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Separation of five flavonoids from tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn) grains *via* off-line two dimensional high-speed counter-current chromatography

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ABSTRACT

An off-line two dimensional (2D) high-speed counter-current chromatography (HSCCC) strategy was successfully used for preparative separation of five flavonoids from tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn) grains with different solvent systems for the first time in this paper. *n*-Hexane–ethyl acetate–methanol–water 3:5:3:5 (v/v) was selected as the first dimension solvent system to purify quercetin (**4**) and kaempferol (**5**). The second dimension solvent system, ethyl acetate–*n*-butanol–water 7:3:10 (v/v), was used to isolate quercetin 3-O-rutinoside-3′-O-β-glucopyranoside (**1**), rutin (**2**) and kaempferol 3-rutinoside (**3**). The purities of these compounds were all above 96.0% and their structures were identified through UV, MS and ^{1}H NMR. The results indicated that the off-line 2D HSCCC is an efficient technique to isolate flavonoids compounds from grains.

1. Introduction

As important dicotyledonous plants of the Polygonaceae family, common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertn) were widely recognised as potential "functional foods" (Kim et al., 2008). The tartary buckwheat received much less attention than common buckwheat because of its bitter taste. Nevertheless, due to its higher levels of bioactive components and stronger pharmacological effects than common buckwheat (Eguchi, Anase, & Osuga, 2009; Liu, Chen, Yang, & Chiang, 2007; Morishita, Yamaguchi, & Degi, 2007), tartary buckwheat had been considered to be more valuable for the medicinal use. The tartary buckwheat has been reported to show excellent benefits for human health such as anti-inflammatory (Ishii et al., 2008), antibacterial (Wang, Yang, Qin, Shan, & Ren, 2013) and antioxidant activity (Kim et al., 2008; Wang, Liu, Gao, Parry, & Wei, 2009). And significant evidence shows that the consumption of tartary buckwheat can reduce the blood glucose level (Yao et al., 2008), regulate the lipid profile (Wang et al., 2009), and lower the total cholesterol level (Qin, Wang, Shan, Hou, & Ren, 2010). It is also used as an important Chinese medicine for antitumour (Guo, Zhu, Zhang, & Yao, 2007, 2010), antidiabetic (Qin et al., 2010) and antifatigue purposes (Jin & Wei, 2011). The above investigation suggests that it would be a very interesting and plentiful source of dietary supplements. And the main bioactive phytochemicals found in the tartary buckwheat are flavonoids, for instance rutin, quercetin, isoquercetin, kaempferol, and so on (Peng et al., 2013; Zhao et al., 2011; Li & Li, 2011). Thus, the preparative separation and purification of flavonoids from this grain for the further chemical research is warranted for new pharmaceutical preparations or functional products and bioassays.

However, the traditional separation methods for complex plant extracts usually require multiple chromatographic steps on silica gel, polyamide, Sephadex LH-20 column and so on, which often lead to low sample recovery and high risk of sample denaturation and irreversible adsorption. To avoid the problems mentioned above, High-speed counter-current chromatography (HSCCC), being a continuous liquid–liquid partition chromatographic method, has been developed and evolved to be an effective technique for the isolation and purification of natural products (Chen et al., 2012; Ito, 2005; Kuang et al., 2013; Yoon, Chin, Yang, & Kim, 2011). However, owing to the limited peak capacity of unidimensional HSCCC, a new strategy – two dimensional (2D) HSCCC has been advanced to be an efficient methods for separating the intractable samples due to its enhanced peak capacity (Lu, Sun, Liu, & Pan, 2007; Lu, Sun, Wang, & Pan, 2007; Yao, Li, & Kong, 2006). Meanwhile, considering the on-line 2D HSCCC usually needs two HSCCC and other special apparatus for separation, a simple

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off-line strategy (Liu et al., 2013) can be realised much more easily and conveniently. In this work, an off-line 2D HSCCC method was applied to separating flavonoids compounds with widely different polarities from crude ethanol extract of tartary buckwheat grains. The critical parameters, including the two-phase solvent systems, the flow rate of mobile phase and revolution speed, were optimised. As a consequence, five flavonoids compounds in milligrams quantities were isolated and identified to be quercetin 3-O-rutinoside-3′-O-β-glucopyranoside (**1**), rutin (**2**), kaempferol 3-rutinoside (**3**), quercetin (**4**) and kaempferol (**5**) through UV, MS/MS, and ^{1}H MNR.

