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Isolation and purification of inflacoumarin A and licochalcone A from licorice by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) technique in semi-preparative scale has been applied to isolate and purify bioactive flavone compounds from the ethanol extract of *Glycyrrhiza inflata* Bat., a particular plant species of licorice. HSCCC separation was performed with a two-phase solvent system composed of *n*-hexane–chloroform–methanol–water (5:6:3:2, v/v) by eluting the lower mobile phase at a flow rate of 1.8 ml/min and a revolution speed of 800 rpm. Purification was performed with a two-phase solvent system composed of *n*-hexane–chloroform–methanol–water (1.5:6:3:2, v/v) by eluting the lower mobile phase at a flow-rate of 1.5 ml/min and a revolution speed of 800 rpm. Two major flavone peaks: inflacoumarin A and licochalcone A were collected and the respective yields of the peaks amount to 6 mg (8.6%, w/w) and 8 mg (11.4%, w/w) from 70 mg of the crude extract sample. The purities of inflacoumarin A and licochalcone A reached 99.6% and 99.1%, respectively, after a sequential purification run. The structures of inflacoumarin A and licochalcone A were positively confirmed by ^{1}H NMR and ^{13}C NMR, ^{1}H–^{13}C-COSY, UV, FT-IR and electron ionization MS analyses.

Keywords: Licorice; *Glycyrrhiza inflata* Bat.; Licochalcone A; Inflacoumarin A

1. Introduction

Licorice has been used as a traditional Chinese medicine for over 2000 years. The herb is frequently used to treat diseases such as phthisis, contagious hepatitis, bronchitis and ague [1]. Flavones extracted from licorice have been extensively studied because many individual species in this compound class have been demonstrated through pharmacological studies and clinical practice to carry significant biological or anti-oxidation activities [2]. *Glycyrrhiza inflate* Bat. is an important species of licorice, and many flavone constituents extracted from it, among them the notable ones such as inflacoumarin and licochalcone, have been reported to carry strong biological activity [3]. Further studies to better understand the biochemical properties of inflacoumarin, licochalcone and other related flavones are needed to ascertain their clinical applications, and this necessitates the development of efficient, preparative-scale separation methods for their isolation and purification. Moreover, the preparation of active compounds or fractions as reference standards for quality control purposes is also in urgent need in research as well as industrial communities.

High-speed counter-current chromatography (HSCCC) is a liquid–liquid chromatographic technique that is gaining increasing interest recently. It relies on the use of centrifugal force for the retention of the stationary phase [4]. Compared to other liquid–liquid techniques, HSCCC is advantageous because of its shorter separation time, wider range of selection of solvent systems and quantitative material recovery. HSCCC has been widely used for the preparative separation of various natural products because of these technical merits [5–8].

In this paper, the development of HSCCC method for the separation and purification of inflacoumarin A and

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licochalcone A from the crude ethanol extract of *G. inflate* Bat. will be described.

2. Experimental

2.1. Materials

Dried licorice root (*G. inflate* Bat.), whose species was identified by Prof. Shouquan Lin (Institute of Medicinal Plants, Chinese Academy Medical Sciences), was provided by ELION Resources Group, Inner Mongolia. Samples of licorice roots were crushed into pieces by a disintegrator made in Hangzhou Chunjiang Pharmacy Machine Co.

Acetonitrile is of chromatographically grade and trifluoroacetic acid is of protein sequencing grade. Both were purchased from Sigma. Water used as the mobile phase in HPLC was prepared with Millipore purifier (Millipore, USA) in our laboratory. All organic solvents used for HSCCC were of analytical grade and purchased from Shanghai chemical reagent corporation, Shanghai, China.

