Journal of Chromatography A, 1218 (2011) 6191–6199

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Preparative isolation of novel antioxidant flavonoids of alfalfa by stop-and-go counter-current chromatography and following on-line liquid chromatography desalination

Junling Liang[a], Zhi Yang[a], Xiaoji Cao[b], Bing Wu[c], Shihua Wu[a,*]

[a] Research Center of Siyuan Natural Pharmacy and Biotoxicology, College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang Province 310058, China
[b] Research Center of Analysis and Measurement, Zhejiang University of Technology, 18 Chaowang Rd, Hangzhou, Zhejiang Province 310014, China
[c] College of Pharmaceutical Science, Zhejiang University, Hangzhou, Zhejiang Province 310058, China

ARTICLE INFO

Article history:
Available online 30 October 2010

Keywords:
Alfalfa
Antioxidant
Counter-current chromatography
Flavonoids
Salting-out
Stop-and-go two-dimensional liquid chromatography

ABSTRACT

In this work, we have established a new stop-and-go two-dimensional chromatography coupling of counter-current chromatography and liquid chromatography (2D CCC × LC) for the preparative separation of two novel antioxidant flavonoids from the extract of alfalfa (*Medicago sativa* L.). The CCC column has been used as the first dimension to purify the target flavonoids using a solvent system of isopropanol and 20% sodium chloride aqueous solution (1:1, v/v) with the stop-and-go flow technique, and the LC column packed with macroporous resin has been employed as the second dimension for on-line absorption, desalination and desorption of the targeting effluents purified from the first CCC dimension. As a result, two novel flavonoids, 6,8-dihydroxy-flavone-7-O-β-D-glucuronide (15.3 mg) and 6-methoxy-8-hydroxy-flavone-7-O-β-D-glucuronide (13.7 mg), have been isolated from 126.8 mg of crude sample pre-enriched by macroporous resin column. Their structures have been identified by electrospray ionization mass spectrometry (ESI-MS), electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) and one- and two-dimensional nuclear magnetic resonance spectra (1D and 2D NMR). Further antioxidant assays showed that the first component possess a strong antioxidant activity. All the results demonstrated that the stop-and-go 2D CCC × LC method is very efficient for the separation of flavonoids of alfalfa and it can also be applied to isolate other comprehensive multi-component natural products.

1. Introduction

Flavonoids, also called bioflavones, are a diverse group of more than 7500 secondary products found throughout the plant kingdom, and accordingly, several major classes have been described to accommodate the enormous number of different structures, such as anthocyanins, flavonones, flavones, flavonols and the isoflavonones [1–3]. They are well known for their various biological activities, such as free radical-scavenging antioxidant activities, metal-ion-chelating activities, anti-tumor or antimitotic activities, and inhibition of a variety of enzymes [4].

Because of their potent biological activities, a series of separation methods, such as counter-current chromatography (CCC), and high-performance liquid chromatography, have been developed for the isolation and purification of flavonoids with different structural groups from a large number of plant resources [5,6]. HPLC can afford a sufficient resolving power for the separation of components using many kinds of solid support matrix while CCC may provide an efficient resolution with a prominent advantage of the support-free liquid–liquid partition chromatography, eliminating irreversible adsorptive loss of samples onto the solid support matrix used in conventional chromatography. Thus CCC method has been accepted and used widely in the analysis and separation of various complexes especially for natural products [7,8]. In addition, the separation efficiency of CCC has been win many straight improvements with the advancements of a series of new CCC assays and apparatus [9–17] for recent years.

Besides the above one-dimensional chromatography, two- or multi-dimensional chromatography can provide more sufficient resolving power for the separation of components in many complex natural samples [18]. Multi-dimensional chromatography is based on the combination among different methods with various mechanisms coupled with different detection mechanisms, such as normal phase, reversed phase, gas chromatography, capillary electrophoresis, size exclusion, ion exchange, affinity chromatography, UV and mass spectrometry [19]. Typically, two-dimensional liquid chromatography (2D-LC) is the most popular method in multi-dimensional chromatography and it has played important roles in

* Corresponding author. Tel.: +86 571 88206287; fax: +86 571 88206287.
E-mail addresses: drwushihua@hotmail.com, drwushihua@zju.edu.cn (S. Wu).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.10.092

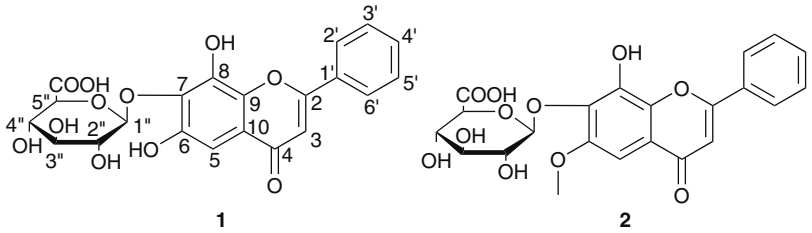


Fig. 1. Chemical structures of the isolated flavonoids. **1**, 6,8-dihydroxy-flavone-7-O-β-D-glucuronide; **2**, 6-methoxy-8-hydroxy-flavone-7-O-β-D-glucuronide.

the separation of various complex samples recently [20]. Comprehensively, 2D-LC implemented by coupling two separations in time presents three available schemes: on-line; stop-and-go; and off-line [19,20] in areas of pharmaceutics, natural products, proteomics analysis, food analysis, and so on [21–26].

Recently, we developed a new hybrid two-dimensional chromatography by on-line coupling of CCC and LC [27]. It is very efficient for high-throughput purification of the one-target component of arctiin from the water extract of *Arctium lappa* L. However, as similar to classical on-line 2D-LC, this system requires that the second-dimensional analysis be completed during the time needed to collect the fraction, transfer and analyze it, and restore the column to the initial conditions of the analysis. This constraint the second-dimension separation to be completed in what is typically a very short amount of time, resulting in a limited separation power and being suitable only for the one-target separation [20]. Therefore, the stop-and-go technology was employed into this system to solve the limitation in operation time for multiple targeting components.

The stop-and-go scheme [20] involves stopping or pausing elution from the first-dimension column while a fraction is transferred to and analyzed on the second-dimension column, and then resuming the elution in the first-dimension. This somewhat alleviates the time constraints of the second-dimension, and provides good chances for separation of more comprehensive components. In addition, recent study [28] indicated that the controlled-cycle CCC process having two individually time periods: flow period and delay period, can receive higher resolution than common CCC operation and this suggests that stop-and-go CCC may overcome some defects in common solid supported two dimensional chromatography, such as the decrease of the separation efficiency caused by axial diffusion of the peaks during their parking time [20].

Therefore, the purpose of this work is to develop a new stop-and-go 2D CCC × LC protocol for the separation of natural antioxidant flavonoids (Fig. 1) from the water extracts of alfalfa (*Medicago sativa* L.). It is well known that alfalfa is thought as "father of all food", and for centuries it has been grown and used as feed for livestock in the form of green feed, hay, or pellets. Moreover, alfalfa sprouts have been widely consumed by humans as garnish and leaf protein concentrates and the dehydrated plant are usually used as the components of many nutritional supplement products. Additionally, alfalfa also contains numerous bioactive secondary metabolites including a large number of flavonoids [29–31] and saponins [32]. Although numbers of chromatographic methods have been used for the separation of components in alfalfa, to the best of our knowledge, this is a first document to demonstrate the application of the stop-and-go CCC × LC separation system for the isolation and purification of flavonoids from the alfalfa.

