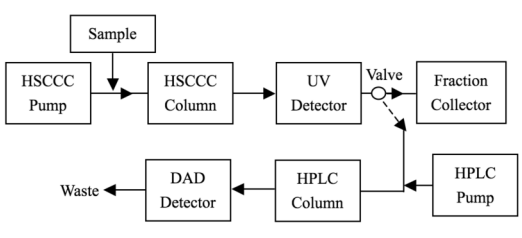


**Figure 2.** Schematic HSCCC–HPLC–DAD instrumental set-up.

suspended in $H_2O$ and then partitioned successively with light petroleum (60–90°C), ethyl acetate, and *n*-BuOH. The combined ethyl acetate parts were evaporated to dryness in vacuum to give a residue (89 g), which was then applied to silica gel (200–300 mesh) column chromatography and eluted with petroleum ether–ethyl acetate mixtures of increasing polarity. The fraction containing 1.2 g of the target components was evaporated separately to dryness under reduced pressure, which was then stored in a refrigerator for subsequent HSCCC separation.

### Preparation of two-phase solvent system and sample solution

The solvent system for HSCCC separation was selected according to the difference of partition coefficients (*K*) of two polymethoxylated flavones in various solvent systems. The *K* was calculated by the HPLC peak area of them in the upper phase and lower phase when a crude sample was separated in a two-phase solvent system. The two-phase solvent system used was composed of *n*-hexane, ethyl acetate, methanol, and water at various volume ratios. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature, and the two phases were separated shortly before use. The upper phase and the lower phase were separated and degassed by sonication for 30 min shortly before use.

The sample solution was prepared by dissolving the enriched extract in the solvent mixture consisting of equal volumes of both upper and lower phases because the sample was not easily dissolved in either phase.

### HSCCC separation procedure

The multi-layer-coiled column was first entirely filled with the upper phase (stationary phase). Then the lower phase (mobile phase) was pumped into the inlet of the column at the flow rate of 1.5 mL/min, while the apparatus was rotated at 800 rpm. After the mobile phase was eluted from the tail outlet and the two phases had established the hydrodynamic equilibrium throughout the column, the sample solution (200 mg of the enriched extract in 20 mL of both phases) was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm, and the chromatogram was recorded. After two peaks were eluted, the centrifuge was stopped, and the column contents were fractionated by continuously eluting the column with the mobile phase.

### HPLC–DAD purity analysis and identification of the fractions

At the suitable time, the valve was switched to collect HPLC fractions for purity analysis, and then, after 1 min, the valve was triggered back. Fractions were conducted on a Symmetry $C_{18}$ column (150 mm × 3.9 mm i.d., 5 µm) using an isocratic elution of acetonitrile–1% acetic acid (51:49, v/v) as the mobile phase. The analysis was carried out using a flow rate of 0.8 mL/min at room temperature. Chromatograms were recorded at 254 nm. The purities of the collected fractions were determined by HPLC, based on the peak area of the target species normalized to the sum of all observed peaks.

Identification of the HSCCC peak fractions was carried out by ESI-MS and $^1$H NMR experiments. ESI–MS data were measured on an Apex III instrument (Bruker Daltonics Corp.). The $^1$H NMR experiments were performed on a Varian Inova-400 (Varian, Palo Alto, CA) NMR spectrometer using DMSO-$d_6$ as solvent.

## Result and Discussion

### Optimization of chromatographic conditions

The optimization procedure was performed with regards to the best possible separation as well as consideration of the run time. Several elution systems were tested in the separation of the enriched extract, such as gradient elution of methanol–water–acetic acid, acetonitrile–water–acetic acid, isocratic elution of methanol–water–acetic acid, acetonitrile–water–acetic acid, etc. When acetonitrile–1% acetic acid was used as the mobile phase in isocratic mode (51:49, v/v), good results could be obtained. The enriched extract from the silica clean-up step of *T. mongolicum* was analyzed by HPLC under the optimum analytical condition, and the chromatogram was given in Figure 3. Two major peaks were separated and detected. The samples can be directly used for HSCCC separation.