2.2. Preparation of the crude ethanol extract of *Glycyrrhiza inflate Bat.*

Roots of *G. inflate* Bat. (0.5 kg) were extracted three times (3 × 45 min), each with 3l of ethanol–water (95:5) by sonication using a SK3200LH ultrasonic cleaning instrument (Shanghai Kudos Ultrasonic Instrument Co., Shanghai, China). The extracts were combined and concentrated under reduced pressure with a Model SENCO R-201 rotary evaporator (Shanghai Shensheng Biotech Co., Shanghai, China). The residue was dissolved in 2% aqueous sodium hydroxide and then filtrated under reduced pressure in a vacuum filtration devise. Two sheets of double-ring brand cellulose qualitative filter paper manufactured by Hangxhou Xinhua Paper Industry (Hangzhou, China) were used. The filtrate was acidified with 2% aqueous hydrochloric acid until sedimentation process stops. The sediment was washed with cool water and freeze-dried with a Model FD-1 freezing drier (Beijing Boyikang Technology, Beijing, China) to get the crude extract of *G. inflate* Bat.

2.3. Preparation of two-phase solvent system and sample solution

Two solvent systems utilized in the HSCCC separation and purification were prepared by mixing *n*-hexane, chloroform, methanol and water, and then thoroughly equilibrating the mixtures in a separator funnel at 25 °C in a self-designed thermostat. Two phases were separated shortly before use. The volume ratios of the four solvents are 5:6:3:2 and 1.5:6:3:2, used respectively for the separation and purification runs.

The sample solutions were prepared by dissolving the crude extract of *G. inflate* Bat. in the upper organic phase at suitable concentrations range of 10–14 mg/ml.

2.4. High-speed counter-current chromatography (HSCCC)

2.4.1. Instrumentation

HSCCC was performed with a Model TBE-300A HSCCC system manufactured by Tauto Biotech Co., Shanghai, China. The multi-layer coil planet centrifuge (CPC) was prepared by winding 1.8 mm i.d. PTFE tubing coaxially onto the column holder with a total capacity of 350 ml. The β-value varied from 0.42 at the internal terminal to 0.63 at the external terminal. $\beta = r/R$ where r is the distant from the coil to the holder shaft, and R is the revolution radius or the distant between the holder axis and central axis of the centrifuge. The rotation speed is adjustable from 500 to 1000 rpm; and 750–900 rpm was used in the present study. Different from ordinary HSCCC systems, the system used in the present study was equipped with a thermostatic jacket. The jacket can keep the CPC at constant temperature with the aim to eliminate the harmful effect of temperature variation on separation efficiency.

The solvent was pumped into the column with a Model S1007 constant-flow pump (Beijing Shengyitong Technology Development, Beijing, China). The effluent was detected on-line by 254 nm absorption with a Model 8823A UV detector (Beijing Institute of New Technology Application). A manual sample injection valve with a 20 ml loop was used to introduce the sample into the column. A Model 3057 portable recorder (Sichuan Instrument Factory, Chongqing, China) was used to record the chromatogram. Moreover, the data was also displayed and analyzed simultaneously on a Model Sepu3000 chromatographic data station provided by Hangzhou Puhui Scientific Technology.

2.4.2. Separation procedure

The multi-layer coiled column was first filled completely with the upper solvent phase (stationary phase). Then, the lower phase (mobile phase) was pumped into the head of the column at a flow rate of 1.8 ml/min while the column was rotating at 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution was injected through the sample injection valve. The column effluent was monitored with a UV detector at 254 nm as stated earlier, and each peak fraction was collected manually according to the chromatographic profile displayed on the recorder.