2. Materials and methods

2.1. Apparatus

The preparative HSCCC was performed using a model TBE-300B HSCCC (Shanghai Tauto Biotechnique Co., Ltd., Shanghai, China), which is composed 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 revolution speed was regulable with a speed controller, ranging from 0 to 1000 rpm. The HSCCC system was equipped with a TBD-2000 UV detector, a TBP-1002 pump, a HX-1050 constant temperature regulator (Beijing Boyikang Lab Implement Co., Ltd., Beijing, China) and a WH V4.0 workstation (Shanghai Wuhao Information Technology Co., Ltd., Shanghai, China). In the separation process, the effluent was collected automatically by BSZ-100 automatic fraction collector (Shanghai Qingpu-Huxi Instruments Factory, Shanghai, China).

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 HPLC 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 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-400 (Bruker Corporation, Germany) NMR spectrometer. The reference compound TMS was used as the internal standard for determination of chemical shifts.

2.2. Reagents and materials

The tartary buckwheat grains were purchased from Liang Shan region in the Sichuan province, China. Ethyl acetate, *n*-hexane, *n*-butanol, methanol and 95% ethanol used for fractions preparation and HSCCC separation were of analytical grade and purchased from Chemical Reagent Factory of Hunan Normal University (Hunan, China). Acetonitrile and acetic acid used for HPLC were of chromatographic grade (Merk, Darmatadt, Germany). Ultra-Pure water (18.2 MΩ resistivity) used in the present work was produced by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Preparation of crude extract

The grains of tartary buckwheat were dried, hulled manually and the seed was ground with a laboratory mill. The pulverised material (100 g) was extracted with 500 mL of 95% ethanol under reflux for 3 h. The extraction procedure was repeated two times. Then the extract solutions were combined, filtered and evaporated to dryness by rotary evaporator at 40 °C under reduced pressure to form the brownish syrup (2.53 g). The concentrated crude extract was stored in a refrigerator and subjected for the subsequent HPLC analysis and HSCCC isolation.

2.4. HPLC–MS conditions

The mobile phase for the chromatographic separation in this study consisted of acetonitrile as mobile phase A and 0.4% (v/v) acetic acid in water as mobile phase B. The gradient programs for the tartary buckwheat crude extract was used according to the following profile: 0–10 min, 5% A; 10–15 min, 5–20% A; 15–30 min, 20% A; 30–40 min, 20–40% A; 40–50 min, 40% A. This was followed by a 8 min re-equilibration. The flow rate was 1.0 mL/min and the injection volume was 10 μL. The 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 Ls. Nitrogen was used as the desolvation and cone gas. Mass detection was performed in full scan mode for m/z in the range 100–1000. All data were acquired and processed using MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.5. Selection of the solvent system

The composition of the two-phase solvent system was evaluated by HPLC according to the partition coefficients (K) of the target compounds. The K-values were measured as follows: a small amount of concentrated crude extract of tartary buckwheat was dropped into a test tube. Then a series of solvent systems with different ratios were prepared and thoroughly mixed. The tube was shaken vigorously for several minutes to thoroughly equilibrate the sample between the two phases. After the two-phase solvents were separated completely, 10 μL of the upper and lower phases were analysed by HPLC at 254 nm, respectively. The K-values were defined as A_U/A_L, where A_U and A_L are the peak areas of objective compounds in the upper and lower phase respectively.

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

According to the volume ratios, the selected two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water (HEMW) and ethyl acetate–*n*-butanol–water (EBW) were prepared for the first and second dimension of the off-line 2D HSCCC separation. The preparation of each two-phase solvent system was performed in a separation funnel and thoroughly equilibrated by shaking at room temperature. The upper phase and lower phase were separated and degassed by sonication for 20 min shortly before use.

The sample solution for the first dimensional HSCCC separation was prepared by dissolving 150 mg of the crude sample in 8 mL of mobile phases. The eluents of 90–125 min in the first dimensional HSCCC were collected and evaporated at 40 °C under vacuum. Then the sample solution for the off-line second dimensional HSCCC was prepared by dissolving the solute in 8 mL of mobile phase.

