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Separation of polyphenols from leaves of *Malus hupehensis* (Pamp.) Rehder by off-line two-dimensional High Speed Counter-Current Chromatography combined with recycling elution mode

Qi Liu [a], Hualiang Zeng [a], Shujing Jiang [a], Li Zhang [a], Fuzhu Yang [a], Xiaoqing Chen [a,b,*], Hua Yang [a,1]

[a] College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China
[b] Collaborative Innovation Center of Resource-conserving & Environment-friendly Society and Ecological Civilization, Changsha 410083, China

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ABSTRACT

In this study, off-line two-dimensional High Speed Counter-Current Chromatography (2D HSCCC) strategy combined with recycling elution mode was developed to isolate compounds from the ethyl acetate extract of a common green tea – leaves of *Malus hupehensis* (Pamp.) Rehder. In the orthogonal separation system, a conventional HSCCC was employed for the first dimension and two recycling HSCCCs were used for the second in parallel. Using a solvent system consisting of *n*-hexane–ethyl acetate–methanol–water (1:4:0.6:4.4, v/v) in the first and second dimension, four compounds including 3-hydroxy-phlorizin (**1**), phloretin (**2**), avicularin (**3**) and kaempferol 3-O-β-D-glucoside (**4**) were obtained. The purities of these four compounds were all over 95.0% as determined by HPLC. And their structures were all identified through UV, MS and ^{1}H NMR. It has been demonstrated that the combination of off-line 2D HSCCC with recycling elution mode is an efficient technique to isolate compounds with similar polarities in natural products.

1. Introduction

With advantages such as high sample recovery, large loading capacity, and low risk of sample denaturation and irreversible adsorption (Ito, 2005), the continuous liquid–liquid partition chromatography-High Speed Counter-Current Chromatography (HSCCC) (Ito & Bowman, 1970) has become a beneficial supplement of Liquid Chromatography (LC). Nevertheless, compared with LC, the relatively lower theoretical plates of HSCCC usually results in insufficient peak capacity and resolution (Berthod, Ruiz-Angel, & Carda-Broch, 2009). Meanwhile, all-pervading existence of constituents with similar structures in natural products (Cragg & Newman, 2013; Mishra & Tiwari, 2011) would incidentally lead to its indiscriminate distribution in two-phase solvent system. Retention time (R_t) of a solute in HSCCC is directly decided by its distribution coefficient (K). Thus, it is common that compounds are co-eluted in one peak when implementing HSCCC in the separation of natural products (Shi et al., 2012). Longer HSCCC column might provide improved separation efficiency when there is little difference in K values. Yet it should also be noted that length of commercial HSCCC columns are constant and limited. In addition, elongated column would bring up issues including higher column pressure, increased experiment cost and higher requirement of equipments. Fortunately, the methodology equivalent to elongating column – recycling strategy could address this problem. As a practical approach to separating compounds with poor resolution, recycling elution mode could remarkably improve the separation potential (Han, Song, Qiao, Wong, & Xu, 2006; Tong, Guan, Yan, Zheng, & Zhao, 2011). Meanwhile, due to the usage of the effluent as the mobile phase and sample solution, it would diminish mobile phase or sample loss during recycling periods.

The most evident feature of the recycling methodology lies in that the effluents of the column would be redirected into the same column, which could increase the risk of peak overlap in recycling process. Consequently, severe peak overlapping usually could be observed for complicated sample when implementing recycling HSCCC (R-HSCCC) separation (Shi et al., 2012). As we know, the crude extract of natural products is extremely complicated, which could probably contain from dozens to hundreds of compounds (Ma, Liang, Jiang, Wang, & Luo, 2012). It is highly demanding to pre-purify the target object before adopting recycling elution mode. Preparative LC, silica gel column chromatography, and HSCCC are commonly used purification methods for natural

* Corresponding author at: College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China. Tel.: +86 731 88830833.
E-mail addresses: xqchen@csu.edu.cn (X. Chen), hychem@csu.edu.cn (H. Yang).
[1] Additional corresponding author. Tel.: +86 731 88830833.

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products. As mentioned before, HSCCC possesses advantages such as low risk of sample denaturation and irreversible adsorption (no solid stationary phase used) and serves as a novel separation technique for bioactive compounds. Undoubtedly, LC and silica gel column chromatography would compromise this unique feature of HSCCC separation. Using HSCCC as the tool to pre-purify the complex crude extract, or namely off-line two dimensional (2D) HSCCC (Liu et al., 2013; Su, Liu, Yang, Yu, & Chen, 2013), would preserve its specialty. 2D HSCCC is committed to separating compounds co-eluted in the first dimensional (1st-D) twice. Usually different solvent system or elution mode is used in the second dimension (2nd-D). Compared with on-line 2D HSCCC (Liu et al., 2014; Lu, Hu, & Pan, 2010; Lu, Sun, Liu, & Pan, 2007; Lu, Sun, Wang, & Pan, 2007), relatively simple off-line HSCCC can be realised much more easily and conveniently.

