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Application of complexation high-speed counter-current chromatography in the separation of 5-hydroxyisoflavone isomers from *Belamcanda chinensis* (L.) DC.

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ABSTRACT

A novel separation technique of complexation high-speed counter-current chromatography (HSCCC) using copper ion as a complexation agent was first developed to isolate 5-hydroxyisoflavone isomers from *Belamcanda chinensis* (L.) DC. According to the partition coefficient and separation factor, the two-phase solvent system composed of light petroleum–ethyl acetate–methanol–water (3:5:3:5, v/v) and copper nitrate (0.10 mol/L in the lower phase) was selected. 9.2 mg isoirigenin (**1**), 46.4 mg irigenin (**2**) and 1.2 mg 5,7,4′-trihydroxy-6,3′,5′-trimethoxyisoflavone (**3**) were simultaneously purified from 100 mg crude extract by HSCCC with the purity of 95.06%, 96.98% and 93.69%, respectively. As evidenced by the results of UV–Vis spectroscopy, the stoichiometries of the copper ion with the three 5-hydroxyisoflavones were all 1:1 and their chelating power was **3** > **2** > **1**. Those explained the complexation HSCCC behavior. It is the first report that includes the practical application of complexation HSCCC and explanation of its chromatographic behavior.

1. Introduction

Rhizome of *Belamcanda chinensis* (L.) DC. (Iridacae) is a famous traditional Chinese medicine and widely distributed in eastern Asia including China, Korea, India and Japan [1]. It was usually used for the treatment of inflammation, asthma as well as throat disorders, e.g. cough, tonsillitis and pharyngitis [2]. A variety of secondary metabolites such as isoflavonoids [3–6], stilbenes [7], xanthones [8], and triterpeniods [9–11] have been found in the medicinal herb. Among them, the isoflavonoids were considered as the major active compounds [12–15], which had a very similar isoflavone skeleton and considerable diversity in the number and substitutional position of hydroxyl and methoxyl groups [1]. There are some reports stating that isoflavone aglycones are superior to isoflavone glucosides in various bioactivities [16–18]. Therefore, a rapidly growing number of studies focused on the activities of isoflavone aglycones in *B. chinensis* [7,13–15]. For example, irigenin, one of the major bioactive constituents, has been found to be anti-inflammatory, antimutagenic and antioxidant activities as demonstrated by the literatures [14,15]. In order to better understand the biochemical properties of isoflavone aglycones and ascertain their pharmacological applications, there is an urgent need to develop efficient separation methods for their isolation and purification. However, in our extensive research on the isoflavone aglycone, three isomers were difficult to be separated by other traditional separation methods in one step.

Complexation chromatography is a high selective separation technology, which is based on the different chelating abilities between receptor and ligand [19]. In its application of natural compounds separation, silver ion chromatography has been largely used to resolve unsaturated compounds containing double bonds [20,21]. Nevertheless, only limited papers focused on isolation of secondary metabolites by complexation chromatography except silver ion chromatography [22,23]. As we know, the hydroxylflavonoids have the ability to chelate with a variety of metal ions such as Cu(II), Fe(III) and Al(III). The main chelating sites occurring in flavonoids are, respectively, the 3-hydroxy-4-keto, the 5-hydroxy-4-keto and ortho-dihydroxyl groups [24]. This property has been extensively used in the determination and purification of metal traces in solution [25–27]. However, utilizing this coordination ability in the separation of flavoniods has not been paid enough attention.

The complexation chromatography usually involves chemically bonding into stationary matrix or adding additives into mobile phase. Since manufacturing such a chromatographic column requires a series of complicated processes and the additive ions are most deleterious to chromatographic system, neither of them is suitable for commercial process. Different from liquid–solid chromatography, HSCCC is a support free liquid–liquid technique, in which the stationary and mobile phases are constituted by two immiscible liquids [28]. Therefore, it is an excellent alternative and can be best applied in complexation chromatography by adding