2. Experimental

2.1. Apparatus

The CCC instrument employed in the present study is a TBE-300A high-speed CCC (Tauto Biotech. Co., Ltd., Shanghai, China) with three multilayer coil separation columns connected in series (I.D. of the tubing, 1.8 mm; total column volume, 260 mL, and extra volume, 10 mL). The instrument is equipped with a 20 mL sample loop and two six-port valves (valves I and II). The CCC instrument revolution radius is 5 cm, and the β values of the multilayer coil varies from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. A TC 1050 constant-temperature circulating instrument (Tauto Biotech. Co., Ltd., Shanghai, China) has been used to control the separation temperature. In addition, this CCC system is equipped with two gradient systems consisting of four P270 metering pumps and two gradient controller, two UV 230$^+$ spectrometers (Elite Analytical Instrument Co., Ltd., Dalian, China), a BSZ-100 fraction collector and an EC2000 ChemStation (Elite Analytical Instrument Co., Ltd., Dalian, China).

An Agilent 1100 system HPLC has been used for the analysis of the crude extract and a number of fractions. It was equipped with a G1379A degasser, a G1311A QuatPump, a G1367A Wpals, a G1316A column oven, a G1315B diode assay detector (DAD), and an Agilent ChemStation for LC.

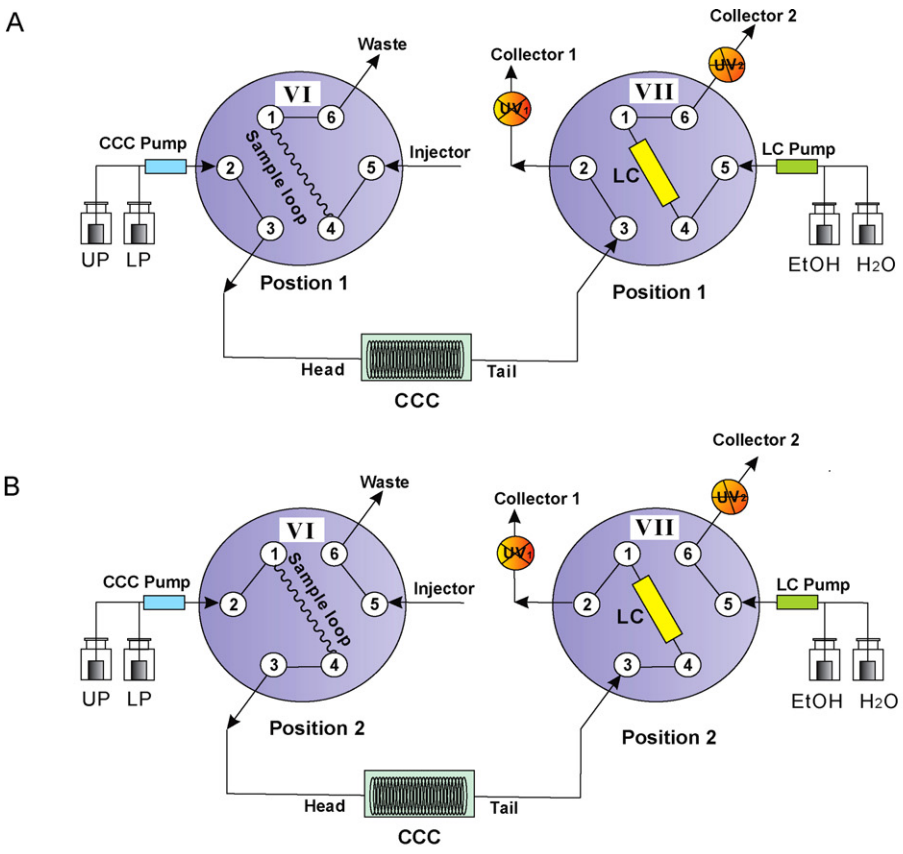
2.2. Reagents

All organic solvents used for CCC were of analytical grade and purchased from Huadong Chemicals, Hangzhou, China. The water was purified by a water purifier (18.2 MΩ) (Wanjie Water Treatment Equipment Co., Ltd., Hangzhou, China) and used for all solutions and dilutions. Methanol used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt, Germany. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Chemicals Co. (St. Louis, MO). Vitamin C and butylated hydroxytoluene (BHT) was purchased from Huadong Chemicals, Hangzhou, China.

The water extract of alfalfa was obtained as a gift sample from Qianjiang drying Co. Ltd., Hangzhou, China, which was a commercial product named "Mucaosu" and it was used widely as animal feed additive for chicken and pigs [33].

2.3. Enrichment of flavonoids from the crude water extract by macroporous resin

25.16 g of the crude water extract of alfalfa was first dissolved in 1 L of water. After centrifugation (3000 rpm) for 15 min, the insoluble components were removed and the supernatant liquid was subjected to the top of a column (1000 mm length × 48 mm I.D.) packed with HP-20 resin. After loading the sample, the column was eluted step-by-step with water, 30%, and 95% ethanol aqueous solutions. As a result, the fraction eluted by 95% ethanol aqueous solution was found to contain the prominent flavonoids and thus it was collected as a sample fraction for further CCC separation.



C

Step	Position		Status			Pumped liquid	
	Valve I (VI)	Valve II (VII)	CCC apparatus	CCC pump	LC pump	CCC column	LC column
1. Preparation & CCC elution							
(1) Pumping SP & Loding sample	1	1	Ready	Run	Ready	SP[a]	
(2) Injecting sample & CCC run	2	1	Run	Run	Run	MP	water
2. Elute the first targeting peak							
(1) CCC elution & LC absorption	2	2	Run	Run	Ready	MP	
(2) LC desalination	2	1	Run	Stop	Run		water
(3) LC desorption	2	1	Run	Stop	Run		Ethanol
(4) LC washing	2	1	Run	Stop	Run		Ethanol
(5) LC equilibrium	2	1	Run	Stop	Run		Water
3. Elute the other component	2	1	Run	Run	Run	MP	ready
4. Elute the last targeting peak							
(1) CCC elution & LC absorption	2	2	Run	Run	Ready	MP	
(2) LC desalination	2	1	Run	Run	Run	MP	water
(3) LC desorption	2	1	Run	Run	Run	MP	Ethanol
(4) LC washing	2	1	Run	Run	Run	MP	Ethanol
(5) LC equilibrium	2	1	Run	Run	Run	MP	Water
5. Extrude last other components							
(1) CCC extrusion & LC washing	2	1	Stop	Run	Run	Ethanol	Ethanol
(2) End	1	1	Stop	Stop	Stop		

[a] SP, stationary phase. MP, mobile phase.

Fig. 2. Scheme of the stop-and-go CCC × LC system coupling between two UV detectors, including two six-port switching valves (V I, and V II). CCC column in the first separation dimension; and macroporous resin column (LC) in the second separation dimension. (A) System configuration in position 1 of valves, (B) system configuration in position 2 of valves and (C) step-by-step system settings for stop-and-go 2D CCC-LC separation.