Malus hupehensis (Pamp.) Rehder, an ornamental tree species belonging to the Rosaceae family, is mainly distributed in southern China. As an important class of tea, leaves of *M. hupehensis* (Pamp.) Rehder is nicknamed "San-Pi-Guan" implying that a pot of tea can be made by using only three pieces of leaves. It has been found to possess important biological activities such as anti-inflammation, anti-fatigue and treating hypoglycemia effect (Boccia, Kopf, & Baratti, 1999; Bradford & Allen, 2007; Janssen et al., 2003; Wang et al., 2013), which are attributed in part to the presence of polyphenols and their related antioxidant capacities. The relatively complicated silica gel column chromatography as well as Sephadex LH-20 and semi-preparative HPLC (Wang et al., 2013) have been employed as the separation technique for *M. hupehensis* (Pamp.) Rehder. In this work, off-line 2D HSCCC strategy was applied to separating compounds with similar structures from extract of leaves of *M. hupehensis* (Pamp.) Rehder. The co-eluted compounds in the 1st-D HSCCC were subjected to the second dimensional (2nd-D) R-HSCCC. As a consequence, four compounds were isolated and identified to be 3-hydroxy-phlorizin, phloretin, avicularin and kaempferol 3-O-β-D-glucoside through UV, MS/MS, and ^{1}H MNR.

2. Experimental

2.1. Apparatus

The preparative HSCCC was performed using a model TBE-300B HSCCC (Shanghai Tauto Biotechnique Co. Ltd., Shanghai, China). The apparatus consists of an upright coil type-J planet centrifuge with three multilayered coils connected in series (diameter of tube, 1.6 mm, total capacity 260 mL) and a 20 mL manual sample loop. The rotation speed is ranged from 0 to 1000 rpm. The HSCCC system was equipped with a TBP-50A LC pump, a TBD-2000 UV detector, a HX-1050 constant temperature regulator (Beijing Boyikang Lab Implement Co. Ltd., Beijing, China), a BSZ-100 automatic fraction collector (Shanghai Qingpu-Huxi Instruments Factory, Shanghai, China) and a WH V4.0 workstation (Shanghai Wuhao Information Technology Co. Ltd., Shanghai, China). The connection of R-HSCCC is shown in Fig. 1, in which two different positions of the 6-port valve represent two separation periods respectively. Position A 1-2 shows conventional non-recycling HSCCC instrument configuration. The 6-port valve was switched to 6-1 to form closed-loop R-HSCCC system when recycling elution mode is needed.

HPLC apparatus (Dionex Ultimate 3000, USA) and a reversed-phase Waters Spherisorb ODS2 (250 mm × 4.6 mm i.d., 5 μm, Waters, Milford, MA, USA) column were employed for analysis. Dionex Ultimate 3000 system is comprised of a SRD-3600 6 degasser channels, a DGP-3600SD binary pump, a WPS-3000SL auto sampler, a TCC-3000SD thermo stated column compartment and

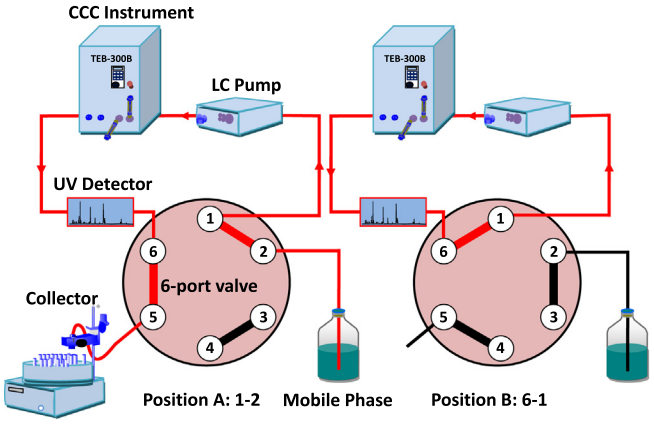


Fig. 1. Instrument configuration of the R-HSCCC.

a DAD-3000 multiple wavelength detector (Dionex, Sunnyvale, CA, USA). MS data were acquired in the negative ion mode from a Micromass® Quattro micro™ API mass spectrometer (Waters Corp., Milford, MA, USA) with an ESI interface. NMR experiments were performed on a Bruker-500 (Bruker Corporation, Germany) NMR spectrometer. The reference compound TMS was used as the internal standard for determination of chemical shifts.

2.2. Materials and reagents

The dried leaves of *M. hupehensis* (Pamp.) Rehder were purchased from Tianjian Drugstore (Yuelu District, Changsha, Hunan, China) and identified by local pharmacist. Petroleum ether (60–90 °C), *n*-hexane, ethyl acetate, *n*-butanol, 95% ethanol and methanol were of analytical grade and obtained from Chemical Reagent Factory of Hunan Normal University (Changsha, Hunan, China). Acetonitrile used for HPLC was of chromatographic grade (Merk, Darmstadt, Germany). Ultrapure water (18.2 MΩ resistivity) used in the present work was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Preparation of crude sample

The dried and chopped leaves of *M. hupehensis* (Pamp.) Rehder (100 g) were extracted with 95% ethanol twice (500 mL × 2). Then the extracts were combined, filtered, and concentrated at 40 °C under reduced pressure to afford the crude mixture (4.30 g). The resulting residue was then diluted with 500 mL of H_2O and extracted successively with 500 mL of petroleum ether, ethyl acetate, and *n*-butanol respectively. The concentrated ethyl acetate extract was stored in a refrigerator (4 °C) and subjected for subsequent HPLC analysis and HSCCC separation.