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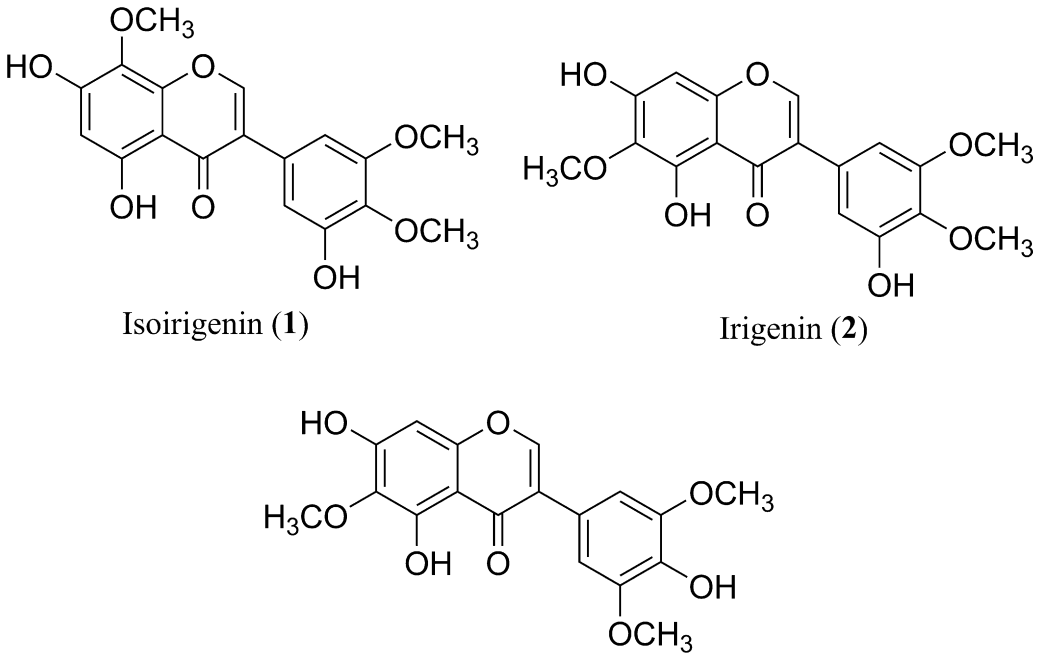

Fig. 1. Chemical structures of isoirigenin (**1**), irigenin (**2**) and 5,7,4′-trihydroxy-6,3′,5′-trimethoxyisoflavone (**3**).

adequate metal ions to the stationary liquid phase. The convenience and applicability have been demonstrated by the applications of ionic liquids and chiral selectors in HSCCC [29–31].

In this paper the extremely original separation method of complexation HSCCC was successfully applied to simultaneous separation of isoirigenin (**1**), irigenin (**2**) and 5,7,4′-trihydroxy-6,3′,5′-trimethoxyisoflavone (**3**) from the extract of *B. chinensis*. Their structures are shown in Fig. 1. The stoichiometry and stability of copper–flavone complexes were also investigated using UV–Vis spectroscopy to explain the separation results. To our best knowledge, it is the first report that includes the practical application of complexation HSCCC and explanation of its chromatographic behavior.

2. Materials and methods

2.1. Reagents and materials

All solvents used for HSCCC and UV–Vis spectroscopy were of analytical grade (Nanjing Chemical Reagent Co., Ltd., Jiangsu, China). RP-C_{18} (40–63 μm, Fuji, Japan) was used for column chromatography. The copper nitrate with the purity of above 99% was purchased from Zhenxin Chemical Factory (Shanghai, China). Methanol used for HPLC was of chromatographic grade (Hanbon Sci. & Tech.) and water used was distilled water. They were filtered and degassed prior to use.

The rhizomes of *B. chinensis* were purchased from traditional Chinese medicine market in Bozhou (Anhui, China). The samples were crushed into pieces before extracted.

2.2. Preparation of crude sample

The dried rhizomes of *B. chinensis* (4.0 kg) were chopped and extracted three times (2 h for each time) with 3 L 95% ethanol. Then the extract was filtered and evaporated to dryness by rotary evaporation at 55 °C under reduced pressure. The concentrated residue was diluted with H_2O and extracted successively with petroleum ether, CH_2Cl_2 and EtOAc. The CH_2Cl_2 extract (11.3 g) was subjected to an ODS chromatography column (35 cm × 3.4 cm) and eluted with 40% (1600 mL) and 50% (4000 mL) methanol, respectively. The eluate of 50% methanol was collected and analyzed by HPLC. The same one was combined to afford the crude sample (1.5 g) for HSCCC.

2.3. HSCCC

2.3.1. Apparatus

HSCCC was performed with a TBE-300 HSCCC (Tauto Biotechnique, Shanghai, China) equipped with three multilayer coils connected in series (diameter of the tube = 1.5 mm, total volume = 300 mL) and a 20 mL sample loop. The β value of the multilayer coil ranged from 0.5 at internal terminal to 0.8 at the external terminal ($\beta = r/R$, $R = 5$ cm, where r is the rotation radius or the distance from the coil to the holder shaft, and R, the revolution radius or the distance 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 between 0 and 1000 rpm. The system was also equipped with an S-1007 constant flow pump (Shenyitong Tech. & Exploitation, Beijing, China), a model 8823B-UV monitor (Bingdayingchuang Sci. & Tech., Beijing, China). N2000 chromatography workstation (Zhejiang University, Hangzhou, China) was employed to record the chromatogram.