2.4. The phase diagram analysis of isopropanol/salt-containing aqueous two-phase system

The phase diagram was made by a simple phase transition method as reported [34] with minor revisions. In brief, the selected salt (sodium chloride, NaCl or ammonium sulfate, $(NH_4)_2SO_4$) with different weight were first dissolved into the equal volume of water to form the salt aqueous solutions with the desired concentrations, such as 3%, 5%, 10%, 15%, 20%, 25%, 30% and 35%. And then isopropanol was subsequently added drop by drop into each tube containing equal volume of salt aqueous solution with the different concentrations on an electric balance for measuring the added isopropanol. After each droplet, the mixture was shaken for 2 min on a vortex mixer. When a new phase emerged in the mixture after mixing and when one more drop salt aqueous solution was added back into the mixture, the produced new phase disappeared, and

the point was the phase transition point, in which two-phase began to form. After this, the volume of new phase got bigger with the increase of the volume of the added isopropanol. The total weight of the added isopropanol was measured accurately and the volume of the isopropanol was calculated and the phase diagram curves were plotted.

2.5. Determination of partition coefficients for the selection of the two-phase system

The two-phase solvent system is selected mainly according to the partition coefficient (K) of each target component, settling time and phase ratio of a system. Based on the data of the phase diagram analysis, several two-phase solvent systems composed of isopropanol/salt aqueous solutions with two-phase volume ratio close to 1 were selected. And then the partition coefficients (K) for the target flavonoids were determined by HPLC analysis as follows: a small amount (1 mg) of the crude sample was dissolved into equal volumes (800 μL) of aqueous phase (lower phase) and organic phase (upper phase) of the thoroughly equilibrated two-phase solvent system in a 2 mL test tube. After the equilibration was established, both the solutions of upper phase and lower phase were directly analyzed by HPLC and the peak area of each component in the upper phase and lower phase was recorded as A_1 and A_2, respectively. The partition coefficient (K) was then calculated by the following equation:

$$K = \frac{A_1}{A_2}$$

2.6. Preparation of two phase solvent and sample solution

Hydrophilic organic/salt-containing aqueous two-phase systems were prepared by thoroughly mixing the desired amount of isopropanol with inorganic salt solutions in a separatory funnel at room temperature, allowing the two clear phases to form. The two phases were separated shortly before use and degassed in an ultrasonic water bath for 10 min.

The CCC sample solutions were prepared by dissolving the sample fraction obtained by 95% ethanol in Section 2.3 in a solvent mixture consisting of equal volumes of both upper and lower phases at a suitable concentration according to the preparative scale of CCC separation.

2.7. Classical CCC separation procedure

The CCC separation used a typical mode of "injection after equilibrium". In brief, the column was first filled with the upper phase as stationary phase, and then the apparatus was rotated at 850 rpm, and the lower phase as mobile phase was pumped through the column at a flow-rate of 1.5 mL/min from the head end of the column to the tail end at the temperature of 30 °C. When a hydrodynamic equilibrium was established in the column and the mobile phase started emerging in the effluent, 5 mL of the sample solution containing 126.8 mg of the enriched fraction was injected through the injection valve. The effluent was monitored by a DAD detector at 280 nm and automatically collected into 20 mL test tube per 7 min using a BSZ-100 fraction collector. Peak fractions were collected according to the elution profile and analytical HPLC detection.

2.8. Stop-and-go 2D CCC × LC system

As shown in Fig. 2, the present stop-and-go 2D CCC × LC system configuration is similar with the on-line CCC × LC system [27] except for some improvements. In brief, the current system is equipped with two gradient pump systems for pumping independently/dependently upper phase and lower phase of the solvent system used for CCC separation, and ethanol and water for LC column absorption, desalination and desorption without frequent change of solvent bottle (Fig. 2A and B). In addition, several time-dependent system configurations have been designed according to the elution sequence of targeting components as shown in Fig. 2C. A representative stop-and-go CCC × LC has been performed as following:

CCC pumps delivered desired upper or lower phase of selected two-phase solvent system for the first dimension of CCC separation. When the first CCC peak containing the first targeting flavonoid emerged, the effluent was switched to the second dimension of LC column (400 mm length × 10 mm I.D.) packed with macroporous resin through switching the second 6-port valve II to position 2. After all effluents of the first CCC peak have been eluted and adsorbed on the macroporous resin column, the CCC is kept on the same rotation while the CCC pump is stopped. Meanwhile, the valve II was switched back to position 1 and LC pumps began to deliver water and 95% ethanol aqueous solution to desalination and desorption of the first targeting flavonoid step-by-step.

Once the first desorption has finished, the CCC pumps were started again at the same flow rate and the other components were eluted successively. When the second CCC peak emerged, the effluent was switched again to the second dimension of LC column through the same valve switch. Then the LC column adsorbing the second targeting flavonoid underwent the same desalination and desorption processes performed by the elution of water and ethanol step-by-step while the CCC pumps were stopped until the desorption process was finished. All effluents were monitored by UV detector at 280 nm and automatically collected into test tube using a BSZ-100 fraction collector.

When each desorption on the second dimension finished, the LC pump delivered water (more than one bed volume) to equilibrate the second dimension LC column, and then the second dimension column got ready for the next effluent from the first dimension. Peak fractions were collected according to the elution profile and analytical HPLC detection. The 95% ethanol desorbed fractions of per CCC peak have been combined and collected respectively as purified targeting flavonoids. The obtained peak fractions with more than 98% purity were collected and used as reference substances for further quantitative HPLC analysis.

2.9. Qualitative and quantitative HPLC analysis

Qualitative and quantitative analysis of flavonoids and several extracts or fractions have been performed on a reversed-phase Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm I.D., 5 μm) with a guard column (10 mm × 4.6 mm I.D., 5 μm). The mobile phase used was methanol (A) and 0.03% trifluoroacetic acid (TFA) aqueous solution (v/v) (B) in a linear gradient mode as follows: A from 30% to 70% and B from 70% to 30% during 0–20 min. The flow-rate of the mobile phase was 1.0 mL/min and the effluents were monitored at 280 nm by a DAD detector. The column temperature was kept at 30 °C.

For quantitative analysis of flavonoids, the working calibration curve based on the reference substances obtained by CCC has been measured. The reference substances were first dissolved in dimethyl sulfoxide (DMSO) to form the stock solution (1 mg/mL) and then diluted to a series of desired concentrations of 5, 10, 20, 40, 80 and 100 μg/mL.

The peaks of the HPLC profile were identified by comparing their retention time with that of each standard substance which was eluted in parallel with a series of mobile phases. In addition, spiking samples with the reference substances further confirmed the identities of the peaks. All samples were centrifuged for 20 min, and 10 μL of supernatant were injected for HPLC analysis.