2.7. HSCCC separation procedure

The HSCCC separation was performed as follows: the separation column of HSCCC was initially filled with the stationary phase (the upper phase) by constant flow pump at 20 mL/min with no rotation. The separation temperature was controlled at 25 °C. Until the separation column was entirely filled with stationary phase, the mobile phase (the lower phase) was pumped into the column at the flow rate of 2.0 mL/min, while the apparatus was rotated at a revolution speed of 850 rpm. In order to calculate the retention of the stationary phase (S_F) in the column, the resulting effluent was collected in a cylinder. After a clear mobile phase eluted at the tail outlet and the hydrodynamic equilibrium was established throughout the coil, the sample solution was injected into the separation column through the injection valve. The effluent from the outlet of column was continuously monitored by a UV detector at 254 nm continuously. And the peak fractions were automatically collected by the automatic fraction collector.

2.8. HPLC analysis and identification of HSCCC peak fractions

Every HSCCC peak fraction was analysed on Dionex Ultimate 3000 and Waters Spherisorb ODS2 under the same conditions as described in Section 2.4. ^{1}H NMR spectroscopic data were obtained from a Bruker-400 (Bruker Corporation, Germany) NMR spectrometer and every concentrated compound was dissolved in deuterated dimethyl sulfoxide (DMSO-d_6).

3. Results and discussion

3.1. Optimisation of HPLC conditions

Previous chemical investigations have revealed (Li, Zhou, Gao, Bian, & Shan, 2009; Ren, Wu, & Zhang, 2013; Ren, Wu, Ren, & Zhang, 2013) that buckwheat species possess abundant flavonoids and minor flavonol glycosides. Since the structural difference of flavonol glycosides is just contributed by the position and number of glucosyl group and the difference of parent structure, many position isomers may be present. Furthermore, the properties of position isomer such as UV absorbance and HPLC pattern are identical or similar, which can lead a significant error during the quantification analysis or purity test. So, the optimal HPLC condition should be evaluated to exclude erroneous results.

Because acid is known to provide better separation for compounds with hydroxyl groups by reducing the tailing of the peaks (Dai et al., 2013), acetic acid is added into the mobile phase in this study. The conditions for the HPLC analysis of the crude extract and the HSCCC fractions including the gradient program of mobile phase, detection wavelength and column temperature were all optimised. The results indicated that the optimum mobile phase composed of acetonitrile (A) and 0.4% (v/v) acetic acid (B) in an appropriate gradient mode (0–10 min, 5% A; 10–15 min, 5–20% A; 15–30 min, 20% A; 30–40 min, 20–40% A; 40–50 min, 40% A) was most suitable for our analysis. The flow rate was 1.0 mL/min and the injection volume was 10 μL. The temperature was controlled at 25 °C. Spectra were recorded from 190 to 400 nm while the chromatogram was acquired at 254 nm. Under the above conditions, the target compounds reached base-line separation in satisfactory analysis time. The HPLC chromatogram of the crude extract of tartary buckwheat is shown in Fig. 1.

3.2. Selection of two-phase solvent system and other conditions of HSCCC

The main difficulty of HSCCC separation arises from the selection of a suitable two-phase solvent system. An appropriate two-phase solvent system can be achieved through the test of partition coefficient, whose value should fall within the range of 0.5–2.0 to get an efficient separation. Moreover, the settling time of selected solvent system should be shorter than 30 s, a satisfactory retention of the stationary phase (S_F > 40%) and a reasonable volume ratios (close to 1:1) of the two phases are also required. According to the rules of selection of the appropriate two-phase solvent system introduced by Ito (Ito, 2005), the regular solvent system HEMW, which has been commonly used in the separation and purification of solutes with wide range of polar, was first employed. The *K*-values of target compounds **1**–**5** in the solvent systems HEMW with different volume ratios of 4:5:4:5, 3:5:3:5 and 2:5:2:5 (v/v) were tested. The experimental results were shown in Table 1, which demonstrated that HEMW at a volume ratio of 3:5:3:5 (v/v) was suitable for the separation of compounds **4** and **5** because appropriate *K*-values were obtained. Further HSCCC separation was performed and the results indicated that the solvent system HEMW 3:5:3:5 (v/v) could provide a satisfactory retention of stationary phase (57.7%) for preparative HSCCC. Thus, the target compounds **4** and **5** could be completely separated within acceptable separation time. However, in the solvent system HEMW 3:5:3:5 (v/v), target compounds **1**, **2** and **3** may be eluted together because of their little *K*-values differences. Therefore, effective separation for all the target compounds could not be achieved only by this solvent system. So, other solvent systems composed of ethyl acetate, *n*-butanol and water at different volume ratios were also conducted *K*-values tests for compounds **1**, **2** and **3**. Results indicated that the *K*-values of these compounds in the EBW 4:1:5 (v/v) were still slightly small. While a more hydrophilic solvent system EBW 7:3:10 (v/v) can provide more suitable *K*-values for compounds **1**, **2** and **3**, which mean it would be a satisfactory experimental solvent system for these compounds. Consequently, the two solvent systems HEMW 3:5:3:5 (v/v) and EBW 7:3:10 (v/v) were ultimately selected for the off-line 2D HSCCC separation of five target compounds in this study.