2.5. High-performance liquid chromatography

The crude ethanol extract of *G. inflate* Bat. and each of the peak fractions collected from HSCCC separation were analyzed by HPLC. The analyses were performed with HP1100 HPLC instrument (Agilent, USA). The column (Kromasil KR100-5C_{18}, 150 mm × 4.6 mm i.d., Dalian Elite Analytical Instruments) was eluted with acetonitrile–0.05% aqueous trifluoroacetic acid at a flow-rate of 0.8 ml/min. The percentage of acetonitrile in the mobile phase was programmed

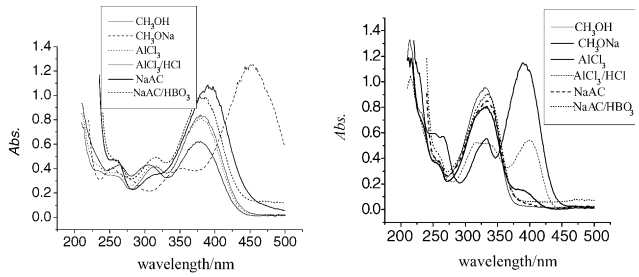

Fig. 1. The UV spectra of licochalcone A (*left*) and inflacoumarin A (*right*).

as follows: 20% (0 min)–40% (5 min)–50% (10 min)–50% (25 min)–80% (35 min). Elution was monitored at 254 nm and 364 nm using diode array detection (DAD). The purity of the isolated constituents was determined based on the peak area normalized to all observed HPLC peak areas.

2.6. Identification of the separated peaks

The ultraviolet (UV) spectra of the separated individual peaks were measured with a Model DU-7400 UV–vis spectrophotometer (Beckman, USA). Different solvents including CH_3OH, CH_3ONa, $AlCl_3$ solution, $AlCl_3$–HCl, CH_3COONa and CH_3COONa–H_3BO_3 were used to identify their structures by shifts and strengthen of the UV spectra, which is shown in Fig. 1 and Tables 5 and 7. The Fourier transform infrared (FT–IR) spectra were recorded on a Model 740SX infrared spectrometer (Nicolet, USA) with the samples prepared as potassium bromide discs. Elemental analyses (EA) were performed with a EA/MA 1110 elemental analyzer (Carlo Erba, Italy). Electron ionization (EI) mass spectra (MS) were measured with a Esquire 3000 plus mass spectrometer (Bruker, USA) in positive ion mode. The ^1H NMR, ^{13}C NMR, and ^1H–^{13}C-COSY spectra were recorded on an Unity 500 MHz nuclear magnetic resonance spectrometer (Varian, USA) with TMS based chemical shifts. Approximately 30 mg each of the purified compounds were dissolved in 500 μl of [$^2H_6$]-acetone in NMR measurement.

Table 1
Factors and levels of the $L_9(3^4)$ orthogonal experiment

Level	Factors		
	T (°C)	Rs (rpm)	Fr (ml/min)
1	20	700	1.5
2	25	800	2.0
3	30	900	2.5

Notes: *T* is abbreviation of temperature, Rs is revolution speed and Fr is flow-rate.

3. Results and discussion

3.1. Selection of solvent system

Suitable solvent system is the key factor for a successful HSCCC separation. A series of experiments were performed to determine the optimum solvent system for HSCCC separation. Under the conditions of 800 rpm revolution speed, 2.0 ml/min flow-rate, and 25 °C CPC temperature, the following five solvent systems were evaluated: (1) *n*-hexane–ethyl acetate–ethanol–water (8:5:10:4, v/v); (2) *n*-hexane–ethyl acetate–ethanol–water (8:7:10:4, v/v); (3) *n*-hexane–ethyl acetate–methanol–water (8:9:10:4, v/v); (4) *n*-hexane–chloroform–methanol–water (1.8:6:3:2, v/v); and (5) *n*-hexane–chloroform–methanol–water (5:6:3:2, v/v). The upper phases were used as the stationary phase while the lower phases were used as the mobile phase in all tests. 72.7%, 57.3%, 15.8%, 77.3% and 59.4% of stationary phase retentions were obtained for solvent systems (1), (2), (3), (4) and (5), respectively. Among these, the *n*-hexane–chloroform–methanol–water (5:6:3:2, v/v) system gave the best separation, as shown in Fig. 2A. The solvent system was therefore used for all HSCCC runs except the ones used in purification runs, for which a solvent system of *n*-hexane–chloroform–methanol–water at a volume ratio of 1.5:6:3:2 was selected based on optimization experiments carried out separately.