2.4. HPLC and HPLC-MS conditions

The chromatographic separation was carried out using a mixture of acetonitrile (A) and water containing 0.4% (v:v) acetic acid (B). The gradient program for the ethyl acetate extract was used according to the following profile: 17% A (0–20 min), 17–25% A (20–30 min), 25% A (30–40 min), 25–40% A (40–45 min), 40% A (45–55 min). The flow rate was 1.0 mL/min while the ambient temperature was controlled at 25 °C. Spectra were recorded from 190 to 400 nm while the chromatogram was acquired at 254 nm.

For HPLC-MS experiments, the stationary phase and the elution gradient were the same as those in the HPLC analysis. Triple-quadrupole tandem mass spectrometric detection was carried out on a Micromass® Quattro micro™ API mass spectrometer

(Waters Corp., Milford, MA, USA) with an electrospray ionisation (ESI) interface. The ESI source was set in negative ionisation mode. The following settings were applied to the instrument: capillary voltage, 3.00 kV; cone voltage, 40.0 V; extractor voltage, 3.00 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas flow, 750 L/h; cone gas flow, 50 L/h, dwell time, 0.05 s. Nitrogen was used as the desolvation and cone gas. Mass detection was performed in full scan mode for m/z in the range 160–800. All data were acquired and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.5. HSCCC separation

2.5.1. Selection of solvent system

In the present study, a series of two-phase solvent systems including *n*-hexane–ethyl acetate–methanol–water (HEMW) in various ratios were tested for their partition capabilities.

The partition coefficients of solutes in two-phase solvent systems were determined by HPLC as follows. The ethyl acetate fraction was firstly dissolved in methanol, which would be used in the following experiments. Then, a series of solvent systems in different ratios were prepared and shaken (methanol was substituted by methanol solution of ethyl acetate extract). After being equilibrated for 20 min, the resulting two layers were separated completely, and each layer was taken out and analysed by HPLC. The *K*-values of target components were calculated according to the equation $K = A_U/A_L$, where A_U and A_L are the peak areas of target compounds in the upper phase and lower phase respectively.

2.5.2. Preparation of two-phase solvent systems and sample solution

After being mixed according to the volume ratios, the selected solvent system HEMW (1:4:0.6:4.4, v/v) was thoroughly equilibrated in a separatory funnel at room temperature. Then the upper phase and lower phase were separated and degassed by sonication for 30 min prior to use.

The sample solution of the off-line 1st-D HSCCC separation was prepared by dissolving 260 mg of ethyl acetate extract in 6 mL of mobile phase. The A and B parts of eluents in the 1st-D HSCCC were collected, combined and then evaporated at 40 °C under vacuum. 28.2 mg and 47.8 mg of solutes were obtained from two parts respectively. Then the sample solutions for two parallel R-HSCCCs were prepared by dissolving these two solutes in 5 mL of mobile phase respectively.

2.5.3. HSCCC separation procedure

In the 1st-D HSCCC, the separation column of HSCCC was entirely filled with the stationary phase (the upper phase) by constant flow pump at 20.0 mL/min. The separation temperature was controlled at 25 °C. After the separation column was filled with stationary phase, the apparatus was rotated at 900 rpm and the mobile phase was pumped into the column at the flow rate of 1.2 mL/min. The retention of the stationary phase (S_F) was calculated when hydrodynamic equilibrium of system was established in the column (the mobile phase emerged at the outlet). Then the sample solution was injected into the separation column through the injection valve. And effluent from the outlet of column was continuously monitored at 254 nm and collected by BSZ-100 automatic fraction collector.

Restricted by the limited peak capacity of HSCCC and their negligible differences of *K* values, compounds in A and B parts of the 1st-D HSCCC were co-eluted. Therefore, two off-line 2nd-D HSCCCs with recycling elution mode were employed in parallel for further separation. The R-HSCCC separation process can be described as follows: firstly, the 6-port switching valve was enforced at 1-2 (the configuration is shown in Fig. 1), and the HSCCC column was entirely filled with the upper phase of solvent system at 20.0 mL/min. Then the apparatus was rotated at 900 rpm, while the lower phase was pumped into the column at 1.2 mL/min. After the hydrodynamic equilibrium was established, the sample solution was injected through the injection valve. The effluent from the outlet of column was continuously monitored at 254 nm. Secondly, immediately after those components without the necessity for recycling were eluted, the 6-port valve was turned to position B 6-1 to form a recycling tube. Finally, when the target compounds were sufficiently separated through several HSCCC cycles, recycling elution mode was stopped by switching the valve back to 1-2 to release the separated samples and collected automatically.

2.6. Analysis and identification of separated compounds

HPLC analysis of every HSCCC peak fraction was performed on Dionex Ultimate 3000 and Waters Spherisorb ODS2 under the same conditions as described in Section 2.4. Every concentrated compound was dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) and submitted to Bruker-500 NMR spectrometer. The reference compound TMS was used as the internal standard for determination of chemical shifts.