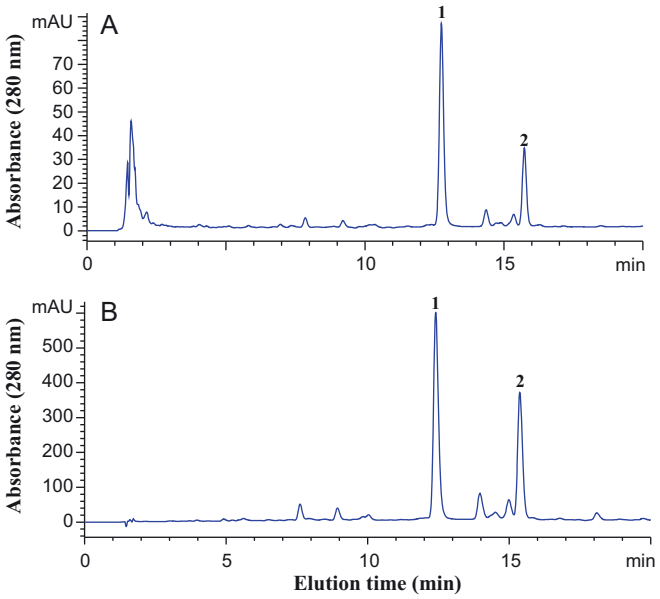


Fig. 3. HPLC analysis of (A) crude water extract of alfalfa and (B) the pre-enriched fraction eluted with 95% ethanol by HP-20 macroporous resin column chromatography. The analysis was performed on a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm I.D., 5 μm) with a guard column (10 mm × 4.6 mm I.D., 5 μm) using a mobile phase of methanol–0.03% TFA in a linear gradient mode as follows: methanol from 30% to 70% and B from 70% to 30% at 0–20 min. The flow-rate of the mobile phase was 1.0 mL/min and the effluents were monitored at 280 nm by a DAD detector. The column temperature was kept at 30 °C.

2.10. Structural identification of the isolated flavonoids

Identification of the CCC fractions was carried out by electrospray ionization mass spectrometry (ESI-MS), electropray ionization time-of-flight mass spectrometry (ESI-TOF-MS), one- and two-dimensional nuclear magnetic resonance spectra (1D- and 2D-NMR) including ^{1}H NMR, ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT) 135, ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple band coherence (HMBC). Positive ESI-MS analysis was performed using a Thermo Finnigan LCQ Deca XP Electrospray-Ion Trap-Mass Spectrometer. Positive high-resolution ESI-TOF-MS analysis was employed an Agilent 6210 Series LC/MSD Time-of-Flight Accurate-Mass spectrometers. 1D- and 2D-NMR experiments were carried out using a Bruker Advanced DMX 500 NMR spectrometer with DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard.

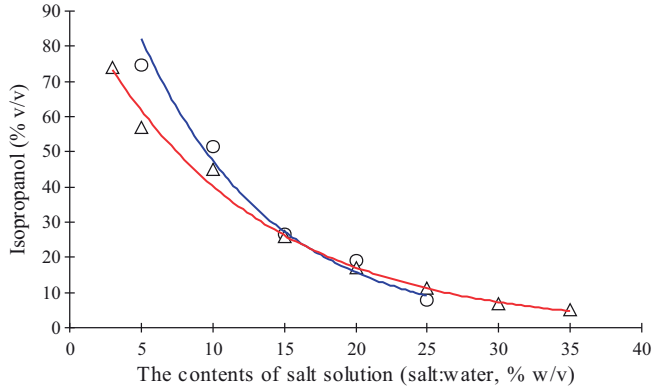


Fig. 4. Phase diagram of isopropanol/system. (○) Isopropanol/NaCl and (△) isopropanol/$(NH_4)_2SO_4$.

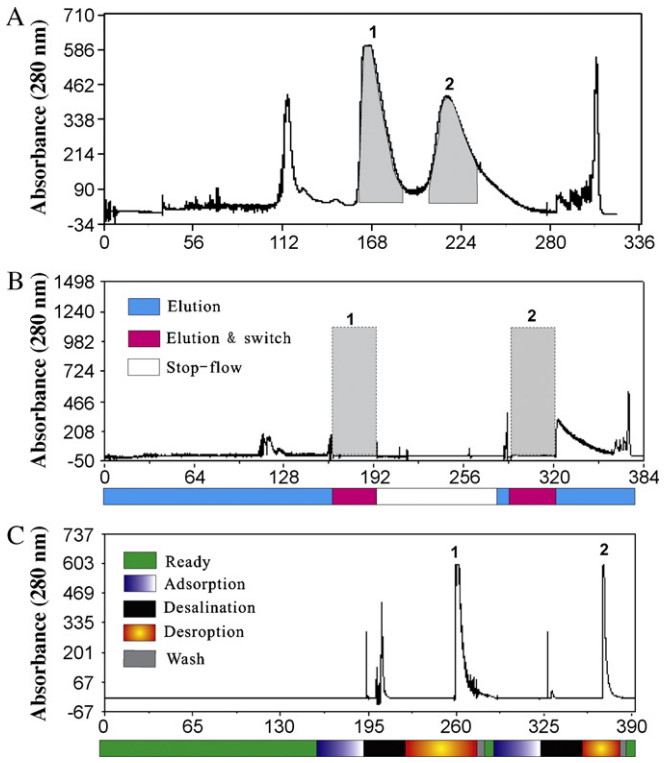


Fig. 5. (A) The classical CCC purification and (B and C) the stop-and-go 2D CCC-LC profiles of alfalfa sample. **1**, 6,8-dihydroxy-flavone-7-O-β-D-glucuronide; **2**, 6-methoxy-8-hydroxy-flavone-7-O-β-D-glucuronide. Conditions: (i) (A and B) CCC conditions: solvent system: isopropanol–20% NaCl aqueous solution (1:1, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; column temperature: 30 °C; flow rate: 1.5 mL/min; rotational speed: 850 r/min; detection wavelength: 280 nm; sample size: 126.8 mg; injection mode: injection after equilibrium; injection volume: 5 ml. (ii) Valve switching time: 160–190 min for the flavonoid (**1**) and 290–320 min for the flavonoid (**2**). (iii) (B) CCC status: 0–190 min, elution with the flow rate of 1.5 mL/min; 190–280 min, CCC stop-flow; 280–380 min, elution. (iv) (C) LC status: 0–160 min, column ready including the first washing and later equilibrium by water before adsorption; 160–190 min, adsorbing the flavonoid (**1**) on the column; 190–210 min, elution with water to remove the salt in the effluents of CCC; 210–270 min, desorption by 95% ethanol; 270–280 min, washing by 95% ethanol; 280–290 min equilibrium by water; 290–320 min, adsorption of the flavonoid (**2**); 320–340 min, elution with water to desalt; 340–380 min, desorption by 95% ethanol.

2.11. DPPH radical scavenging activity of isolated phenolic compounds

The DPPH radical scavenging activity of the isolated compounds was evaluated as the analytical protocols of von Gadow et al. [35] and Huang et al. [36] with minor modifications. Briefly, 2 mL of DPPH (79.36 μg/mL) in methanol was added into 2 mL of sample solution of the isolated flavonoids. After incubation for 30 min at 25 °C, the absorbance of the reacted mixture was measured at 515 nm by a UV spectrophotometer. The percent radical scavenging activity was determined by comparison with a DMSO-containing control prepared without adding flavonoids and calculated by the following equation:

$$E(\%) = \frac{A_C - (A_X - A_{X0})}{A_C} \times 100$$

where A_C is the absorbance of the control unreacted mixture without the tested compounds, A_X is the absorbance of the reacted mixture containing the tested compounds, and A_{X0} is the absorbance of the unreacted mixture of sample and DMSO. EC_{50} values represent the concentration that causes a decrease in the initial DPPH concentrations by 50% and are expressed as means

Table 1
Partition coefficient of flavonoids of selected solvent system for the CCC separation.