2.3.2. Selection of two-phase solvent system

The two-phase solvent system was selected according to the partition coefficient (K) value, which was determined by HPLC as follows. A series of solvent systems with different ratios were prepared and equilibrated. Then the two-phase solvents were separated shortly before use. Different concentrations of Cu^{2+} (0.050 mol/L, 0.075 mol/L and 0.100 mol/L) and suitable amount of crude sample were put into a test tube, to which 3 mL of each phase was added and shaken violently. After the two-phases separated

completely, 1 mL of each layer was taken out and analyzed by HPLC. The *K*-values of target components were calculated according to the equation $K = A_L/A_U$, where A_L is the peak area of target compounds in the lower phase, and A_U, the upper phase.

2.3.3. Preparation of HSCCC solvent systems and sample solutions

Different concentrations of Cu^{2+} (0 mol/L, 0.050 mol/L, 0.075 mol/L and 0.100 mol/L) on HSCCC separation were investigated using the selected two-phase solvent system. At first the solvent system was added into a separation funnel according to the volume ratios and fully equilibrated by shaking repeatedly. Before using, the two-phases were separated and a certain amounts of copper nitrate were added. Then the upper and lower phases were degassed by supersonic for 30 min. The sample solution was prepared by dissolving 100 mg of crude extracts in 10 mL upper phase for HSCCC separation.

2.3.4. HSCCC separation procedure

The multilayer coiled column was first entirely filled with the lower phase as a stationary phase. Then, the apparatus rotated at 850 rpm and the upper phase was pumped into the column at a flow-rate of 1.0 mL/min with a tail-to-head elution model at the same time. After the hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution was injected through the sample port. The effluent was continuously monitored with a UV detector at 254 nm and the fractions were collected manually according to the chromatographic peak profiles displayed on the recorder. Each collection was evaporated under reduced pressure and the residue was dissolved in methanol for further purity analysis by HPLC.

2.3.5. Recovery of copper salt

After the separation, the solvents in the column were pumped out by pressurized nitrogen gas and collected. The lower phase was concentrated under vacuum at 50 °C until the volume is 50 mL. The residue was extracted with equal volume of CH_2Cl_2 for three times. At last, the upper aqueous phase was concentrated under vacuum at 80 °C to dryness.

2.3.6. Purification of peaks I, II and III

In order to remove the minor copper salt in peaks I, II and III, the collected fractions were evaporated under reduced pressure, respectively. Each residue was dissolved in 20 mL CH_2Cl_2 and extracted three times by equal volume of acidic water, which was prepared by dissolving 0.5 mL concentrated hydrochloric acid in 20 mL distilled water. Then the organic layers were evaporated to give the target compounds, respectively.

2.4. HPLC analysis and identification of isolated peaks

The crude sample and each peak fraction from HSCCC were analyzed by HPLC. The HPLC equipment was Shimadzu LC-2010 C HT system and Shimadzu HPLC workstation (Shimadzu, Japan). The column used was an Ultimate TM XB-C_{18} column (250 mm × 4.6 mm, 5 μm, Welch Materials) with a pre-column at temperature of 30 °C. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) (A)–methanol (B) in gradient mode as follows: 0–15 min, 40–50% B; 15–35 min, 50–57% B; 35–45 min, 57–65%. The effluent was monitored at 254 nm and the flow rate was kept at 1.0 mL/min constantly.

The structural identification was carried out by ESI-MS, ^1H NMR and ^{13}C NMR. ESI-MS used was Agilent 1100 series LC-MS Trap SL (Agilent, USA) and NMR was Bruker AM-500 (Bruker, Switzerland).

2.5. Investigation of stoichiometry and stability for copper–flavone complexes

UV–Vis spectra were performed on a Shimadzu UV-2450 spectrophotometer using quartz cell with 1 cm optical path length at room temperature. 5-Hydroxyisoflavones (5-HIFs) including compounds **1**, **2** and **3** were purified by complexation HSCCC with the purity of above 95%.

The stoichiometry of the complex was determined by the molar ratio method [32]. In this method, the concentration of each 5-HIF in methanol was kept constant (5×10^{-5} M), while the concentration of copper nitrate was varied (from 5×10^{-6} to 2×10^{-4} M). The absorbance at 390 nm against the $[Cu^{2+}]/[5\text{-HIF}]$ ratio was plotted.

The stability constant of the complex was determined by the Bent–French method [33]. According to this method, two kinds of solutions were prepared:

a. Solutions containing a constant concentration of 5-HIFs (5×10^{-5} M) and varied concentration of copper nitrate (from 5×10^{-6} M to 3×10^{-5} M) were prepared. The absorbance (A_i) at 390 nm of each solution was recorded.
b. Solutions containing a constant concentration of 5-HIFs (5×10^{-5} M) and copper nitrate (2.5×10^{-4} M) was prepared. The absorbance (A_{max}) at 390 nm was recorded.