### Selection of two-phase solvent system

The appropriate solvent system plays an important role in separation by HSCCC. Partition coefficient (*K*) is the most important parameter in solvent system selection, which should be close to 1 to get an efficient separation and a suitable run time. If it is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in loss of peak resolution; if the *K* value is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time (25). In the experiment, partition coefficient was tested with various solvent systems, such as ethyl acetate–methanol–water and *n*-hexane–ethyl acetate–methanol–water and at various volume ratios.

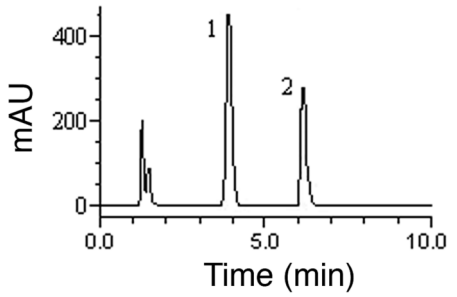


**Figure 3.** HPLC chromatogram of enriched extract from the silica clean-up step. HPLC conditions: reversed-phase Symmetry $C_{18}$ column (150 mm × 3.9 mm i.d., 5 µm); mobile phase, acetonitrile–1% acetic acid (51:49, v/v); flow rate, 0.8 mL/min; UV wavelength, 254 nm; column temperature, at room temperature. Peaks 1 and 2 correspond to 3',4',7-trimethoxyquercetin and artemetin, respectively.

When the two-phase solvent system composed of ethyl acetate–methanol–water was used, the two target compounds were mostly distributed in the upper organic phase, which results in a long elution time to elute the target compounds and a broad peak. Thus, the two-phase solvent system was changed by adding *n*-hexane to reducing the "dissolvability" of the target components in the upper phase. Then, different ratios of volume based on *n*-hexane–ethyl acetate–methanol–water were used to conduct partition coefficient tests. The measured *K* values for two target components are shown in Table I. Among the solvent systems, the two-phase solvent system of *n*-hexane–ethyl acetate–methanol–water at a ratio of (6:5:6:5, v/v/v/v) was found to be suitable for the separation of two target polymethoxylated flavones from the enriched extract.

Other factors, such as the revolution speed of the separation column and the flow rate of the mobile phase, were also investigated. The results showed that when the flow rate was 1.5 mL/min, revolution speed was 800 rpm, retention percentage of the stationary phase could reach 58%, and good separation results could be obtained.

**Table I. The Partition Coefficients (*K*) of the Target Compounds in Different Ratios of Volume in the Solvent System**

| | | *K* | |
|---|---|---|---|
| **Solvent system*** | **Ratio** | **3',4',7-TQ** | **Artemitin** |
| *n*-H–EA–M–W | 6:5:5:6 | 2.09 | 2.63 |
| *n*-H–EA–M–W | 6:5:5.5:5.5 | 1.40 | 1.89 |
| *n*-H–EA–M–W | 6:5:6:5 | 0.62 | 1.21 |
| *n*-H–EA–M–W | 6:5:6.5:4.5 | 0.34 | 0.70 |

* *n*-H–EA–M–W = *n*-hexane–ethyl acetate–methanol–water
† 3',4',7-trimethoxyquercetin = 3',4',7-TQ

### Purification of target compounds by HSCCC–HPLC–DAD

Under the optimum conditions, the enriched sample from *T. mongolicum* was separated and purified. The valve was switched to collect HPLC fractions for on-line purity analysis when the HSCCC elution times reached 178 (first point), 195 (second point), 213 (third point), 230 (fourth point), 251 (fifth point), 269 (sixth point), and 285 (seventh point) min. Two fractions (I and II) were obtained in one-step elution in less than 5 h, which is 84.2 mg of fraction I (collected during 175–220 min) and 52.3 mg of fraction II (collected during 250–280 min). The on-line HPLC purity monitoring of each HSCCC fraction revealed that the purity of fractions I and II was over 98%. Figure 4 shows the preparative HSCCC separation with on-line HPLC chromatograms at several representative sites.

### The structural identification

The structural identification of peak fractions was all performed with ESI–MS and $^1$H NMR spectra.