No.	Solvent system	Phase ratio (upper:lower, v/v)	Settling time (s)	Partition coefficient (K) of flavonoids	
				1	**2**
Ammonium sulfate–isopropanol					
1	15%[a], 2:1[b]	1:1	39	6.15	7.63
2	15%, 5:5	1.2:0.8	15	26.07	49.25
3	35%, 6:4	0.9:1.1	16	46.51	89.84
Sodium chloride–isopropanol					
4	15%, 5:5	1:1	18	1.09	1.24
5	15%, 3:5	1.5:0.5	10	1.02	1.32
6	20%, 5:5	1:1	8	1.05	1.42

[a] The concentration of salt aqueous solution (salt:water, w/v).
[b] Volume ratio of salt-containing aqueous solution and isopropanol.

(standard deviation of three separate experiments). Vitamin C and BHT were used as positive controls.

3. Results and discussion

3.1. HPLC analysis of the crude water extract

Using the acidic methanol–TFA aqueous solution as mobile phase, the components of crude water extract of alfalfa can be well resolved. As shown in Fig. 3A, although numbers of components such as flavonoids [29–31] and saponins [32] have been found in the alfalfa, the HPLC analysis showed that the present commercial water extract of alfalfa had only a few major absorbance peaks at 280 nm. This may be attributed to two major reasons: one is that strong polarity of water as extracting solvent results in the minor solubility of low polarity of components with high UV absorbance, and the other reason is for the low UV absorbance of components in the extract. For example, a large number of reported saponins [32] showed very weak absorbance at less than 220 nm, and almost no absorbance at 280 nm.

For quantitative analysis of flavonoids (**1** and **2**), the working calibration curve based on the reference substances obtained by CCC was measured and showed a good linearity over the range of 0.005–0.1 mg/mL for the two flavonoids. The regression lines for two flavonoids (**1** and **2**) were $y_1 = 18.1150x_1 - 7.4789$ ($R^2 = 0.9996$, $n = 5$) and $y_2 = 8.2868x_2 - 1.8851$ ($R^2 = 0.9999$, $n = 5$), respectively, where y was the peak area of flavonoids while x was the concentration (μg/mL). As a result, the contents of favonoids **1** and **2** in the crude water extract of alfafa were about 12.6 mg/g and 10.0 mg/g, respectively.

3.2. Enrichment of flavonoids from crude water extraction by macroporous resin

Macroporous resin column chromatography has been widely used for absorption and enrichment of numbers of natural products, especially some hydrophilic flavonoids and glycosides [37,38]. After scheduled screening of appropriate macroporous resins by comparing their static and dynamic absorption capacities with each other, the HP-20 resin was selected as the most suitable for absorption of the two flavonoids. As shown in Fig. 3B, two flavonoids could be well absorbed on the HP-20 macroporous resin until 95% ethanol was used as desorption solvent. Quantitative analysis showed that the contents of two flavonoids (**1** and **2**) in the fraction of 95% ethanol were 132.7 mg/g and 103.5 mg/g, respectively, which was 10-fold higher than that in crude extract. Clearly, macroporous resin column chromatographic purification is an efficient step for the enrichment of bioactive flavonoids and may be introduced into industrial plants to produce larger amount of feed additives.

3.3. Selection of suitable two-phase solvent system

Although multi-component organic/aqueous solvent systems, such as hexane-ethyl-acetate–methanol/ethanol–water, chloroform–methanol–water and ethyl acetate-*n*-butanol–water, have won popular applications [7], the one-component organic/salt-containing aqueous solution system showed some competitive advantages, such as low cost for use and reused application as well as the relatively low environmental toxicity [27]. In this work, two one-component/salt-containing solvent systems have been investigated. Isopropanol is another promising solvent besides ethanol and widely used as solvent or extract agent for food, commodity and chemical engineering products. Here we evaluated the feasibility of the two solvent systems of isopropanol/NaCl and isopropanol/$(NH_4)_2SO_4$ for separation of flavonoids in alfalfa.

As shown in Fig. 4, the volume ratio of isopropanol decreased with the increase of the content of both NaCl and $(NH_4)_2SO_4$. In addition, the settling time of formed two-phase also decrease with the increase of the content of salt. Thus we attend to choose the systems with high salt contents for saving the amounts of organic solvent used. However, it should be noted that higher salt contents decrease the usable volume range of isopropanol due to the formation of the precipitate. Thus several solvent systems with short settling time and good two-phase ratio close to 1 have been selected to measure the partition coefficients of flavonoids.

Table 2
MS data of the isolated flavonoids.

	Flavonoids	
	1	**2**
Molecular weight	446	460
Molecular formular	$C_{21}H_{18}O_{11}$	$C_{22}H_{20}O_{11}$
ESI-MS$^-$	445 $[M-H]^-$, 891 $[2M-H]^-$	459 $[M-H]^-$, 919 $[2M-H]^-$
MS^2	445 (60%), 269 (100%), 175 (20%)	459 (100%), 416 (10%)
ESI-MS$^+$	447 $[M+H]^+$, 915 $[2M+Na]^+$	461 $[M+H]^+$
MS^{2+}	447 (20%), 271 (100%)	461 (10%), 285 (100%)
MS^{3+}	271 (15%), 253 (100%), 225 (50%), 169 (30%), 123 (20%), 103 (10%)	
ESI-TOF-MS		
Ion formular	$C_{21}H_{19}O_{11}$ $[M+H]^+$	$C_{22}H_{21}O_{11}$ $[M+H]^+$
Cal m/z	447.0927	461.1084
m/z	447.0921	461.1083
Error	1.42	0.19
Ion formular	$C_{21}H_{18}O_{11}Na$ $[M+Na]^+$	$C_{22}H_{20}O_{11}Na$ $[M+Na]^+$
Cal m/z	469.0747	483.0903
m/z	469.0737	483.0895
Error	2.09	1.72