3. Results and discussion

3.1. Two-phase solvent system in HSCCC

3.1.1. Selection of the two-phase solvent system

One of the most important steps in performing an HSCCC separation is the selection of an appropriate solvent system, which is not an exception in complexation HSCCC. The partition coefficients of target compounds should be in the range of 0.2–2.0 and the separation factor ought to be greater than 1.5 in a suitable solvent system [34]. According to the literature [35], the solvent system chloroform–methanol–water and *n*-hexane–ethyl acetate–methanol–water (HEMW) were the most proposed system using for separation of flavonoids. Since the *K*-values were too big or too small to fall in that range in the chloroform–methanol–water (2:1:1), light petroleum–ethyl acetate–methanol–water (4:4:4:4) and light petroleum–ethyl acetate–methanol–water (2:5:2:5) solvent systems (Table 1), they were not suitable for the separation even with some modification. In the tested light petroleum–ethyl acetate–methanol–water system, the volume ratio of 3:5:4:4 and 3:5:3:5 gave adequate *K*-values. However, the separation factors between compounds **1** and **2** were smaller than 1.5 and they cannot be separated at all by conventional HSCCC.

Particular properties should be taken into consideration in the selection of an adequate metal ion. First, it should cooperate with target compounds and the chelating power should be discrepant enough so that their separation factor could be modified. Second, the metal salts should be easily dissolved in the stationary phase of HSCCC. Comparing with zinc chloride, aluminum muriate and copper sulfate, the copper nitrate was ultimately selected in our preliminary experiment according to the above discussions. When the copper nitrate was added, the partition coefficients increased dramatically and the separation factors between compounds **1** and **2** were also improved. Compared with the light petroleum–ethyl acetate–methanol–water (3:5:4:4), the light petroleum–ethyl acetate–methanol–water (3:5:3:5) with copper nitrate in the lower phase gave optimum distribution ratio. The separation factor, however, did not change remarkably with the concentration of the copper nitrate from 0.05 mol/L to 0.10 mol/L. It is quite possible that the method searching for the two-phase sol-

Table 1
The *K* (partition coefficient) values of target compounds in different solvent systems.

Solvent system	*K*-values[a]		
	1	**2**	**3**
$CHCl_3$–MeOH–H_2O (2:1:1)	>5	>5	>5
PE–EtOAC–MeOH–H_2O (4:4:4:4)	2.49	2.68	4.42
PE–EtOAC–MeOH–H_2O (3:5:4:4)	1.10	1.11	1.77
PE–EtOAC–MeOH–H_2O (3:5:3:5)	0.22	0.25	0.39
PE–EtOAC–MeOH–H_2O (2:5:2:5)	<0.2	<0.2	<0.2
PE–EtOAC–MeOH–H_2O (3:5:4:4, 0.050 mol/L $Cu(NO_3)_2$ in the lower phase)	1.24	2.70	4.60
PE–EtOAC–MeOH–H_2O (3:5:4:4, 0.075 mol/L $Cu(NO_3)_2$ in the lower phase)	1.39	2.99	4.99
PE–EtOAC–MeOH–H_2O (3:5:4:4, 0.100 mol/L $Cu(NO_3)_2$ in the lower phase)	1.43	3.15	5.26
PE–EtOAC–MeOH–H_2O (3:5:3:5, 0.050 mol/L $Cu(NO_3)_2$ in the lower phase)	0.34	0.66	1.06
PE–EtOAC–MeOH–H_2O (3:5:3:5, 0.075 mol/L $Cu(NO_3)_2$ in the lower phase)	0.41	0.80	1.29
PE–EtOAC–MeOH–H_2O (3:5:3:5, 0.100 mol/L $Cu(NO_3)_2$ in the lower phase)	0.45	0.87	1.40

[a] Expressed as: A_L/A_U, where A_L is the peak area of target compound in the lower phase, and A_U, the upper phase.

vent systems by HPLC is focused on hydrophobicity of the solvent system. An additional adjustment is required with respect to the ionic strength of the solvent system and at present this can only be possible by a trial and error method [36]. So further studies are still essential in optimizing the concentration of the copper nitrate by HSCCC.