3. Results and discussion

3.1. Optimisation of HPLC conditions

Different mobile phases with different flow rates were optimised for the HPLC analysis of ethyl acetate fraction of leaves of *M. hupehensis* (Pamp.) Rehder. And acetic acid was added into the mobile phase in this study, as acid has been known to provide better peak symmetry for compounds with hydroxyl groups (Dai et al., 2013). The results indicated that the mobile phase was composed of acetonitrile (A) and water containing 0.4% (v:v) acetic acid (B) in a appropriate gradient mode (17% A (0–20 min), 17–25% A (20–30 min), 25% A (30–40 min), 25–40% A (40–45 min), 40% A (45–55 min)) and flow rate 1.0 mL/min were most suitable for this analysis. Column temperature and detection wavelength were routinely set at 25 °C and 254 nm, and spectra were recorded from 190 to 400 nm respectively. Under the above conditions, a relatively satisfactory separation of target compounds was obtained, even though some co-eluted peaks still could not be completely eliminated due to existence of compounds with similar structures. The chromatogram of ethyl acetate extract is shown in Fig. 2, in which two compounds were eluted in a fork-like peak at around 24.0 min. After 1st-D HSCCC separation, compound **2** and **4** were also co-eluted in a single peak at 31.0 min. Other examined mobile phase compositions or gradient modes still could not provide a satisfactory result, although some mobile phase compositions with lower organic phase ratio could give higher separation factors of these compounds with much longer retention times. It should be noticed that the negligible retention differences of these compounds in the HPLC chromatogram also verified the difficulty of this HSCCC separation.

3.2. Two-phase solvent systems and separation conditions

Being one of the most important steps in HSCCC separation, selection of a suitable two-phase solvent system can be achieved through testing partition coefficients (*K*) of solutes. Generally, ideal *K* values are expected to be between 0.5 and 2.0 (Ito, 2005). Furthermore, satisfactory retention of the stationary phase ($S_F > 40\%$), reasonable volume ratios of the two phases (close to 1:1) and short settling time (<30 s) of selected solvent system are also required. Considering the polarities of target compounds, the

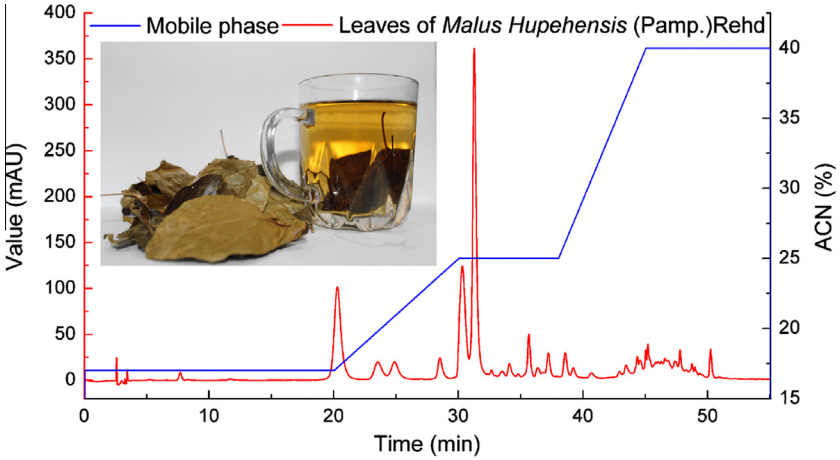


Fig. 2. HPLC chromatogram and mobile phase gradient of leaves of *Malus hupehensis* (Pamp.) Rehder.

regular solvent system – HEMW, which was commonly used in the separation and purification of solutes with wide range of polarities in HSCCC (Ito, 2005), was employed. K values of solutes in HEMW solvent systems with different volume ratios including 1:2:1:2, 1:5:1:5 and 1:4:1:4 (v/v) were tested. Results showed smaller *K* values of compounds are presented in 1:2:1:2 (v/v), while large *K*-values for most compounds were obtained in 1:5:1:5 (v/v). It is well known that low *K*-values result in poor peak resolution and large *K*-values usually lead to elongated retention times and excessive sample band broadening. Thus, HEMW solvent systems in the ratios of 1:2:1:2 and 1:5:1:5 (v/v) were not suitable for our HSCCC separation. In the ratio of 1:4:1:4 (v/v), K values for most compounds were marginally larger than the desired value (1.0). Therefore, slight adjustment of volume ratios of the solvent system was executed in order to decrease *K* values modestly. The proportion of menthol was cut from 1.0 to 0.6. However, the proportion of water was increased to 4.4 simultaneously to secure the volume ratio of upper and lower phase close to 1:1. Thus, HEMW 1:4:0.6:4.4 (v/v) was selected as the experimental solvent system. Furthermore, according to the basic formula between S_F, *K*-value and R_t, the higher the retention level, the better the peak resolution. So the factors influencing S_F including flow rate and revolution speed were also optimised. It is clear that higher flow rate is unfavorable to the retention of the stationary phase, while lower flow rate gives higher S_F and concurrently produces a good separation with longer separation time. Taking S_F and experimental duration into account, a flow rate of 1.2 mL/min was ultimately used. Considering higher rotary speed can increase S_F value and might produce excessive sample band broadening, a relative high revolution speed of 900 rpm was chosen for the HSCCC separation. Under above separation conditions, S_F reached up to 64.6%.