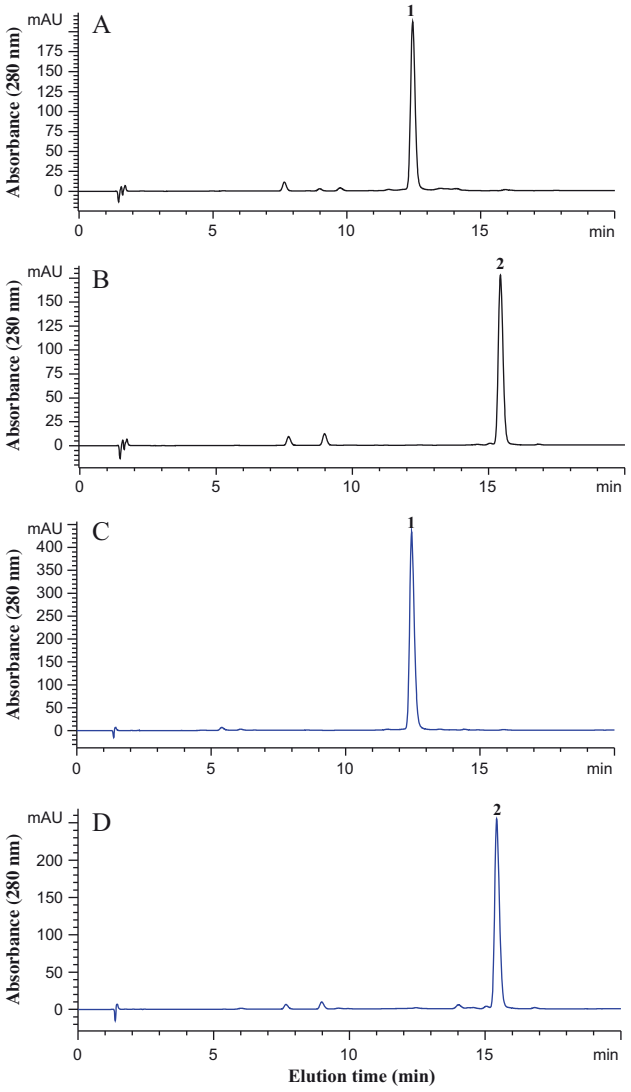


Fig. 6. HPLC analyses of the flavonoids purified by (A and B) the classical CCC and (C and D) the stop-and-go 2D CCC-LC. **1**, 6,8-dihydroxy-flavone-7-O-β-D-glucuronide; **2**, 6-methoxy-8-hydroxy-flavone-7-O-β-D-glucuronide. HPLC analytical conditions as described in Fig. 3 or Section 2.9. Purity: (A) 93.2%, (B) 90.3%, (C) 96.8 and (D) 90.8%.

As shown in Table 1, in the selected systems of isopropanol/$(NH_4)_2SO_4$, two flavonoids are major distributed in upper phase while only minor amount was found in lower phase, which may be due to that large water distributed into the upper isopropanol phase which resulted in the high solubility of two flavonoids in the upper phase. By contrast, the two phases of the selected systems of isopropanol/NaCl showed almost equal properties resulting in the good partition coefficients (in the range of 0.5–2) for two flavonoids. Thus, the system of isopropanol/NaCl is acceptable for CCC separation. Taking accounts of the separation factor of targeting flavonoids, such as settling time and volume ratio of two phases, we selected the system composed of isopropanol and 20% NaCl aqueous solution (1:1, v/v) as the solvent system for further CCC separation.

3.4. Classical CCC separation

Using the optimized solvent system, the classical CCC separation has been performed. Fig. 5A shows a typical separation. Clearly, two major flavonoids have been well resolved and peak

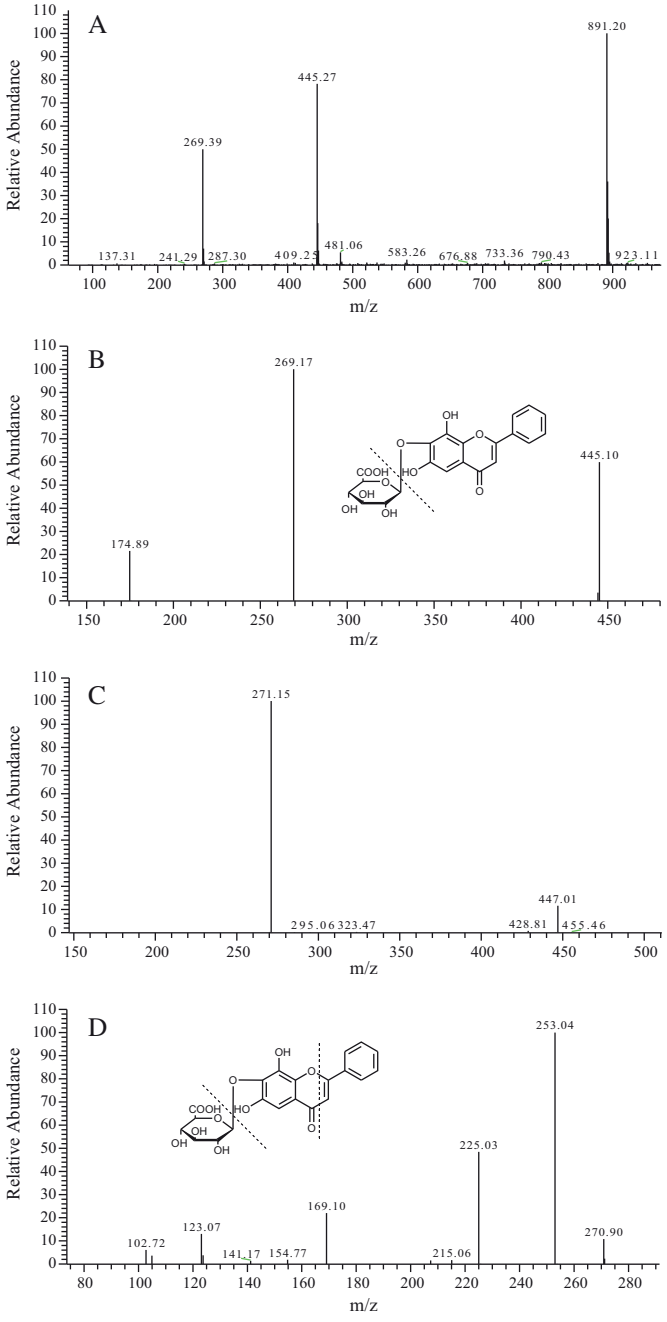


Fig. 7. The ESI-MS^n spectra of the isolated flavonoid 6,8-dihydroxy-flavone-7-O-β-D-glucuronide (**1**). Negative (A) ESI-MS and (B) ESI-MS^2, and positive (C) ESI-MS^2 and ESI-MS^3 (m/z 447 → 271 →).

1 has the retention time at 175 min with the peak width of 30 min (160–190 min) while the peak **2** has the retention time at 215 min with the peak width at 200–230 min. The effluents between the two peaks (**1** and **2**) were found to be a mixture of the two targeting flavonoids but no other components. The HPLC analysis showed that the two flavonoids fractions obtained by CCC both have a purity of more than 90% at 280 nm, except for NaCl (Fig. 6A and B).

3.5. Stop-and-go 2D CCC × LC separation

As described above, although two flavonoids could be purified by off-line or classical CCC separation, the subsequent process, such as back-extraction of organic solvent or further off-line

Table 3
One- and two-dimensional NMR data of the isolated flavonoids (in DMSO).