3.1.2. Optimizing the concentration of the copper nitrate

Different concentrations of copper nitrate, including 0 mol/L, 0.050 mol/L, 0.075 mol/L and 0.100 mol/L were added into the lower phase of light petroleum–ethyl acetate–methanol–water (3:5:3:5, v/v). They were investigated under the same conditions. The partition coefficient (K) and separation factor (α) were calculated from the HSCCC chromatograms as follows [37]:

$$K = \frac{V_R - V_M}{V_C - V_M} \quad (1)$$

$$\alpha = \frac{K_2}{K_1}(K_2 > K_1) \quad (2)$$

where V_R and V_M indicate the retention volume of compound and mobile phase and V_C equals to 300 mL, respectively.

From the above equations, the results of HSCCC experiments were calculated and shown in Fig. 2. Generally, the partition coefficients of flavones all increased with the increasing of Cu^{2+} concentration, but at different extents of different compounds, resulting in the large difference of separation factors.

In the Cu^{2+}-free separation, peaks I and II could not be separated at all. The baseline separation also was not achieved between them and peak III. As the $[Cu^{2+}]$ increased, all peaks eluted later because the partition coefficients increased. The separation factor between peaks I and II (α_1), peaks II and III (α_2) also increased. When the $[Cu^{2+}]$ was 0.10 mol/L, α_1 (1.61) reached the maximum. The α_2 (1.55) reached the maximum when $[Cu^{2+}]$ was 0.05 mol/L. When the $[Cu^{2+}]$ was sequentially increased over 0.10 mol/L, it was difficult to dissolve in the stationary phase of HSCCC. In order to obtain good separation results in the HSCCC separation, the $[Cu^{2+}]$ of 0.10 mol/L was selected at last.

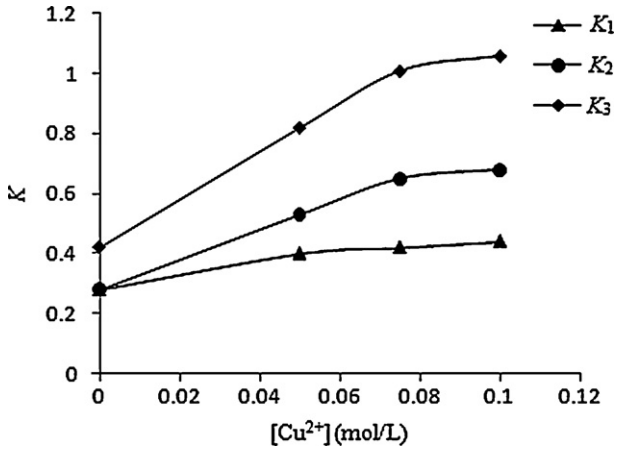

Fig. 2. Effect of the concentration of Cu^{2+} on partition coefficient (K) for compounds **1** (K_1), **2** (K_2) and **3** (K_3).

3.2. HSCCC procedure

Based on the above discussions, the solvent system was selected as light petroleum–ethyl acetate–methanol–water (3:5:3:5, v/v) with 0.1 mol/L copper nitrate in the lower phase. The influences of revolution speed and flow rate of the mobile phase were also investigated. Good results were obtained when the revolution speed was 850 rpm and the flow rate was 1.0 mL/min. Under the optimum conditions, peaks I (9.2 mg), II (46.4 mg) and III (1.2 mg) were obtained from 100 mg crude extract. The HSCCC chromatogram is given in Fig. 3. The retention of the stationary phase was 78.3%. The copper salt was recovered with high recovery (95.7%) and can be recycled in further experiments, which could reduce environmental pollution and limit the cost of the experiments. Although the copper nitrate is retained in the stationary phase, it may be carried over by the mobile phase and the target fractions may contain minor copper nitrate. Purification of peaks I, II and III was carried out to

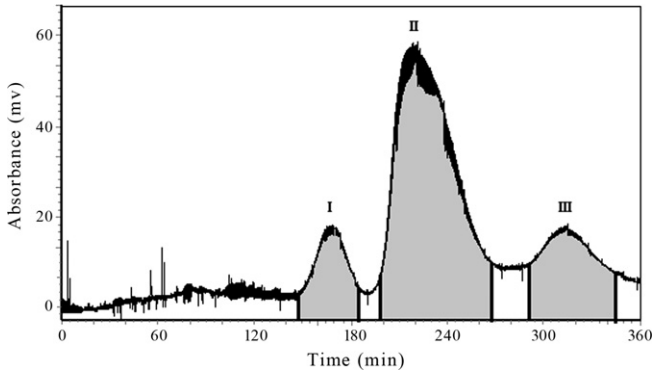

Fig. 3. HSCCC chromatogram of crude extract from *B. chinensis* by adding copper ion. Two-phase solvent system: light petroleum–ethyl acetate–methanol–water (3:5:3:5, v/v) with 0.10 mol/L copper nitrate in the lower phase; stationary phase: the lower aqueous phase; mobile phase: the upper organic phase; elution mode: tail to head; flow rate: 1.0 mL/min; revolution speed: 850 rpm; detection wavelength: 254 nm; sample size: 100 mg of crude extract dissolved in 10 mL of the upper phase; retention percentage of the stationary phase: 78.3%.

remove the small amount of copper nitrate and its recovery was in the range of 93.0–95.0%.