3.2. Selection of operational parameters

The preliminary experiments showed that CPC temperature, revolution speed and flow-rate all affect separation

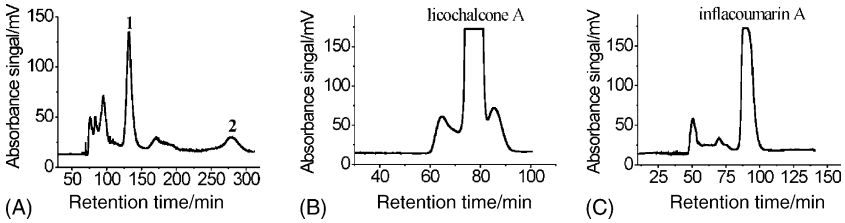

Fig. 2. HSCCC chromatograms of (A) the crude ethanol extract of licorice crude extract; (B) peak 1 of A; (C) peak 2 of A; solvent systems: (1) *n*-hexane–chloroform–methanol–water (5:6:3:2, v/v) for (A) and (2) *n*-hexane–chloroform–methanol–water (1.5:6:3:2, v/v) for (B) and (C); stationary phase: upper phase; mobile phase: lower phase; flow-rate: 1.8 ml/min for (A) and 1.5 ml/min for (B) and (C); revolution speed: 800 rpm; temperature: 25 °C; sample size: 54.8 mg dissolved in 5 ml lower phase of solvent system (1) for (A), 15 mg of peak 1 in 1 ml lower phase solvent system (2) for (B), and 20 mg of peak 2 in 1 ml lower phase of solvent system (2) for (C); detection at 254 nm.

Table 2
Effects of operational factors on separation time

Source	Sum of squares of variance	Degree of freedom	Estimator of variance	*F*-value	Significance
Temperature (°C)	2.4	2	1.2	3	
Revolution speed (rpm)	0.7	2	0.4	1	
Flow-rate (ml/min)	280	2	140	335	***
Error	0.8	2	0.4		
Total	284	8	142		

Notes: $F_{0.01}(2, 2) = 99.00$(***); $F_{0.05}(2, 2) = 19.00$(**); $F_{0.1}(2, 2) = 9.00$(*).

performance. To optimize process conditions, orthogonal array design (OAD), which is a fractional factorial experimental design technique [9], was adopted. Orthogonal here means balanced, separable or not mixed, i.e., when the effect of a particular factor is being calculated, the influence of all other factors is canceled out, and hence effects of individual factors can be sorted out independently. The 4-factor, 3-level, usually noted as $L_9(3^4)$, orthogonal array experiments were applied for which the details were listed in Table 1. The effects of the above three factors on retention time and peak resolution were analyzed by analysis of variance (ANOVA), as shown in Tables 2 and 3. Range analyses (Table 4) indicate that the highest separation efficiency and best resolution were achievable within the temperature range of 20–30 °C. Tables 2–4 also indicate that the resolution increased with increasing rotary speed, but then high rotary speed resulted in the loss of the stationary phase material due to the mixing of the solvent system by emulsification. A comprised 800 rpm was therefore selected for operation.

Within the flow rate range of 1.5–2.5 ml/min, the flow-rate affects greatly the overall run time but only slightly on resolution. In order to shorten separation time while still maintaining adequate resolution to meet different analytical objectives, 1.8 ml/min was used in preliminary sample separation experiments while 1.5 ml/min was used for the refining, peak purification operations. Typical run time for sample separation was 330 min.

Sample size affects the separation resolution. When sample was over 70 mg, the purities of the separated peaks 1 and 2 in Fig. 2A reduced obviously. Thus, the sample size is set to be no more than 70 mg.