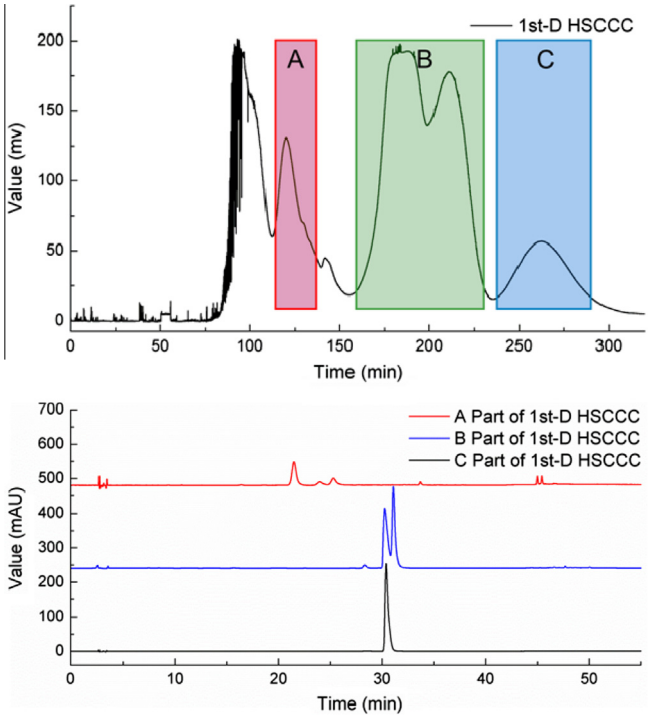


Fig. 3. HSCCC chromatogram of ethyl acetate extract of the leaves of *Malus hupehensis* (Pamp.) Rehder, and HPLC chromatograms of the three HSCCC fractions A, B and C. HSCCC conditions: solvent system: HEMW (1:4:0.6:4.4, v/v); mobile phase: lower phase; stationary phase: upper phase; revolution speed: 900 rpm; sample loading: 260 mg of ethyl acetate extract dissolved in 6 mL of lower phase; detection wavelength: 254 nm; flow rate: 1.2 mL/min; separation temperature: 25 °C; retention of the stationary phase: 64.6%.

3.3. HSCCC separation process

As shown in Fig. 3, four main peaks presented in the HSCCC chromatogram of ethyl acetate extract through a separation process of five hours. It was observed that *K* values of those compounds fell in a reasonable range and therefore could be eluted successfully. However, restricted by the limited peak capacity of HSCCC and existence of compounds with similar structures, one peak with a shoulder and one folk-like peak were presented in the A and B parts of the HSCCC chromatogram respectively, indicating that those compounds were separated unsuccessfully. Results of HPLC analysis (shown in Fig. 3) verified this inference: Only compound **4** (0.45 mg) was successfully purified in part C. Compound **1** was eluted with other several impurities in part A, while two main compounds **2** and **3** were presented in the HPLC chromatogram of B part. Typically, it is extremely difficult to separate these compounds through just altering volume ratio of solvent system (Shi et al., 2012). One possible solution is to introduce complexation agents such as copper (Liu, Luo, & Kong, 2011) or silver ion (Wen et al., 2009) into solvent system. However, due to the specificity of complexation agents, searching and optimising the species and concentrations of complexation agents are usually complicated and time-consuming. Noticeably, the existence of the peak with a shoulder and the fork-like peak in the HSCCC chromatogram also indicates that compounds in A and B parts could be partially separated by the solvent system. And their

resolutions were just less than 1.5. Be committed to separating intractable compounds with repeated HSCCC processes, recycling elution mode has been proven feasible in separating compounds with small distribution coefficient differences (Han et al., 2006; Tong et al., 2011). In our previous work, compounds with the separation factor of 1.30 were successfully separated by adopting recycling strategy (Liu et al., 2013). It is predictable that a better separation could be achieved through adopting recycling elution mode. Thus, off-line 2nd-D R-HSCCC strategy was adopted for further separation of A and B parts, which were collected from the 1st-D HSCCC separation.

Part A containing compound **1** was firstly subjected to a 2nd-D R-HSCCC separation. And the recycling elution mode was executed immediately before the target peak was about to emerge (at 88 min). Under the same separation conditions, part A was eluted as it in the 1st-D HSCCC separation (Fig. 4). A single peak with slight shoulder was presented, which suggested that compound **1** was eluted with other impurities simultaneously. However, separation factor of these two peaks was obviously improved accompanied with increase of recycling pass: after one cycle, the shoulder becomes increasingly clear, and the single peak was intended to be split into two peaks; two main peaks were presented clearly *via* five cycles. Meanwhile, the peak range was extended from 50 min to 140 min after six cycles. Considering the end of the first peak in the sixth cycle would overlap the front of the next peak in seventh cycle, the recycling elution mode was stopped at 695 min although no baseline separation was obtained. Compounds in those peaks were then released, collected automatically, and combined sequentially. HPLC chromatograms (Fig. 4) of the two peaks indicated that compound **1** (with amount of 15.7 mg) was successfully isolated from the mixture A. It also should be noticed that a small peak emerged before the first peak and its retention time in HSCCC was different from that of the main peak. This impurity peak was released and completely separated from the first peak after the forth cycle was accomplished. This also verified that the recycling HSCCC usually can afford the separated compounds with higher degree of purities.

Similarly, part B was also subjected to a 2nd-D R-HSCCC separation. The recycling elution mode was enforced at 150 min, and a fork-like peak showed up as expected (Fig. 5). Due to the relative higher separation factor between compounds **2** and **3**, two peaks were separated by baseline after only one recycling pass. The peak width was also extended from 90 min to 150 min. Those separated compounds were collected automatically, and then subjected to HPLC.