3.3. Purity analysis and structural identification of HSCCC peaks

Each fraction obtained by HSCCC was analyzed by HPLC. The purities of peaks I, II and III in Fig. 4 were 95.06%, 96.98%, and 93.69%, respectively.

The structural identification of each peak fraction in Fig. 4 was performed with ESI-MS, ^{1}H NMR and ^{13}C NMR spectra as follows:

Peak I: ESI-MS: m/z 359 $[M-H]^-$; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 12.63 (1H, s, 5-OH), 8.46 (1H, s, H-2), 6.73 (1H, d, J = 2.0 Hz, H-2′), 6.68 (1H, d, J = 2.0 Hz, H-6′), 6.33 (1H, s, H-6), 3.81 (3H, s, 8-OCH_3), 3.78 (3H, s, 4′-OCH_3), 3.71 (3H, s, 5′-OCH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 154.7 (C-2), 122.1 (C-3), 180.1 (C-4), 157.5 (C-5), 99.3 (C-6), 156.8 (C-7), 127.5 (C-8), 149.8 (C-9), 104.1 (C-10), 126.0 (C-1′), 110.4 (C-2′), 150.3 (C-3′), 136.4 (C-4′), 152.9 (C-5′), 104.6 (C-6′), 59.9 (8-OMe), 60.9 (4′-OMe), 55.8 (5′-OMe). The spectral data were similar to those in Ref. [38], and peak I corresponded to isoirigenin.

Peak II: ESI-MS: m/z 359 $[M-H]^-$; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 13.04 (1H, s, 5-OH), 10.82 (1H, s, 7-OH), 9.29 (1H, s, 3′-OH), 8.38 (1H, s, H-2), 6.72 (1H, d, J = 1.8 Hz, H-2′), 6.67 (1H, d, J = 1.8 Hz, H-6′), 6.52 (1H, s, H-8), 3.80 (3H, s, 5′-OCH_3), 3.76 (3H, s, 4′-OCH_3), 3.71 (3H, s, 6-OCH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 155.3 (C-2), 122.2 (C-3), 180.8 (C-4), 153.8 (C-5), 132.0 (C-6), 158.0 (C-7), 94.4 (C-8), 153.4 (C-9), 105.3 (C-10), 126.6 (C-1′), 105.0 (C-2′), 150.7 (C-3′), 136.9 (C-4′), 153.1 (C-5′), 110.8 (C-6′), 60.4 (6-OMe), 60.4 (4′-OMe), 56.3 (5′-OMe). The spectral data were similar to those in Ref. [39], and peak II corresponded to irigenin.

Peak III: ESI-MS: m/z 359 $[M-H]^-$; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 13.07 (1H, s, 5-OH), 8.39 (1H, s, H-2), 6.86 (2H, s, H-2′, H-6′), 6.50 (1H, s, H-8), 3.79 (6H, s, 3′-OMe, 5′-OMe), 3.76 (3H, s, 6-OMe); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 154.5 (C-2), 122.0 (C-3), 180.5 (C-4), 153.3 (C-5), 131.6 (C-6), 158.0 (C-7), 94.0 (C-8), 152.8 (C-9), 104.8 (C-10), 120.8 (C-1′), 106.9 (C-2′), 147.8 (C-3′), 135.9 (C-4′), 147.8 (C-5′), 106.9 (C-6′), 60.0 (6-OMe), 56.2 (3′-OMe, 5′-OMe). The spectral data were similar to those in Ref. [38], and peak III corresponded to 5,7,4′-trihydroxy-6,3′,5′-trimethoxyisoflavone.