Table 3
Effects of operational factors on resolution of peaks 1 and 2

Source	Sum of squares of variance	Degree of freedom	Estimator of variance	*F*-value
Temperature (°C)	0.21	2	0.11	0.30
Revolution speed (rpm)	0.74	2	0.37	1.04
Flow-rate (ml/min)	0.56	2	0.28	0.79
Error	0.71	2	0.35	
Total	2.21	8	1.11	

Table 4
Results and range analysis of orthogonal experiments

Order	A	B	C	Time (min)	Resolution
1	1	1	1	341	3.84
2	1	2	2	254	4.29
3	1	3	3	206	4.28
4	2	1	2	261	4.21
5	2	2	3	197	3.68
6	2	3	1	335	5.51
7	3	1	3	209	4.24
8	3	2	1	345	4.67
9	3	3	2	275	4.47
Time (min)					
$K_1/3$	267	270	340		
$K_2/3$	264	265	263		
$K_3/3$	276	272	204		
Optimal			$A_2B_2C_3$		
Resolution					
$K_1/3$	4.14	4.10	4.67		
$K_2/3$	4.47	4.21	4.32		
$K_3/3$	4.46	4.75	4.07		
Optimal			$A_{3/2}B_3C_1$		

For real sample runs, the crude extract can be dissolved in either the upper or the lower phase. Sample solubility is higher in the upper phase than those in the lower phase. However, if the sample was dissolved in the upper phase or a mixture of upper and lower phases (1:1, v/v), striping loss of the stationary phase occurs, resulting in baseline drifting problems. On the other hand, if lower phases were used for sample dissolution, higher volume of solvent would be needed because of its lower solubility towards the sample. The injection of large sample size would then degrade the separating resolution. Experimental results indicated that resolution is a

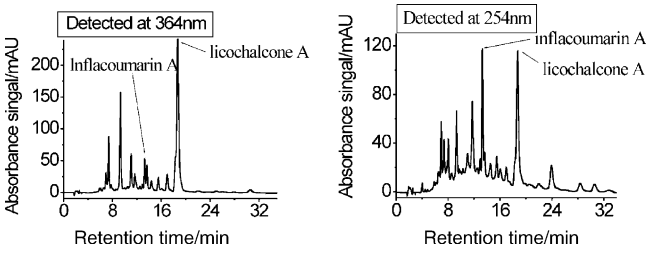

Fig. 3. HPLC chromatograms of the crude extract of licorice; column: Kromasil KR100-5C_{18} (150 mm × 4.6 mm) at room temperature; elution: acetonitrile–water (added 0.05% trifluoroacetic); flow-rate: 0.8 ml/min; detection at 364 nm for the left part of the figure and 254 nm for the right row.

