Research article

Phytochemical Analysis

Received: 9 March 2015, Revised: 16 May 2015, Accepted: 18 May 2015 Published online in Wiley Online Library: 23 June 2015

(wileyonlinelibrary.com) DOI 10.1002/pca.2579

Rapid separation of three proanthocyanidin dimers from *Iris lactea* Pall. *var*. *Chinensis* (Fisch.) Koidz by high-speed counter-current chromatography with continuous sample load and double-pump balancing mode

Huanhuan Lv,[a,b] Zhenzhen Yuan,[a,b] Xiaoyan Wang,[a] Zhenhua Wang,[a] Yourui Suo[a] and Honglun Wang[a]*

ABSTRACT:

Introduction – The dried seeds of *Iris lactea* have been used in traditional Chinese medicine. Previous studies have been focused on irisquinones while other chemical components are rarely reported.

Objective – To establish an efficient high-speed counter-current chromatography (HSCCC) separation method with continuous sample load (CSL) and double-pump balancing (DPB) mode to isolate proanthocyanidins from *I. lactea*.

Methods – Firstly, an ethyl acetate extract of *I. lactea* was pre-fractionated by silica column chromatography for the enrichment of proanthocyanidins. Secondly, the enriched proanthocyanidins sample (EPS) was further fractionated by HSCCC with a two-phase solvent system ethyl acetate:*n*-butanol:water (9:1:10, *v*/*v*/*v*) using DPB mode. The flow rate of the two phases was 2.2 mL/min, the revolution speed was 900 rpm, the separation temperature was 30 °C and the detection wavelength was 280 nm. Finally, the structures of the three isolated proanthocyanidins were elucidated by spectroscopic methods and compared with published data.

Results – Under the optimized conditions, 600 mg of the EPS with six continuous injections (100 mg/time) was fractionated, yielding 57 mg of prodelphinidin B3, 198 mg of procyanidin B3, and 162 mg of procyanidin B1, at purities of 97.2%, 98.1% and 97.3%, respectively.

Conclusions – The HSCCC separation method with CSL and DPB proved to be rapid, convenient and economical, constituting an efficient strategy for the isolation of proanthocyanidins.

Keywords: *Iris lactea* Pall. *var. chinensis* (Fisch.) Koidz; proanthocyanidins; HSCCC; continuous sample load; double-pump balancing mode

Introduction

Iris lactea Pall. *var. chinensis* (Fisch.) Koidz, which belongs to the family of Iridaceae, is a herbaceous perennial plant widely distributed in China. The dried seeds of *I. lactea* have the effects of clearing heat, eliminating dampness and stanching bleed. It has been used as a folk medicine for the treatment of jaundice, diarrhoea, leucorrhoea, pharyngitis, inflammation and carbuncle swollen in traditional Chinese medicine (Shen *et al*., 2008). Concerning the chemical constituents of *I. lactea*, previous studies have been essentially focused on its irisquinone components while other chemical components are rarely reported (Wu and Yang, 1980, 1981). The high performance liquid chromatography profile and the chemical-identification reaction of the ethyl acetate extracts have revealed the presence of proanthocyanidins in the seed coats of *I. lactea*. Proanthocyanidins are a group of naturally occurring oligomeric and polymeric flavan-3-ols that have been associated with a broad spectrum of pharmacological effects (Dauer *et al*., 2003; DalBó *et al*., 2006; Fan *et al*., 2007; Yousef *et al*., 2009; Dong *et al*., 2013; Kim *et al*., 2013). Most studies on proanthocyanidins have focused on the more important sources, such as tea, cocoas and grapes (Molan *et al*., 2004; Gürbüz *et al*., 2007; Kothe *et al*., 2013). Before further pharmacological evaluation of the proanthocyanidins from *I. lactea* can take place development of an efficient method for the isolation of pure proanthocyanidins is critical.