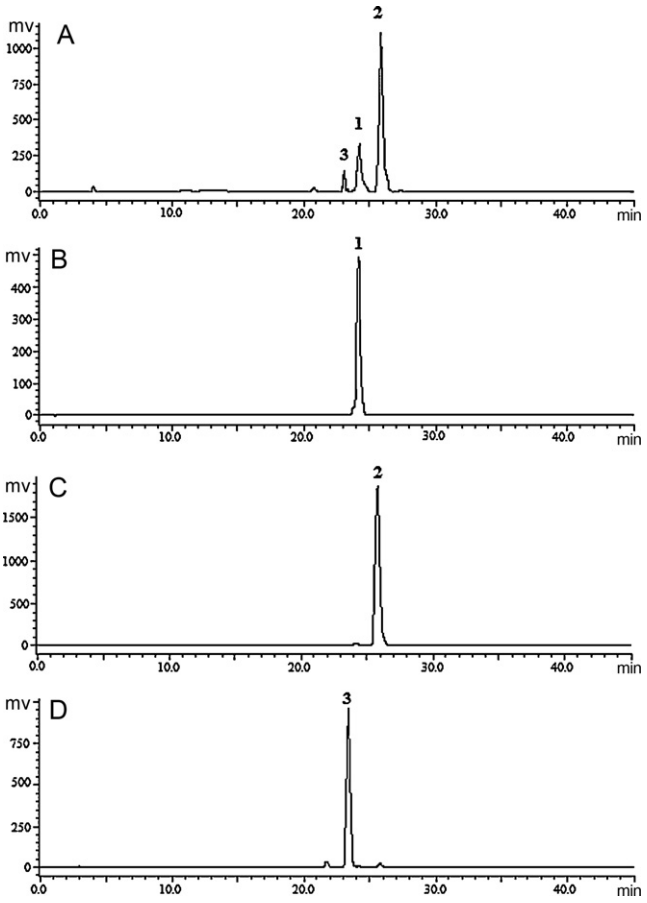

Fig. 4. HPLC chromatograms. (A) Crude extract from *B. chinensis*; (B) HSCCC fraction of compound **1**; (C) HSCCC fraction of compound **2**; (D) HSCCC fraction of compound **3**. Column: ultimate TM XB-C_{18} column (250 mm × 4.6 mm, 5 μm, Welch Materials); mobile phase: 0.1% TFA (A)–methanol (B) in gradient mode as follows: 0–15 min, 40–50% B; 15–35 min, 50–57% B; 35–45 min, 57–65%; detection wavelength: 254 nm; flow rate: 1 mL/min.

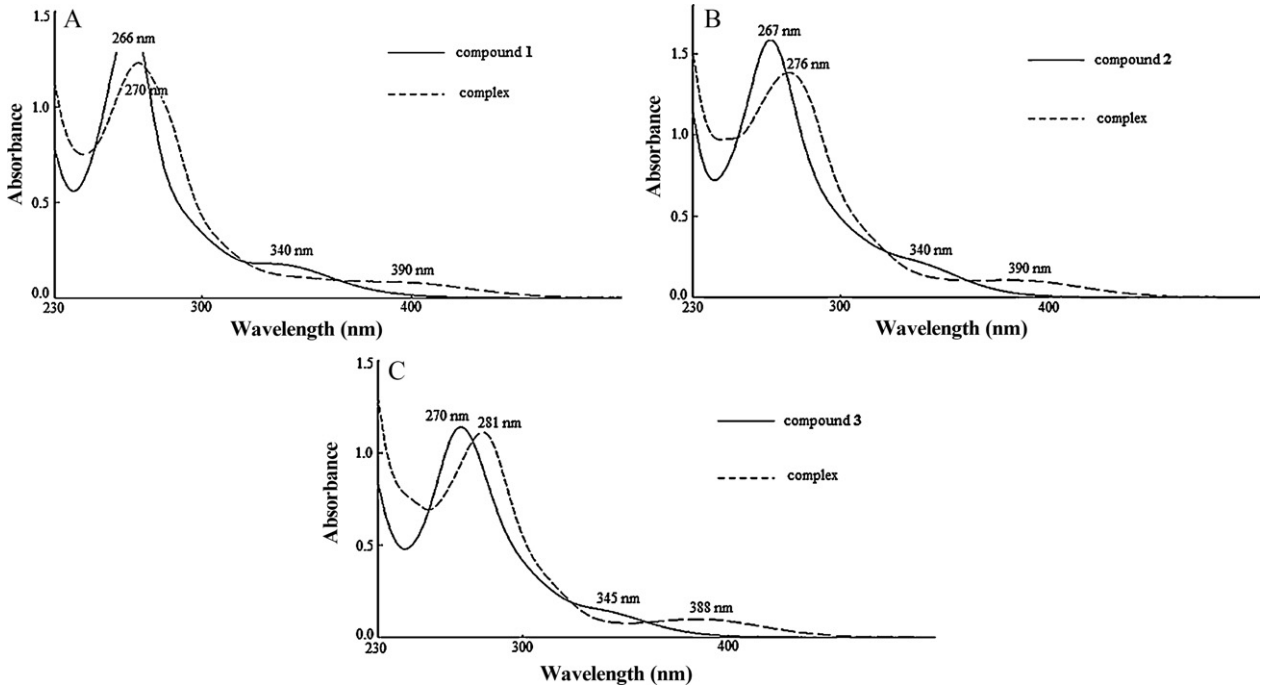

Fig. 5. UV–Vis spectra of 5-hydroxyflavones free and complexed with copper nitrate in methanol. (A) Compound **1**; (B) compound **2**; (C) compound **3**.