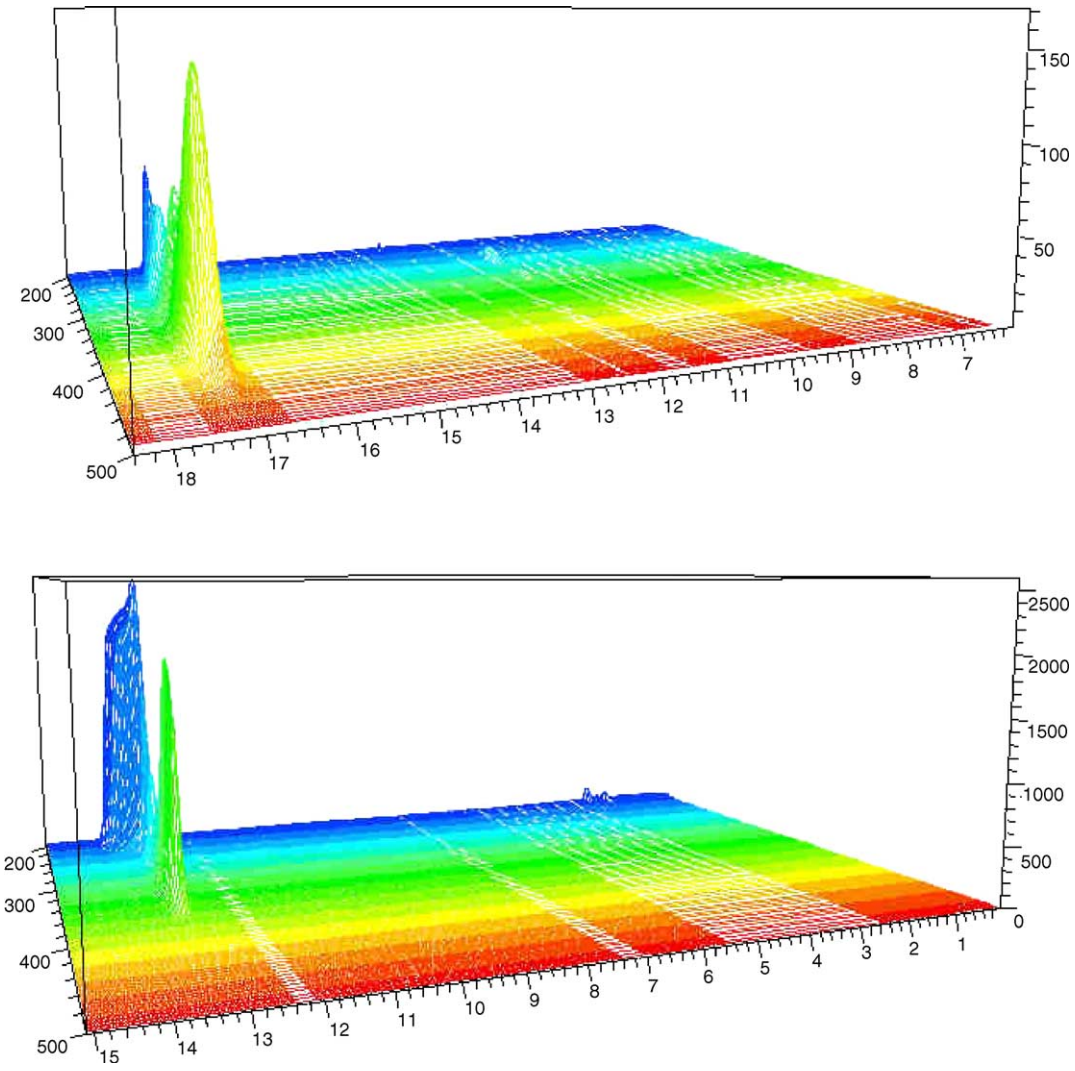

Fig. 4. The 3-D HPLC-DAD chromatograms of licochalcone A (*top*) and inflacoumarin A (*bottom*).

more important factor in maintaining the peak purities (peaks 1 and 2 in Fig. 2A) than the drifting baseline. Thus, the upper phase was selected as the sample solvent to dissolve the crude extract.

3.3. Purification of the separated constituents

Fig. 3 shows the HPLC chromatograms of the crude extract from *G. inflata* Bat. It can be seen that the crude extract gives many peaks among which licochalcone A and inflacoumarin A were the two major ones. The peaks 1 and 2 in Fig. 2A were individually collected and their dry weights obtained after solvent evaporation. The run yielded 8 mg (11.4%, w/w) of licochalcone A and 6 mg of inflacoumarin A (8.6%, w/w), respectively. The purities of the collected licochalcone A and inflacoumarin A fractions were checked by HPLC, and their three-dimensional chromatograms were displayed on Fig. 4. Normalized against total observed UV absorptions in the chromatographic runs, the purities were calculated to be 95.0% for licochalcone A, and 98.8% for inflacoumarin A. To further purify the fractions, a second purification run on HSCCC was made with a two-phase solvent system composed of *n*-hexane–chloroform–methanol–water (1.5:6:3:2, v/v) by eluting the lower phase at a flow-rate of 1.5 ml/min and a revolution speed of 800 rpm. The purities of licochalcone A and inflacoumarin A after the second purification run were 99.1% and 99.9%, respectively. The HSCCC chromatograms for