As shown in Fig. 5, peak area percentages of two main peaks in the HPLC chromatograms were all over 98%, indicating the unique advantage of R-HSCCC in achieving effective separations among compounds with negligible *K* values distinction in this experiment. Considering the general existence of structural similarity and relative lower peak capacity of HSCCC, R-HSCCC may find broader application prospect in the separation of natural products in the near future.

3.4. Structural identification of separated compounds

The structures (Fig. 6) of those isolated compounds were characterised by UV, MS and ^{1}H NMR. As data shown below, UV spectra of compounds **1** and **2** were similar, indicating they have little structural differences. By comparing their UV, MS and ^{1}H NMR data with reported reference (Xü, Lü, Qü, Shan, & Song, 2010), compounds **1** and **2** were assigned as 3-hydroxyphlorizin (**1**), phloretin (**2**) respectively. Meanwhile, with two absorbance near 260 nm and 350 nm, typical spectra of flavonoids were observed in UV spectrograms of compounds **3** and **4**. The two compounds were then identified as avicularin (**3**) and kaempferol 3-O-β-D-glucoside (**4**) by further comparing their UV, MS and ^{1}H NMR with the references (Ren, Wu, Ren, & Zhang, 2013; Sirat, Rezali, & Ujang, 2010).

Compound **1**. UV max (nm): 222, 285; ESI-MS *m*/*z*: 451.12 $[M-H]^-$, ^{1}H NMR (500 MHz, DMSO-d_6): 13.78 (1H, s), 6.63 (1H,

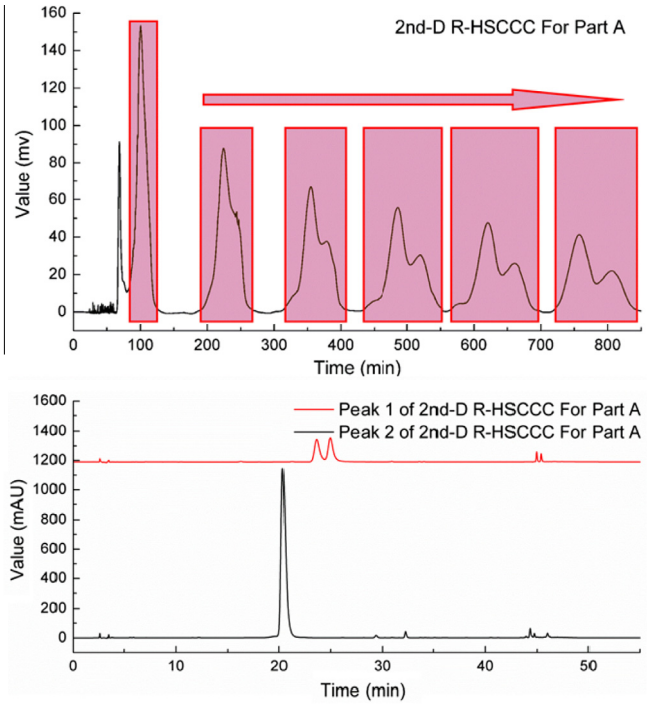


Fig. 4. Recycling chromatogram of the 2nd-D HSCCC for separation of part A in 1st-D HSCCC, and HPLC chromatograms of the two peaks in the off-line 2nd-D R-HSCCC for part A. HSCCC conditions is the same as it in the 1st-D HSCCC.

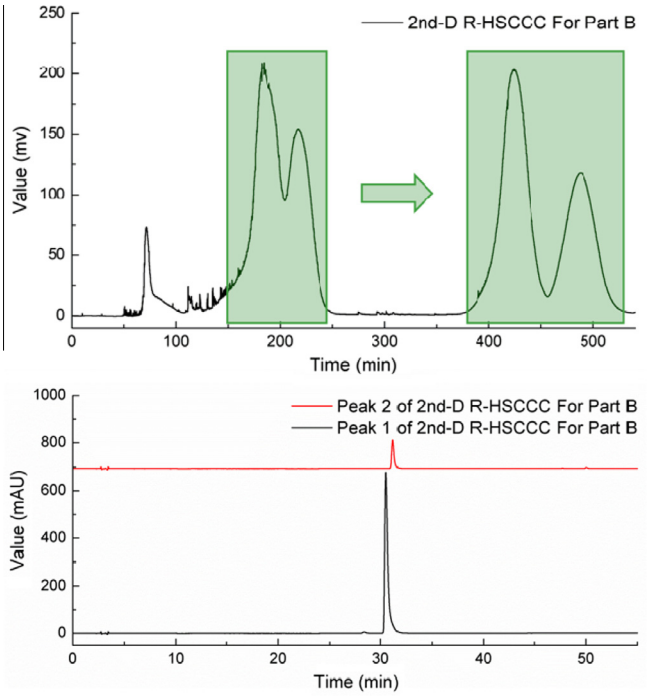
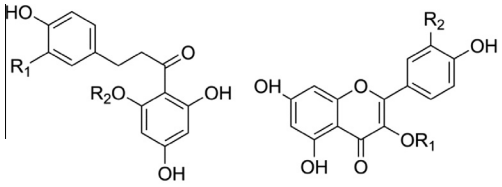


Fig. 5. Recycling chromatogram of the 2nd-D HSCCC for separation of part B in 1st-D HSCCC, and HPLC chromatograms of the two peaks in the off-line 2nd-D HSCCC for part B. HSCCC conditions is the same as it in the 1st-D HSCCC.