High-speed counter-current chromatography (HSCCC) uses no solid matrix and can provide excellent resolution and greater recovery of natural components at lower costs (Wu *et al*., 2012). In recent years, HSCCC has been widely used in the isolation and purification of various kinds of natural products (Ma *et al*., 2010; Jin *et al*., 2013; Han *et al*., 2014; Xia *et al*., 2014). A number of articles have reported the HSCCC separation of proanthocyanidins from various plant sources, such as grape seed and tea extracts (Kohler

* Correspondence to: H. Wang, Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, 23 Xinning Road, Xining 810001, China.
E-mail: hlwang@nwipb.cas.cn

[a] Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, 810001, China

[b] University of Chinese Academy of Sciences, Beijing, 100049, China

et al., 2008; Savitri Kumar *et al*., 2009; Grace *et al*., 2014). However, most of them reported the HSCCC separation of proanthocyanidins associated with the issues of purity and operation time. In addition, preparative HPLC is usually required to make further purification. Hence, a rapid and efficient HSCCC method to separate proanthocyanidins with high yield and purity is desirable

The continuous sample load (CSL) in HSCCC means that after the separation process of the first loaded sample, the second sample is consecutively loaded into the system at an appropriate time without re-establishing hydrodynamic equilibrium and pushing out the solvents, this can reduce the separation time and improve the separation efficiency (Wang *et al*., 2010; Tong *et al*., 2011). In this study, an HSCCC separation method that combines CSL with double-pump balancing (DPB) mode was developed. The normal HSCCC separation usually uses one constant flow pump in the whole separation, and the separation time tends to be relatively long. The DPB mode, which uses two constant flow pumps to respectively pump the upper phase and the lower phase into the HSCCC system at the same time, can largely reduce the separation time.

The objective of this study was to develop an HSCCC method for the separation and purification of three proanthocyanidin dimers (structures are shown in Fig. 1) with high purity from the seed coat extracts of *I. lactea*. Meanwhile, a procedure based on combining CSL and DPB mode was employed in the HSCCC separation of three proanthocyanidins for the first time.

Methods

Apparatus

The HSCCC separation was performed on a TBE-300C HSCCC instrument (Tauto Biotechnique Company, Shanghai, China). The apparatus was equipped with three preparative coils connected in series (diameter of polytetrafluoroethylene (PTFE) tube = 1.9 mm; total volume = 320 mL, including the 300 mL separation volume and a 20 mL sample loop). The revolution speed of the instrument was adjustable, ranging from 0 to 1000 rpm. The system was also equipped with two TBP5002 constant flow pumps (Tauto Biotechnique Company), a UV2000D detector model (Shanghai Sanotac Scientific Instrument Co., Ltd, Shanghai, China) and a DC0506 low constant temperature bath (Tauto Biotechnique Company). EasyChrom-1000 chromatography workstation (Shanghai Sanotac Scientific Instrument Co., Ltd) was employed to record the chromatograms.

An Agilent 1260 HPLC system was used, equipped with a quaternary pump (G1311C), an auto-sampler (G1329B), a thermostated column compartment (G1316A), a diode array detector (G1315D), a Zorbax Eclipse XDB-C18 analytical column (4.6 × 250 mm, 5 μm), and an HPLC workstation.

NMR experiments were performed on a Varian INOVA 600 NMR spectrometer.

Reagents and materials

All organic solvents used for fractionation and HSCCC separation were of analytical grade and purchased from Tianjin Baishi Chemical Co., Ltd (Tianjin, China). Methanol used for HPLC was of chromatographic grade (Shandong Yuwang Industrial Co., Ltd, Dezhou, China).

The seeds of *I. lactea* were collected from Malian Lake of Alxa League of Inner Mongolia, China, in August 2013. The plant was identified by Dr Yuhu Wu (Northwest Institute of Plateau Biology, Chinese Academy of Sciences, China) and authenticated using the voucher specimen in the Qinghai-Tibetan Plateau Museum of Biology (Xining, China) (reference No.158719) and the Chinese Virtual Herbarium (http://qtpmb.cvh.org.cn).