Table 2
The stoichiometry and stability constant of the complexes formed between 5-hydroxyflavones and copper nitrate.

Compound	Stoichiometry	Linear regression analysis		
		Intercept value	Slope value	Correlation coefficient
1	1:1	3.0529	0.7865	0.9668
2	1:1	3.4253	0.9216	0.9738
3	1:1	3.7589	0.9757	0.9830

3.4. Evaluation of stoichiometry and stability for copper–flavone complexes to explain the chromatographic results

The UV–Vis spectra of compounds **1**, **2**, and **3** in MeOH (Fig. 5) are characterized by isoflavone absorption bands with a maximum around 267 nm (band II) and a shoulder around 340 nm (band I). With the addition of copper nitrate, compound **1** showed bathochromic shifts of band II from 266 nm to 270 nm and band I from 340 nm to 390 nm. Likewise, compounds **2** and **3** also yielded bathochromic shifting trends due to the similarity of structures. The band observed at 390 nm could be presumed to be the formation of a new ring system between the metal and oxygen atoms from the 4-keto and 5-OH positions.

The stoichiometry and stability constant of the complex were determined applying the molar ratio method and Bent–French method, respectively [32,33]. The data obtained are summarized in Table 2. All of the three compounds formed 1:1 complexes and the chelating power is **3** > **2** > **1**.

Based on the above analysis of stoichiometry and stability for copper–flavone complexes, a simple mathematic model in Fig. 6 could facilitate understanding the separation results of complexation HSCCC [40]. In the aqueous stationary phase different ligands (L_1 and L_2) and the metal ion (M) are in equilibrium with their chelates (L_1M and L_2M), where both M and its complexes are assumed to be insoluble in the mobile phase. Therefore, only free ligands, L_1 and L_2, are in partition equilibrium between the two phases as shown by the vertical arrows. Under these conditions, distribution ratios of L_1 and L_2 are expressed in the following equations:

$$D_1 = D_a\{1 + [M]_{aq}K_{f1}\} \quad (3)$$

$$D_2 = D_b\{1 + [M]_{aq}K_{f2}\} \quad (4)$$

where D_a and D_b are the partition coefficients of L_1 and L_2 in the metal-free two-phase solvent system, while K_{f1} and K_{f2} indicate the complexation constants of L_1M and L_2M complexes, respectively.

From Eq. (3) and (4), the separation factor is given by

$$\alpha = \frac{D_1}{D_2} = \frac{D_a\{1 + [M]_{aq}K_{f1}\}}{D_b\{1 + [M]_{aq}K_{f2}\}} \quad (5)$$

Eq. (5) indicates that the separation factor increases with the metal ion concentration and the magnitude of the K_{f1}/K_{f2} ratio in a selected solvent system, where the D_a and D_b are constant. The results of our experiments in Section 3.1.2 are basically in accordance with the mathematical analysis. Since the complexation constant magnitude of the three 5-HIFs is **3** > **2** > **1**, their separation factors are improved with the increasing of the copper ion concentration. However, it needs to point out that this increasing trend is within certain range in the complexation HSCCC.

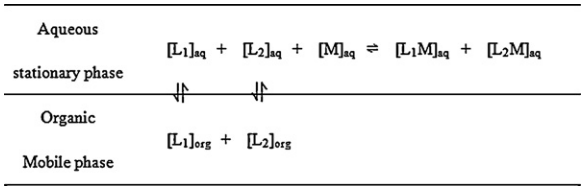

Fig. 6. Schematic diagram of chemdynamic equilibrium between different ligands (L_1 and L_2) and the metal (M) in the separation column.

4. Conclusions

It is well known that the separation of natural compounds with very similar structures is extremely difficult. Conventional column chromatography was frequently proved to be ineffective and required multiple steps. The novel separation technique of complexation HSCCC utilized the long known ability of copper ion to form complexes with hydroxyflavones. This method is quite effective and fit for difficultly separated flavonoids. Three 5-HIFs isomers, isoirigenin, irigenin and 5,7,4′-trihydroxy-6,3′,5′-trimethoxyisoflavone were simultaneously isolated with a two-phase solvent system composed of light petroleum–ethyl acetate–methanol–water (3:5:3:5, v/v) and copper nitrate (0.10 mol/L in the lower phase). The additive Cu^{2+} has different chelating power with 5-HIFs as elucidated by UV–Vis spectroscopy modifying the retention behavior of 5-HIFs in HSCCC. We believe that this developed technology could be used as a more powerful tool for the separation of bioactive compounds.

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