(**1**) R_1 = OH, R_2 = Glucose (**3**) R_1 = Glucose, R_2 = H

(**2**) R_1 = H, R_2 = H (**4**) R_1 = Arabinose , R_2 = OH

Fig. 6. Structures of isolated compounds **1**–**4**.

s), 6.58 (1H, d, *J* = 8.0 Hz), 6.47 (1H, d, *J* = 8.0 Hz), 6.02 (1H, s), 5.78 (1H, s), 4.89 (1H, t, *J* = 3.5 Hz), 3.70 (1H, d, *J* = 5.5 Hz), 3.50 (1H, dd, *J* = 6.0, 12.0 Hz), 3.28 (2H, dd, *J* = 4.5, 7.5 Hz), 3.18–3.21 (2H, m), 2.66–2.76 (2H, m).

Compound **2**. UV max (nm): 222, 287; ESI-MS *m*/*z*: 273.08 $[M-H]^-$, ^{1}H NMR (500 MHz, DMSO-d_6): 12.19 (2H, s), 10.33 (1H, s), 9.12 (1H, s), 6.97 (2H, d, *J* = 8.0 Hz), 6.53 (2H, d, *J* = 8.0 Hz), 6.05 (1H, s), 5.91 (1H, s), 3.16–3.21 (2H, m), 2.47–2.58 (2H, m).

Compound **3**. UV max (nm): 256, 348; ESI-MS *m*/*z*: 447.10 $[M-H]^-$, ^{1}H NMR (500 MHz, DMSO-d_6): 13.62 (1H, s), 9.13 (1H, s), 7.05 (2H, d, *J* = 8.5 Hz), 6.64 (2H, d, *J* = 8.5 Hz), 6.07 (1H, s), 5.86 (1H, s), 5.30 (1H, s), 5.17 (1H, s), 5.07 (1H, s), 4.92 (1H, d, *J* = 7.5 Hz), 4.61 (1H, s), 3.70 (1H, d, *J* = 11.5 Hz), 3.51 (1H, dd, *J* = 5.0, 11.5 Hz), 3.19 (1H, t, *J* = 7.5 Hz), 2.79 (2H, t, *J* = 7.5 Hz).

Compound **4**. UV max (nm): 256, 353; ESI-MS *m*/*z*: 433.08 $[M-H]^-$, ^{1}H NMR (500 MHz, DMSO-d_6): 13.48 (1H, s), 9.01 (1H, s), 7.52 (1H, s), 6.92 (2H, d, *J* = 8.0 Hz), 6.41 (1H, s), 6.22 (1H, s), 5.48 (1H, s), 4.35 (1H, m), 3.77–3.93 (3H, m), 3.51 (2H, m).

4. Conclusions

Restricted by relatively limited peak capacity of HSCCC and existence of compounds with similar structures, peak overlapping is a general and troublesome problem in HSCCC separation of natural products. To address this problem, a simple and easily-attainable off-line 2D HSCCC strategy combined with recycling elution mode in the 2nd-D HSCCC was established in this work. Four compounds 3-hydroxy-phlorizin, phloretin, avicularin and kaempferol 3-O-β-D-glucoside were successfully purified from the traditional Chinese tea – leaves of *M. hupehensis* (Pamp.) Rehder. In this experiment, R-HSCCC also has been proven to be a powerful tool for separating compounds with little distribution difference in two-phase solvent systems although pre-purification is usually prerequisite. It is unneglectable that, owing to its extended peak width and increased peak overlap between two neighbouring cycles, R-HSCCC would be unsuitable for simultaneously separating several compounds. Compared with the traditional purification technique such as LC and silica gel column chromatography, HSCCC itself possesses the unique feature of no irreversible adsorption. Using HSCCC to pre-purify the target compounds will preserve its specialty. It can be anticipated that the off-line 2D-HSCCC combined with R-HSCCC would serve as a general and efficient pathway in the separation of natural products.

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References

Berthod, A., Ruiz-Angel, M. J., & Carda-Broch, S. (2009). Countercurrent chromatography: People and applications. *Journal of Chromatography A, 1216*(19), 4206–4217.

Boccia, M., Kopf, S., & Baratti, C. (1999). Phlorizin, a competitive inhibitor of glucose transport, facilitates memory storage in mice. *Neurobiology of Learning and Memory, 71*(1), 104–112.

Bradford, B., & Allen, M. (2007). Phlorizin induces lipolysis and alters meal patterns in both early-and late-lactation dairy cows. *Journal of Dairy Science, 90*(4), 1810–1815.

Cragg, G. M., & Newman, D. J. (2013). Natural products: A continuing source of novel drug leads. *Biochimica et Biophysica Acta (BBA) General Subjects, 1830*(6), 3670–3695.

Dai, X., Huang, Q., Zhou, B., Gong, Z., Liu, Z., & Shi, S. (2013). Preparative isolation and purification of seven main antioxidants from *Eucommia ulmoides* Oliv. (Du-zhong) leaves using HSCCC guided by DPPH-HPLC experiment. *Food Chemistry, 139*(1–4), 563–570.