Atom	**1**					**2**	
	δ_C (ppm)	DEPT[a]	HMQC δ_H (ppm)	HMBC correlation with	^{1}H-^{1}H COSY correlation with	δ_C (ppm)	δ_H (ppm)
2	164.03	q		H-2′, H-6′, H-3		164.05	
3	105.25	CH	6.98 (1H, s)			105.75	6.70 (1H, s)
4	183.06	q[b]		H-5, H-3		182.89	
5	94.26	CH	7.04 (1H, s)			99.26	7.06 (1H, s)
6	131.34	q		H-5		131.24	
7	151.82	q		H-5, H-1″		156.68	
8	147.25	q				149.68	
9	149.70	q		H-5		156.50	
10	106.61	q		H-5, H-3		106.80	
1′	131.11	q		H-3′, H-5′, H-3		129.69	
2′, 6′	126.88	CH	8.06 (2H, d, J = 7.00 Hz)	H-6 and H-4′ for C-2′, H-2′ and H-4′ for C-6′	H-3′ for H-2′, H-5′ for H-6′	126.93	8.08 (2H, d, J = 6.75 Hz)
3′, 5′	129.68	CH	7.57 (2H, d, J = 6.17 Hz)	H-5′ for C-3′, H-3′ for C-5′	H-2′ and H-4′ for H-3′, H-6′ and H-4′ for H-5′	129.80	7.61 (2H, d, J = 7.15 Hz)
4′	132.56	CH	7.61 (1H, s)	H-2′, H-6′	H-3′, H-5′	132.78	7.62 (1H, s)
1″	100.47	CH	5.18 (1H, d, J = 7.30 Hz)	H-2″, H-3″	H-2″	100.28	5.23 (1H, d, J = 5.85 Hz)
2″	73.30	CH	3.50 (1H, m)	H-3″	H-1″, H-3″	73.46	3.50 (1H, m)
3″	75.88	CH	3.36 (1H, m)	H-5″, H-4″, H-2″	H-2″, H-4″	76.43	3.38 (1H, m)
4″	71.87	CH	3.45 (1H, m)	H-5″, H-3″	H-3″, H-5″	71.97	3.45 (1H, m)
5″	75.78	CH	4.04 (1H, d, J = 9.60 Hz)	H-4″	H-4″	75.36	4.0 (1H, m)
6″	170.72	q		H-5″		171.09	
OCH_3						61.90	3.84 (3H, s)

[a] DEPT 135 experiments.
[b] Quaternary carbon.

column chromatography purification, removing salt in the targeting flavonoids fractions obtained by CCC, are still tedious and labor-consuming. Thus a stop-and-go 2D CCC × LC system was established. Fig. 2 shows a representative configuration of the system. After scheduled operations by switching valves I and II, two target flavonoids (**1** and **2**) were purified without more options, such as the extra processes of desalination, desorption, collection and HPLC analysis.

As shown in Fig. 5B and C, after injecting the sample, the CCC first underwent a classical elution. Once the first target flavonoid (**1**) emerged, the CCC effluents (between 160 and 190 min) were switched on to the LC column, on which, the effluents were adsorbed, desalted by water and desorbed by 95% ethanol, and meanwhile the flow-rate of CCC was zero. After desorption of flavonoid (**1**) has finished, the LC was washed by ethanol and water successively to the initial state for the adsorption of flavonoid (**2**). Then, CCC pump began to run and eluted the effluents between the peaks 1 and 2 in Fig. 5A until the peak of flavonoid (**2**) emerged. And then the effluent of flavonoid (**2**) (between 290 and 320 min) was switched on to the second dimensional LC column and under the same processes of adsorption, desalination and desorption. As a result, 15.3 mg of flavonoid (**1**) and 13.7 mg of flavonoid (**2**) have been obtained by the stop-and-go CCC × LC system from 126.8 mg of crude sample of alfalfa. HPLC analysis showed that their purity were both more than 90% (Fig. 6C and D).

3.6. Identification of target compounds

The structures of the isolated flavonoids by stop-and-go CCC × LC system have been determined by several spectral analyses. The MS and NMR data have been summarized in Tables 2 and 3.

As shown in Fig. 7 and Table 2, ESI-MS of the flavonoid (**1**) showed that it had prominent ions of $[M-H]^-$ at m/z 445, $[2M-H]^-$ at m/z 891 and $[M+H]^+$ at m/z 447, suggesting that its molecular weight was 446. Further high resolution ESI-TOF-MS analysis gave its molecular formula was $C_{21}H_{18}O_{11}$. The prominent fragments at m/z 269 in the negative ESI-MS^2 and at m/z 271 in the positive ESI-MS^2 was closely corresponding the loss of the uronic acid from the parent molecule. The ^{13}C NMR spectrum (Table 3) showed the presence of 21 carbons, 15 of which corresponded to an aglycon molecule. Similarly, in the ^{1}H NMR spectrum, seven proton signals in the range of 6.97–8.06 corresponded to the aglycon part of the molecule. The remaining five proton signals corresponded to a glucuronic acid molecule. All chemical shifts, and ^{1}H-^{1}H COSY and DEPT spectra suggested that flavonoid (**1**) was a analogue of baicalin, a flavonoid component obtained from *Oroxylum indicum* [39] and *Scutellaria baicalensis* Georgi. However, HMBC spectra (see Supplementary figures for details) markedly indicated that its aglycon molecule was not a baicalein: there were two cross-peaks observed between C-4 (δ 183.06) → H-5 (δ 7.04) and H-3 (δ 6.98), and H-5 also had other cross peaks with C-10 (δ 106.61), C-6 (δ 131.34), C-9 (δ 149.70) and C-7 (δ 151.82) while H-3 had other cross peaks with C-10, C-2 (δ 164.03) and C-1′ (δ 131.11). In addition, the characteristic OH signal was not observed if it is C-5 OH. These evidences suggested that the aglycon had C-8 OH. The position of the sugar was confirmed by HMBC spectra: cross-peaks were observed between H-1″ of the sugar (δ 5.18) and C-7 of aglycon (δ 151.82), and H-5 and C-7 of aglycon. The configuration of the sugar was determined as β by the coupling constant of H-1″ (J = 7.30 Hz). Thus, the structure of **1** was established as 6,8-dihydroxy-flavone-7-O-β-D-glucuronide (Fig. 1).

Compound **2** had similar spectra characteristics. Its ESI-MS spectra (Table 2) showed prominent ions at m/z 459, $[2M-H]^-$ at m/z 919 and $[M+H]^+$ at m/z 461, suggesting that its molecular weight was 460. Further high resolution ESI-TOF-MS analysis gave its molecular formula $C_{22}H_{20}O_{11}$. The prominent fragments at m/z 285 in the positive ESI-MS^2 is closely corresponding the loss of the uronic acid from the parent molecule. The ^{13}C NMR spectrum (Table 3) showed the presence of 22 carbons, 15 of which corresponded to an aglycon molecule with chemical shifts consistent with those obtained for flavonoid (**1**), and 1 of which corresponded to a replacement of a methyl group. Similarly, in the ^{1}H NMR spectrum, seven proton signals in the range of 6.70–8.08 corresponded to the aglycon part of the molecule. The remaining five proton sig-

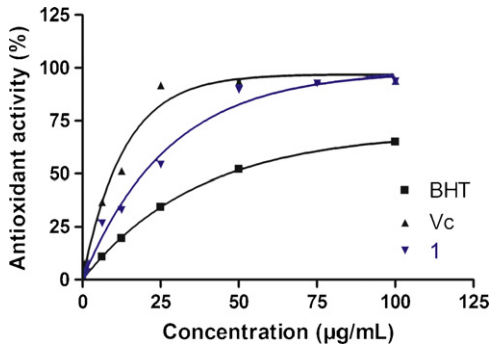


Fig. 8. The DPPH radical scavenging activities of the isolated flavonoids and control sample. **1**, 6,8-dihydroxy-flavone-7-O-β-D-glucuronide, Vc, Vitamin C, and BHT, butylated hydroxytoluene.

nals corresponded to a glucuronic acid molecule. The configuration of the sugar was determined as β by the coupling constant of H-1″ (J = 5.85 Hz). Thus, the structure of **2** was established as 6-methoxy-8-hydroxy-flavone-7-O-β-D-glucuronide (Fig. 1).