Furthermore, the factors influencing S_F including the flow rate of the mobile phase and revolution speed of the separation column were also investigated in this study. According to the basic formula between S_F, *K*-value and R_t, the higher the retention level, the better the peak resolution. Obviously, lower flow rate can provide higher retention of the stationary phase, but it means longer separation time and extended peak width, while higher flow rate is unfavorable to the S_F. High rotary speed can increase the S_F, and concurrently result in better peak resolution, but it may produce excessive sample band broadening. Taking the S_F and experimental duration into account, a flow rate of 2.0 mL/min and a revolution speed of 850 rpm were ultimately selected for the off-line 2D HSCCC separation.

3.3. HSCCC separation

The crude extract (150 mg) was first subjected for HSCCC separation with HEMW at a volume ratio of 3:5:3:5 (v/v) as the solvent system. Conventional normal-phase elution mode was employed. A satisfactory S_F of 57.7% was obtained in the separation. In consequence, as the HSCCC chromatogram shown in Fig. 2A, compounds **4** (20.5 mg, collected between 140 and 170 min) and **5** (2.7 mg, collected between 210 and 245 min) were separated successfully. However, due to the limited peak capacity of unidimensional HSCCC and the close *K*-values for compounds **1** (0.17), **2** (0.25) and **3** (0.31), these compounds were co-eluted, resulting in a poor peak resolution. Because peak capacity can be increased by one order of magnitude in 2D HSCCC, allowing co-eluted compounds to be well-separated. Furthermore, the *K*-values of compounds **1** (0.57), **2** (1.74) and **3** (2.98) also increased as expected in the more