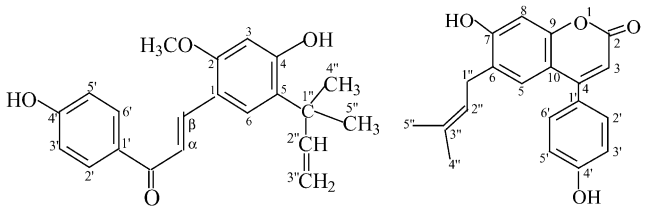

Fig. 5. The structures of licochalcone A (*left*) and inflacoumarin A (*right*).

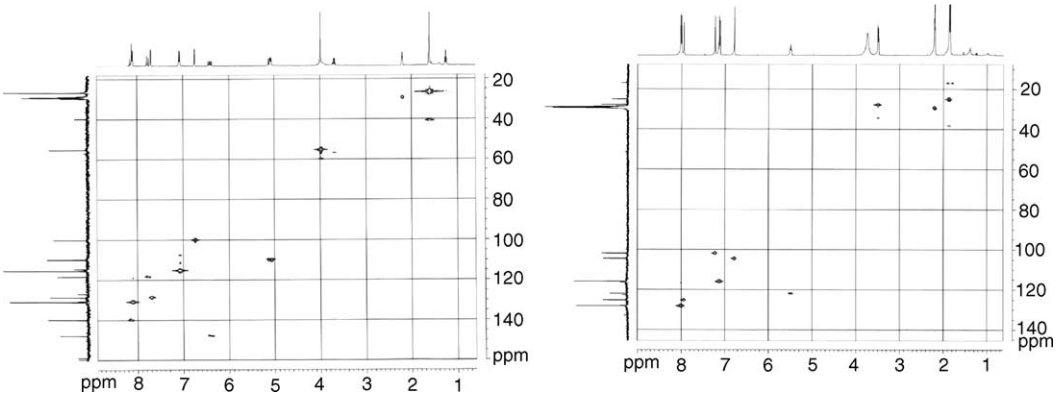

Fig. 6. ^{1}H–^{13}C-COSY spectra of licochalcone A (*left*) and inflacoumarin A (*right*).

Table 5
EA, FT-IR, UV and EIMS results of licochalcone A (compound **1**)

Techniques	Observed values	Reference values	Ref.
EA	C 73.8%, H 6.01%	C 74.6%, H 6.5%	
FTIR (KBr) (cm^{-1})	3437, 2965, 2925, 1637, 1603, 1560, 1446, 1344, 1288, 1215, 1166	3430, 2964, 1640, 1602, 1584, 1553, 1444, 1342, 1288, 1213, 1163	[10]
UV λ_{max} (nm)	378, 305, 263(CH_3OH); 451, 348, 252 (CH_3ONa); 379, 313, 261 ($AlCl_3$); 381, 315, 262($AlCl_3$/HCl); 391, 311, 260 (NaOAc); 380, 315, 264 (NaOAc/H_3BO_3)	378, 308, 262(CH_3OH); 423, 348, 250 (CH_3ONa) 371, 310, 250 ($AlCl_3$); 371, 310, 251 ($AlCl_3$/HCl); 390, 311, 259 (NaOAc); 387, 310, 254 (NaOAc/H_3BO_3)	[10,11]
MS *m/z*	339 ($[M + H]^+$), 297, 283, 191, 121, 107	338 (M^+), 323, 307, 216, 192, 121	[11]

Table 6
Chemical shifts of licochalcone A (compound **1**)