3.7. Antioxidant activities of the isolated flavonoids

The H-transfer reactions are monitored by UV–vis spectroscopy recording the decay of the DPPH visible adsorption band (λ = 515 nm in MeOH) that reflected the conversion of the DPPH radical into the corresponding colorless hydrazine (DPPH-H) by the antioxidant. The antioxidant activities of the isolated flavonoids and control standards were shown in Fig. 8. The DPPH radical scavenging capacity of flavonoid (**1**) was markedly stronger than that of a famous antioxidant BHT and a little less than that of vitamin C. The half inhibition percentage (EC_{50}) of these compounds were found to be 19.5, 9.8, 46.8 μg/mL for flavonoid (**1**), Vc and BHT, respectively. Clearly, flavonoid (**1**) is a very promising potent antioxidant. However, the flavonoid (**2**) showed very weak DPPH radical scavenging activity. The antioxidant activity of high concentration flavonoid (**2**) (300 μg/mL) is only up to 15%, which may be due to its structural methylation originated from flavonoid (**1**).

4. Conclusions

In this work, we have developed a new stop-and-go 2D CCC × LC system for the preparative separation of two novel antioxidant flavonoids from the extract of alfafa. Compared with conventional one-dimensional LC or CCC separation, the stop-and-go CCC × LC system provides simpler and more efficient separation without other extra steps to remove salt. In addition, it can also provide a versatile separation for multiple-target components compared with the on-line CCC × LC system developed recently [27] which is suitable for one-target separation. Moreover, all of solvents used in the work are green, low toxicity and environment-friendly. In conclusion, our results indicated that the stop-and-go 2D CCC × LC using a system of isopropanol/20% NaCl aqueous solution (1:1, v/v) is very efficient for the separation of flavonoids of alfalfa and can be applied to isolate the comprehensive multi-component natural products.

Acknowledgements

This work was supported in part by Natural Science Foundation of China (grant nos.: 20602031 and 20972136) and Zhejiang Province (grant no.: Y4080353), Science and Technology Program of Ningbo City (grant no.: 2007C10029), and the Fundamental Research Funds for the Central Universities.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.092.

References

[1] J.B. Harborne, C.A. Williams, Phytochemistry 55 (2000) 481.
[2] C.A. Williams, R.J. Grayer, Nat. Prod. Rep. 21 (2004) 539.
[3] N.C. Veitch, R.E.J. Grayer, Nat. Prod. Rep. (2008) 555.
[4] B.H. Havsteen, Pharmacol. Ther. 96 (2002) 67.
[5] J. Valls, S. Millan, M.P. Marti, E. Borras, L. Arola, J. Chromatogr. A 1216 (2009) 7143.
[6] F.D. Costa, G.G. Leitao, J. Sep. Sci. 33 (2010) 336.
[7] G.F. Pauli, S.M. Pro, J.B. Friesen, J. Nat. Prod. 71 (2008) 1489.
[8] I.A. Sutherland, D. Fisher, J. Chromatogr. A 1216 (2009) 740.
[9] A. Berthod, M.J. Ruiz-Angel, S. Carda-Broch, Anal. Chem. 75 (2003) 5886.
[10] A. Berthod, J.B. Friesen, T. Inui, G.F. Pauli, Anal. Chem. 79 (2007) 3371.
[11] J.B. Friesen, G.F. Pauli, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 2777.
[12] S.H. Wu, L. Yang, Y.A. Gao, X.Y. Liu, F.Y. Liu, J. Chromatogr. A 1180 (2008) 99.
[13] A. Berthod, S. Ignatova, I.A. Sutherland, J. Chromatogr. A 1216 (2009) 4169.
[14] I. Sutherland, P. Hewitson, S. Ignatova, J. Chromatogr. A 1216 (2009) 4201.
[15] L. Chen, Q. Zhang, G. Yang, L. Fan, J. Tang, I. Garrard, S. Ignatova, D. Fisher, I.A. Sutherland, J. Chromatogr. A 1163 (2007) 337.
[16] Y. Yuan, B.Q. Wang, L.J. Chen, H.D. Luo, D. Fisher, I.A. Sutherland, Y.Q. Wei, J. Chromatogr. A 1194 (2008) 192.
[17] H.Y. Ye, S. Ignatova, H.D. Luo, Y.F. Li, A.H. Peng, L.J. Chen, I. Sutherland, J. Chromatogr. A 1213 (2008) 145.
[18] P. Dugo, F. Cacciola, T. Kumm, G. Dugo, L. Mondello, J. Chromatogr. A 1184 (2008) 353.
[19] G. Guiochon, N. Marchetti, K. Mriziq, R.A. Shalliker, J. Chromatogr. A 1189 (2008) 109.
[20] J.N. Fairchild, K. Horvath, G. Guiochon, J. Chromatogr. A 1216 (2009) 1363.
[21] S.P. Dixon, I.D. Pitfield, D. Perrett, Biomed. Chromatogr. 20 (2006) 508.
[22] P.Q. Tranchida, P. Dugo, G. Dugo, L. Mondello, J. Chromatogr. A 1054 (2004) 3.
[23] S.J. Kok, T. Hankemeier, P.J. Schoenmakers, J. Chromatogr. A 1098 (2005) 104.
[24] H.J. Issaq, K.C. Chan, G.M. Janini, T.P. Conrads, T.D. Veenstra, J. Chromatogr. B 817 (2005) 35.
[25] A. Guttman, M. Varoglu, J. Khandurina, Drug Discov. Today 9 (2004) 136.
[26] K. Horvath, J. Fairchild, G. Guiochon, J. Chromatogr. A 1216 (2009) 2511.
[27] M. Guo, J. Liang, S. Wu, J. Chromatogr. A 1217 (2010) 5398.
[28] A.E. Kostanyan, J. Chromatogr. A 1211 (2008) 55.
[29] C. He, Z. Li, W. Gao, J. Tong, Chin. Pharm. J. 41 (2006) 565.
[30] A. Stochmal, S. Piacente, C. Pizza, F. De Riccardis, R. Leitz, W. Oleszek, J. Agric. Food Chem. 49 (2001) 753.
[31] A. Stochmal, A.M. Simonet, F.A. Macias, W. Oleszek, J. Agric. Food Chem. 49 (2001) 5310.
[32] C. He, W. Gao, J. Tong, Chin. Agric. Sci. Bull. 21 (2005) 107.
[33] Z. Lv, Y. Su, G. Zhao, J. Li, J. Li, W. Xu, Zhongguo Siliao Tianjiaji (2010) 1.
[34] T.W. Tan, Q. Huo, Q. Ling, Biotechnol. Lett. 24 (2002) 1417.
[35] A. von Gadow, E. Joubert, C.F. Hansmann, J. Agric. Food Chem. 45 (1997) 632.
[36] D.J. Huang, B.X. Ou, R.L. Prior, J. Agric. Food Chem. 53 (2005) 1841.
[37] B. Fu, J. Liu, H. Li, L. Li, F.S.C. Lee, X. Wang, J. Chromatogr. A 1089 (2005) 18.
[38] G. Jia, X. Lu, J. Chromatogr. A 1193 (2008) 136.
[39] L.J. Chen, D.E. Games, J. Jones, J. Chromatogr. A 988 (2003) 95.