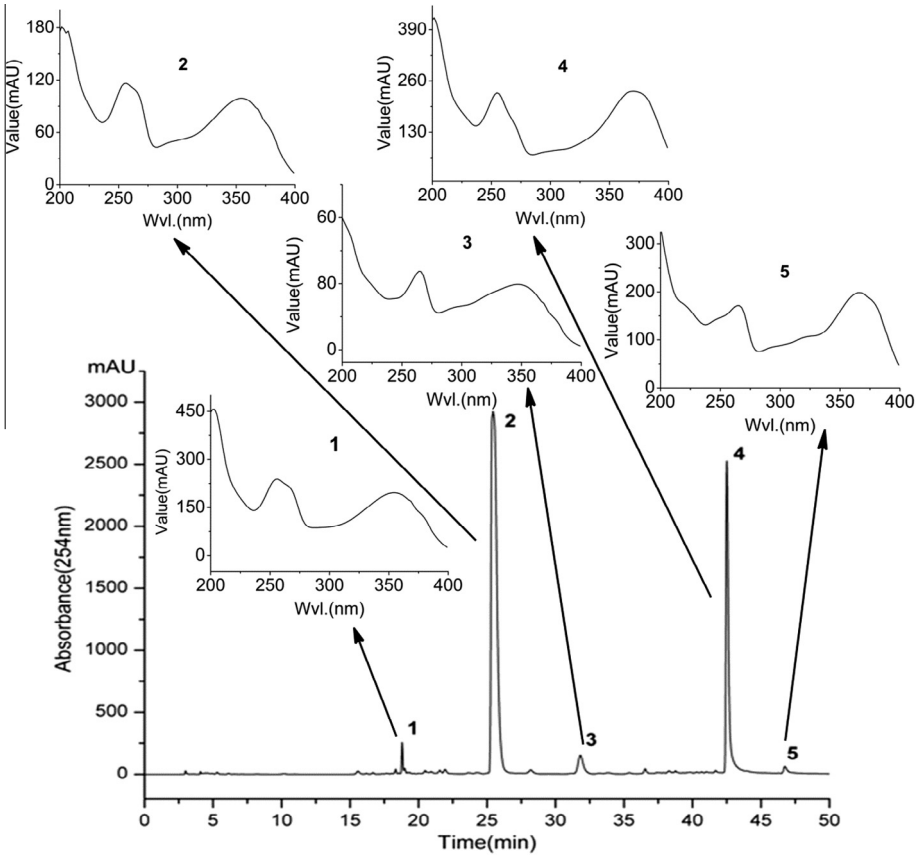


Fig. 1. HPLC chromatogram of the ethanol extract of tartary buckwheat grains and the UV spectrums of compounds **1**–**5**.

Table 1
The *K*-values of target components measured in different solvent systems.

Solvent system	Ratio (v/v)	*K*-values[a,b]				
		Compound **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**
HEMW	4:5:4:5	-	-	-	1.18	1.62
	3:5:3:5	0.17	0.25	0.31	1.36	2.31
	2:5:2:5	0.22	0.30	0.39	1.59	3.15
EBW	4:1:5	0.43	1.17	2.03	--	–
	7:3:10	0.57	1.74	2.98	--	–

[a] Expressed as: A_U/A_L, where A_U and A_L are the peak areas of target compound in the upper and lower phase respectively.
[b] '--' and '-' strand for that the *K*-values were too large and too small, respectively.

hydrophilic solvent system EBW 7:3:10 (v/v), and better separation factors were obtained concurrently (as shown in Table 1). So, using eluate collected between 90 and 125 min (the shaded part of the peak in Fig. 2A) in the first dimensional HSCCC as the sample, an off-line second dimensional HSCCC using EBW 7:3:10 (v/v) as the solvent system was implemented. In the separation process, a satisfactory retention of the stationary phase (50.0%) was obtained. And four peaks were presented clearly in the chromatogram (as shown in Fig. 2B) of the second dimension. Those separated peaks were collected automatically, and then subjected to HPLC. Results indicated that, excepting the first solvent peak, compounds **1** (3.2 mg, collected between 90 and 100 min), **2** (51.0 mg, collected between 160 and 200 min) and **3** (4.1 mg, collected between 235 and 285 min) were obtained successfully in the later three peaks respectively.

Each HSCCC peak fraction was subjected to HPLC for purity assessment, and the chromatograms were shown in Fig. 3A–E. The results showed that the purities of these compounds were 96.1%, 98.9%, 97.7%, 97.1% and 96.7% (determined by HPLC peak area percentage), respectively, which indicated that the two-phase solvent systems composed of HEMW 3:5:3:5 (v/v) and EBW 7:3:10 (v/v) were well suitable for the off-line 2D HSCCC isolation and purification of those five compounds from tartary buckwheat.

3.4. Structural identification

Compound **1**: UV max (nm): 256, 355; ESI-MS *m*/*z*: 771 $[M-H]^-$, ^1H NMR (400 MHz, DMSO-d_6): 4.83 (1H, d, J = 6.8 Hz), 5.26 (1H, d, J = 7.0 Hz), 6.21 (1H, d, J = 2.0 Hz), 6.45 (1H, d, J = 2.0 Hz), 6.90 (1H, d, J = 8.4 Hz), 7.82 (1H, d, J = 2.0 Hz), 7.87 (2H, dd, J = 8.4, 2.0 Hz).

Compound **2**: UV max (nm): 256, 355; ESI-MS *m*/*z*: 609 $[M-H]^-$, ^1H NMR (400 MHz, DMSO-d_6): 1.01 (3H, d, J = 6.4 Hz), 5.02 (1H, d, J = 1.2 Hz), 5.32 (1H, d, J = 7.6 Hz), 6.17 (1H, d, J = 2.0 Hz), 6.36 (1H, d, J = 2.0 Hz), 6.82 (1H, d, J = 8.2 Hz), 7.54 (2H, dd, J = 8.2, 2.0 Hz), 7.55 (1H, d, J = 2.0 Hz).