Preparation of the enriched proanthocyanidins sample (EPS)

Air-dried seed coats (7 kg) of *I. lactea* were powdered and firstly extracted by supercritical carbon dioxide (CO_2) in an HA221-40(50)-(10 + X) supercritical extractor (Nantong Yichuang Experimental Apparatus Co. Ltd, Nantong, China). Then the residue was extracted with 80% ethanol (3 × 25 L, each 3 h) at 60 °C. The combined filtrate was concentrated to afford the ethanol extract. The ethanol extract was suspended in water and partitioned in sequence with ethyl acetate and *n*-butanol to afford an ethyl acetate fraction (100 g). This latter fraction was subjected to column chromatographic fractionation over silica gel and eluted with light petroleum-ethyl acetate gradient (from 5:1 to 0:1). According to the HPLC profile and the UV spectra, the fraction (5.53 g) eluted with light petroleum and ethyl acetate in a ratio of 5:5 containing an enriched proanthocyanidin extract (EPS) was collected and prepared for subsequent HSCCC separation.

Measurement of partition coefficient

A series of experiments were conducted to evaluate the partition coefficients of the target compounds in different two-phase solvent systems as determined by HPLC. About 4 mL of each phase of pre-equilibrated two-phase solvent system was mixed with 2 mg of the EPS in a test tube. The test tube was shaken vigorously and left to stand at room temperature until two phases have a clear separation layer. Then the two phases were collected, dried and re-dissolved in methanol, and analysed by HPLC recording at 280 nm to obtain the partition coefficients of the target compounds respectively. The partition coefficient (*K*) is expressed as

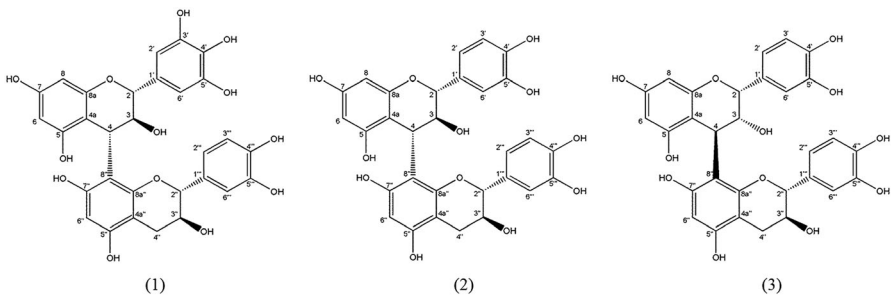

Figure 1. Chemical structures of (**1**) prodelphinidin B3, (**2**) procyanidin B3, and (**3**) procyanidin B1.

the peak area of the target compound(s) in the upper phase divided by that of the lower phase.

Preparation of two-phase solvent system and sample solution

The selected two-phase solvent system (ethyl acetate:*n*-butanol: water, 9:1:10, *v*/*v*/*v*) was prepared by adding all the solvents into a separation funnel at the selected volume ratios. Then the solvent system was thoroughly equilibrated by shaking repeatedly the separation funnel at room temperature. After being thoroughly equilibrated, the upper and lower phases were collected and degassed by sonication for 20 min prior to use. The sample solution was prepared by dissolving 100 mg of the EPS in 5 mL of the upper phase and 5 mL of the lower phase.

HSCCC separation procedure

In the HSCCC separation procedure, the two phases of the solvent system ethyl acetate:*n*-butanol:water (9:1:10, *v*/*v*/*v*) were firstly pumped into the coil column at a flow rate of 20 mL/min with two constant flow pumps, which were connected with a three-way valve, at the same time. After the column was entirely filled with the solvent system and rotating at 900 rpm, the flow rate of both the two phases was adjusted to 2.2 mL/min. Only the lower phase was eluted out from the column in the equilibrium process. Equilibrium was established when the two phases eluted from the outlet of the column had the same volume. After equilibrium, about 10 mL of the sample solution containing 100 mg of the EPS was continuously loaded with a time interval of 73 min. The column temperature was controlled at 30 °C. The effluents were continuously monitored with a UV detector at 280 nm and the chromatogram was recorded. Each peak fraction was manually collected according to the elution profile and concentrated under reduced pressure.