Han, Q. B., Song, J. Z., Qiao, C. F., Wong, L., & Xu, H. X. (2006). Preparative separation of gambogic acid and its C-2 epimer using recycling high-speed counter-current chromatography. *Journal of Chromatography A, 1127*(1), 298–301.

Ito, Y. (2005). Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *Journal of Chromatography A, 1065*(2), 145–168.

Ito, Y., & Bowman, R. L. (1970). Countercurrent chromatography: Liquid–liquid partition chromatography without solid support. *Science (New York, N.Y.), 167*(3916), 281–283.

Janssen, S., Martens, G., Sweep, C., Span, P., Verhofstad, A., & Hermus, A. (2003). Phlorizin treatment prevents the decrease in plasma insulin levels but not the progressive histopathological changes in the pancreatic islets during aging of Zucker diabetic fatty rats. *Journal of Endocrinological Investigation, 26*(6), 508–515.

Liu, Q., Shi, S., Liu, L., Yang, H., Su, W., & Chen, X. (2013). Separation and purification of bovine serum albumin binders from Fructus polygoni orientalis using off-line two-dimensional complexation high-speed counter-current chromatography target-guided by ligand fishing. *Journal of Chromatography A, 1304*, 183–193.

Liu, Q., Zhou, J., Yu, J., Xie, Y., Jiang, X., Yang, H., et al. (2014). Systematic and efficient separation of 11 compounds from Rhizoma Chuanxiong via counter-current chromatography – solid phase extraction – counter-current chromatography hyphenation. *Journal of Chromatography A*. <http://dx.doi.org/10.1016/j.chroma.2014.08.087>.

Liu, W., Luo, J., & Kong, L. (2011). Application of complexation high-speed counter-current chromatography in the separation of 5-hydroxyisoflavone isomers from *Belamcanda chinensis* (L.) DC. *Journal of Chromatography A, 1218*(14), 1842–1848.

Lu, Y., Hu, R., & Pan, Y. (2010). Integrated countercurrent extraction of natural products: A combination of liquid and solid supports. *Analytical Chemistry, 82*(7), 3081–3085.

Lu, Y., Sun, C., Liu, R., & Pan, Y. (2007). Effective two-dimensional counter-current chromatographic method for simultaneous isolation and purification of oridonin and ponicidin from the crude extract of *Rabdosia rubescens*. *Journal of Chromatography A, 1146*(1), 125–130.

Lu, Y., Sun, C., Wang, Y., & Pan, Y. (2007). Two-dimensional counter-current chromatography for the preparative separation of prenylflavonoids from *Artocarpus altilis*. *Journal of Chromatography A, 1151*(1–2), 31–36.

Ma, S., Liang, Q., Jiang, Z., Wang, Y., & Luo, G. (2012). A simple way to configure on-line two-dimensional liquid chromatography for complex sample analysis: Acquisition of four-dimensional data. *Talanta, 97*, 150–156.

Mishra, B. B., & Tiwari, V. K. (2011). Natural products: An evolving role in future drug discovery. *European Journal of Medicinal Chemistry, 46*(10), 4769–4807.

Ren, Q., Wu, C., Ren, Y., & Zhang, J. (2013). Characterization and identification of the chemical constituents from tartary buckwheat (*Fagopyrum tataricum* Gaertn) by high performance liquid chromatography/photodiode array detector/linear ion trap FTICR hybrid mass spectrometry. *Food Chemistry, 136*(3–4), 1377–1389.

Shi, S., Ma, Y., Zhang, Y., Liu, L., Liu, Q., Peng, M., et al. (2012). Systematic separation and purification of 18 antioxidants from *Pueraria lobata* flower using HSCCC target-guided by DPPH–HPLC experiment. *Separation and Purification Technology, 89*, 225–233.

Sirat, H. M., Rezali, M. F., & Ujang, Z. (2010). Isolation and identification of radical scavenging and tyrosinase inhibition of polyphenols from *Tibouchina semidecandra* L.. *Journal of Agricultural and Food Chemistry, 58*(19), 10404–10409.

Su, W., Liu, Q., Yang, Q., Yu, J., & Chen, X. (2013). Separation and purification of four compounds from *Desmodium styracifolium* using off-line two-dimensional high-speed counter-current chromatography. *Journal of Separation Science, 36*(20), 3338–3344.

Tong, S., Guan, Y.-X., Yan, J., Zheng, B., & Zhao, L. (2011). Enantiomeric separation of (R,S)-naproxen by recycling high speed counter-current chromatography with hydroxypropyl-beta-cyclodextrin as chiral selector. *Journal of Chromatography A, 1218*(32), 5434–5440.

Wang, S. Q., Zhu, X. F., Wang, X. N., Shen, T., Xiang, F., & Lou, H. X. (2013). Flavonoids from *Malus hupehensis* and their cardioprotective effects against doxorubicin-induced toxicity in H9c2 cells. *Phytochemistry, 87*, 119–125.

Wen, Y., Wang, J., Chen, X., Le, Z., Chen, Y., & Zheng, W. (2009). Application of silver ion in the separation of macrolide antibiotic components by high-speed counter-current chromatography. *Journal of Chromatography A, 1216*(22), 4668–4672.

Xü, K., Lü, H., Qü, B., Shan, H., & Song, J. (2010). High-speed counter-current chromatography preparative separation and purification of phloretin from apple tree bark. *Separation and Purification Technology, 72*(3), 406–409.