Fraction I: ESI–MS *m*/z: 345 $[M+H]^+$; $^1$H-NMR (DMSO-$d_6$, 400 MHz): ∂ 12.66 ($^1$H, br s, 5-OH), 9.89 ($^1$H, br s, 3-OH), 7.68 ($^1$H, d, *J* = 2.0 Hz, H-2'), 7.63 ($^1$H, dd, *J* = 8.4, 2.4 Hz, H-6'), 6.97 ($^1$H, *J* = 8.4 Hz, H-5'), 6.77 ($^1$H, d, *J* = 2.0 Hz, H-6), 6.38 ($^1$H, d, *J* = 2.0 Hz, H-8), 3.97 (6H, s, 3',4'-di-$OCH_3$), 3.81 ($^3$H, s, 7-$OCH_3$). Fraction I was identified as 3',4',7-trimethoxyquercetin according to the literature (26).

Fraction II: ESI-MS *m*/z: 389 $[M+H]^+$; $^1$H-NMR (DMSO-$d_6$, 400 MHz): ∂ 7.73 ($^1$H, dd, *J* = 8.8, 2.4 Hz, H-6'), 7.67 ($^1$H, d, *J* = 2.4 Hz, H-2'), 7.17 ($^1$H, *J* = 8.8 Hz, H-5'), 6.93 ($^1$H, s, H-8), 3.93, 3.87 (4',3'-OMe), 3.86 (7-OMe), 3.83 (6-OMe), 3.74 (3-OMe). Fraction II was identified as artemitin according to the literature (27).

## Conclusion

The HSCCC–HPLC–DAD method was first applied to the preparative isolation and on-line purity monitoring of the polymethoxylated flavones 3',4',7-trimethoxyquercetin and artemitin from the TCM *T. mongolicum*. The results obtained in the present study demonstrate that HSCCC is a powerful technique for the isolation of bioactive compounds from plant materials, and on-line purity monitoring can improve the efficiency of the overall purification process. Moreover, 3',4',7-trimethoxyquercetin was isolated from the genus *Taraxacum* for the first time.

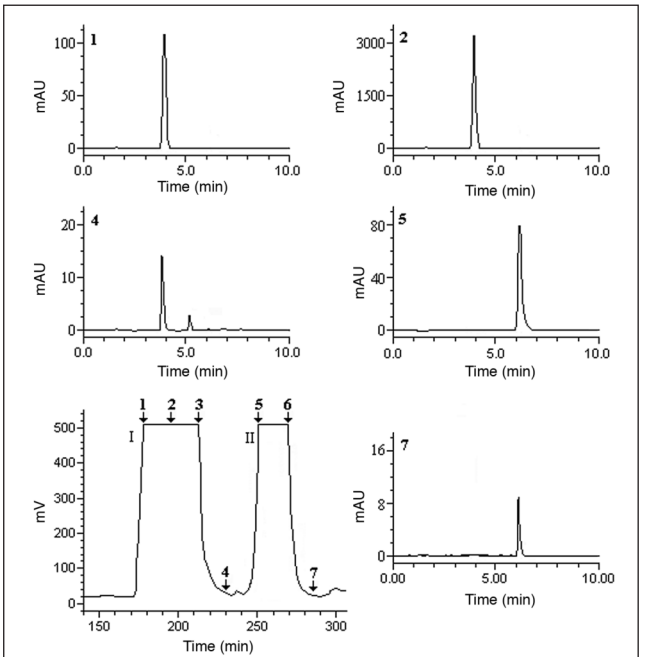


**Figure 4.** HSCCC–HPLC–DAD chromatogram of the enriched extract from *T. mongolicum*. Experimental conditions: revolution speed, 800 rpm; solvent system, *n*-hexane–ethyl acetate–methanol–water (6:5:6:5, v/v/v/v); stationary phase, upper phase; mobile phase, lower phase; flow rate, 1.5 mL/min; retention of the stationary phase, 58%; detection wavelength, 254 nm. Fractions I and II correspond to 3',4',7-trimethoxyquercetin and artemetin, respectively. HPLC–DAD analysis conditions are the same as those in Figure 3. The time point for on-line analysis: 1, 178 min; 2, 195 min; 4, 230 min; 5, 251 min; 7, 285 min.

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