Compound **3**: UV max (nm): 264, 347; ESI-MS *m*/*z*: 593 $[M-H]^-$, ^1H NMR (400 MHz, DMSO-d_6): 1.10 (3H, d, J = 6.1 Hz),

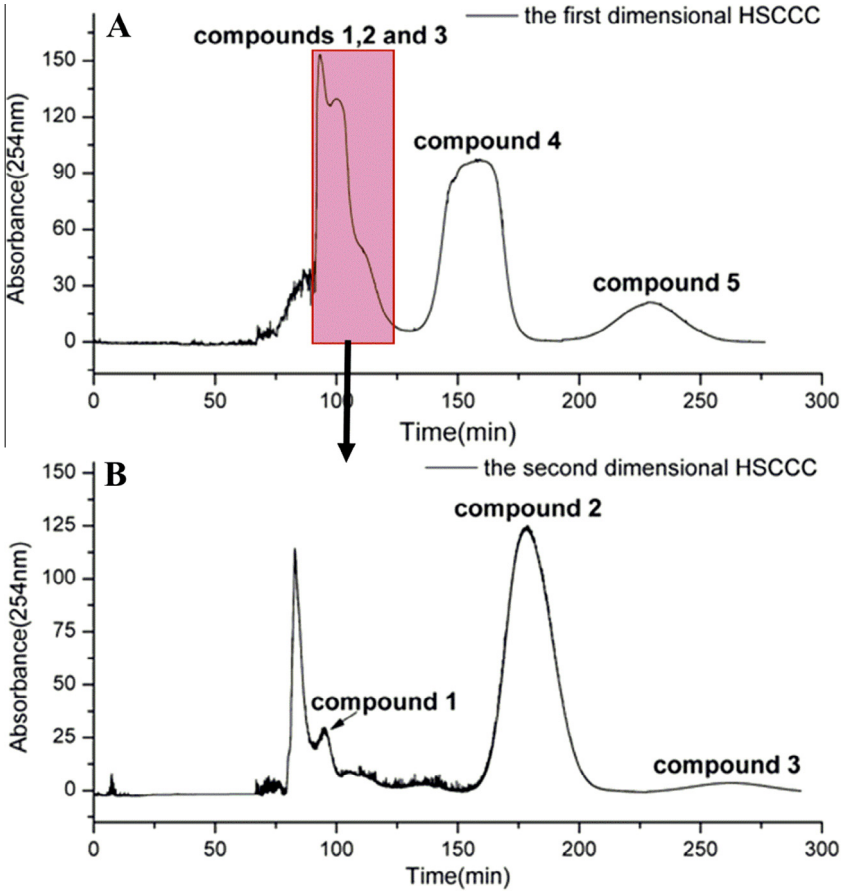


Fig. 2. Off-line 2D HSCCC chromatograms of the ethanol extract of tartary buckwheat grains. (A) Chromatogram of the first dimensional HSCCC separation: two-phase solvent system, HEMW (3:5:3:5, v/v); (B) chromatogram of the second dimensional HSCCC separation: two-phase solvent system, EBW (7:3:10, v/v). Other conditions of HSCCC: stationary phase, upper organic phase; mobile phase, lower aqueous phase; flow rate, 2.0 mL/min; revolution speed, 850 rpm; injection volume, 8 mL; detection wavelength, 254 nm; separation temperature, 25 °C; S_F, 57.7% (A) and 50.0% (B).