Position	Observed		Reference values [10,11]	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
1	–	115.8	–	115.7
2	–	159.7	–	158.8
3	6.59	101.0	6.43	101.0
4	9.52 (O*H*)	160.0	–	159.6
5	–	126.8	–	125.0
6	7.56	129.3	7.45	128.9
α	7.64	119.4	7.58	119.7
β	8.02	140.0	8.03	141.8
1′	–	131.5	–	130.5
2′	7.97	131.7	7.98	131.3
3′	6.93	116.0	6.98	116.1
4′	9.52 (O*H*)	162.3	–	161.5
5′	6.93	116.0	6.98	116.1
6′	7.97	131.7	7.98	131.3
1″	–	40.6	–	39.8
2″	6.27	148.6	6.24	147.7
3″	5.12, 5.07	110.8	5.26, 5.20	113.4
4″	1.45	27.4	1.44	27.0
5″	1.45	27.4	1.44	27.0
OCH_3	3.85	55.9	3.83	55.6
C=O	–	188.5	–	191.2

the second purification runs can be found in Fig. 2B and C.

3.4. Identification of licochalcone A and inflacoumarin A

The two flavones discussed in this paper have been previously identified in literature reports [10–13]. To confirm the identities of the two HSCCC peaks as described above, the isolated material were collected and measured by EA, FT-IR, UV spectrometry, MS and both Proton and ^{13}C NMR. The results were tabulated in Tables 5 and 6 for compound **1**, and Tables 7 and 8 for compound **2**. Based on the results, compound **1** is positively identified as licochalcone A and compound **2** as inflacoumarin A. The structures of the two compounds are shown in Fig. 5. The ^{13}C–^{1}H-COSY NMR spectra for the two compounds are given in Fig. 6.

Table 7
EA, FTIR, UV and EIMS results of inflacoumarin A (compound **2**)

Techniques	Observed	Reference values [12]
EA	C 74.42%, H 5.51%	C 74.53%, H 5.59%
FTIR (KBr) (cm^{-1})	3429, 2923, 1621, 1569, 1478, 833	3338, 2926, 1622, 1561, 1512, 1474, 829
UV λ_{max} (nm)	330, 259, 230, 215 (CH_3OH); 392, 332, 266 (CH_3ONa); 333, 257, 230 ($AlCl_3$); 400, 328, 215 ($AlCl_3$/HCl); 334, 257 (NaOAc); 335 (NaOAc/H_3BO_3)	330, 269, 258, 230
MS *m/z*	323 ($[M + H]^+$), 267, 238, 149	322, 307, 293, 267, 238, 189,149,105, 93

Table 8
The NMR chemical shifts of inflacoumarin A (compound **2**)

Position	Observed		Reference values [13]	
	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
2	–	163.5	–	162.1
3	6.60	105.7	6.70	104.4
4	–	177.3	–	176.1
5	7.83	126.1	7.65	124.5
6	–	128.3	–	127.2
7	10.29	161.0	10.86 (—OH)	160.2
8	7.06	102.8	6.96	101.7
9	–	157.0	–	155.5
10	–	116.7	–	115.7
1′	–	123.9	–	121.8
2′, 6′	7.91	128.8	7.88	127.9
3′, 5′	7.01	117.6	6.90	115.8
4′	9.73	161.3	10.25 (—OH)	160.4
1″	3.41	28.5	3.28	27.6
2″	5.39	122.7	5.31	121.7
3″	–	133.4	–	132.3
4″	1.74	17.8	1.67	17.7
5″	1.76	25.8	1.72	25.7

4. Conclusion

HSCCC has been developed and successfully applied to the separation and purification of licochalcone A and inflacoumarin A in crude extract of licorice. The results illustrate the effectiveness of HSCCC as a semi-preparative separation technique for the isolation and purification of bioactive components from licorice. In a typical run, tens of mgs of samples can be separated with high efficiency to yield mgs of purified materials with over 95% purity. HSCCC thus provides an attractive alternative to HPLC for the semi-preparative scale separation and purification of bioactive components in herbal extracts. A systematic comparison of the two techniques in terms of separation efficiency, cost and capacity is on-going in this laboratory.

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