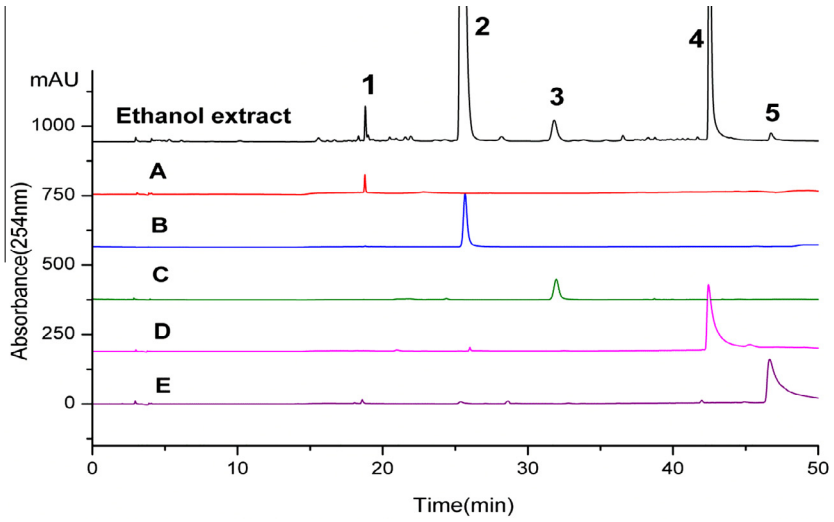


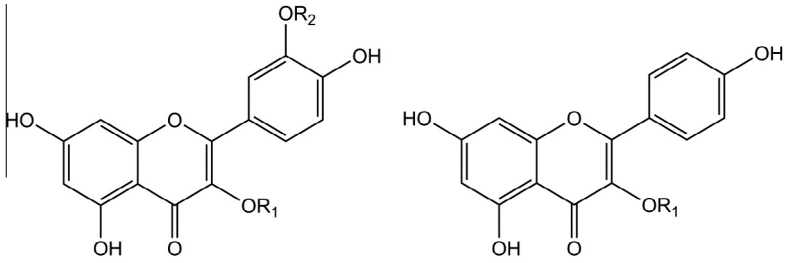
Fig. 3. HPLC chromatograms of HSCCC peak fractions. (A–C), peaks of I–III in Fig. 2B; (D and E), peaks of II and III in Fig. 2A.

4.50 (1H, d, J = 1.5 Hz), 5.12 (1H, d, J = 7.4 Hz), 6.20 (1H, d, J = 2.0 Hz), 6.35 (1H, d, J = 2.0 Hz), 6.80 (2H, dd, J = 8.8,2.0 Hz), 8.05 (2H, dd, J = 8.8, 2.0 Hz).

Compound **4**: UV max (nm): 254, 370; ESI-MS m/z: 301 $[M-H]^-$, ^{1}H NMR (400 MHz, DMSO-d_6): 6.18 (1H, d, J = 2.0 Hz), 6.39 (1H, d, J = 2.0 Hz), 6.88 (1H, d, J = 8.5 Hz), 7.52 (2H, dd, J = 8.5, 2.2 Hz), 7.66 (1H, d, J = 2.2 Hz), 12.40 (1H, s).

Compound **5**: UV max (nm): 264, 367; ESI-MS m/z: 285 $[M-H]^-$, ^{1}H NMR (400 MHz, DMSO-d_6): 6.09 (1H, d, J = 2.1 Hz), 6.18 (1H, d, J = 2.1 Hz), 6.83 (2H, dd, J = 8.8, 2.0 Hz), 8.04 (2H, dd, J = 8.8, 2.0 Hz).

Chemical structures of compounds **1**–**5** (Shown in Fig. 4) were identified as quercetin 3-O-rutinoside-3′-O-β-glucopyranoside (**1**), rutin (**2**), kaempferol 3-rutinoside (**3**), quercetin (**4**) and



(**1**) R_1=Rutinose, R_2=Glucose
(**2**) R_1=Rutinose, R_2=H
(**4**) R_1=H, R_2=H

(**3**) R_1=Rutinose
(**5**) R_1=H

Fig. 4. Chemical structures of the identified flavonoids from tartary buckwheat grains: quercetin 3-O-rutinoside-3′-O-β-glucopyranoside (**1**), rutin (**2**), kaempferol 3-rutinoside (**3**), quercetin (**4**), kaempferol (**5**).

kaempferol (**5**) by comparing their UV, MS and ^{1}H NMR with the references (Dai et al., 2013; Li & Li, 2011; Singh et al., 2008).

4. Conclusions

In the experiment, a simple and rapid off-line 2D HSCCC method with significantly enhanced peak capacity was developed for the separation of compounds from the crude extract of tartary buckwheat grains. Using HEMW 3:5:3:5 (v/v) and EBW 7:3:10 (v/v) as the two-phase solvent systems in the first and second dimension respectively, five flavonoids, including quercetin 3-O-rutinoside-3′-O-β-glucopyranoside (**1**), rutin (**2**), kaempferol 3-rutinoside (**3**), quercetin (**4**) and kaempferol (**5**), were successfully isolated with high purities (over 96%). Considering the relative lower peak capacity of unidimensional HSCCC, 2D HSCCC can improve both column capacity and peak resolution, and has promising application prospect in preparative separation of natural products. Compared with on-line 2D HSCCC, relatively simple off-line 2D HSCCC can be realised easily and conveniently. The overall results indicated that the off-line 2D HSCCC method could be a useful pattern for isolation of compounds with widely different polarities from crude plant extracts. And we also believe that the present investigation could promote the future research for tartary buckwheat gains on new pharmaceutical preparations or functional products and bioassays.

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