HPLC analyses of HSCCC peak fractions

The HPLC analyses of the EPS, partition coefficients and each peak fraction obtained from HSCCC were conducted on a reversed-phase Eclipse Zorbax XDB C_{18} column (4.6 × 250 mm, 5 μm) with gradient elution. The mobile phase was composed

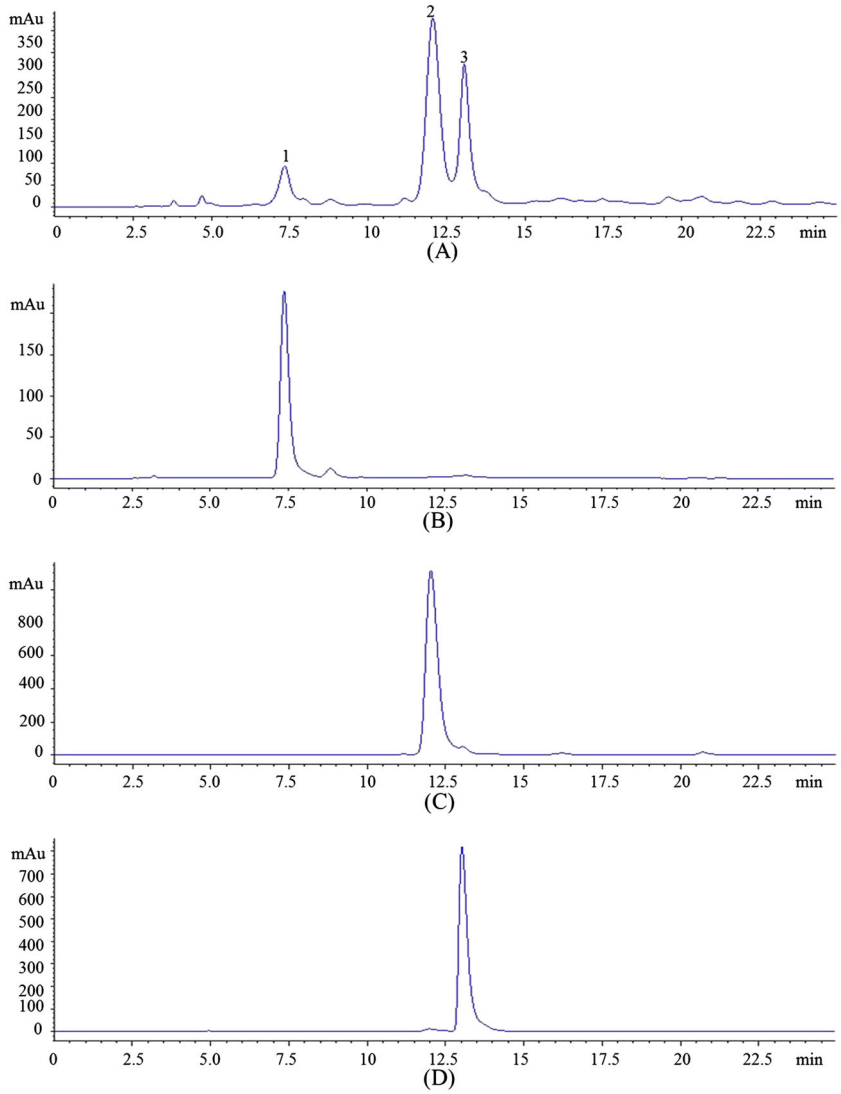

Figure 2. HPLC chromatograms of the EPS (A) and HSCCC peak fractions: (B) prodelphinidin B3; (C) procyanidin B3; (D) procyanidin B1. Column: Eclipse XDB-C_{18} analytical column (5 μm, 4.6 mm × 250 mm); mobile phase: methanol:water (0–25 min, 10–25% methanol); flow rate: 1.0 mL/min; detection wavelength: 280 nm.

of water and methanol using a linear gradient elution as follows: 0 min, 90:10 water:methanol, *v/v*; 25 min, 75:25 water:methanol, *v/v*. The flow rate was maintained at 1.0 mL/min and the column temperature was set at 30 °C. The detection wavelength was 280 nm.

Identification of HSCCC peak fractions by NMR

The identification of the HSCCC peak fractions was carried out by ^{1}H NMR (600 MHz), ^{13}C NMR (150 MHz) and compared with published data.

Table 1. *K*-Values of the target compounds measured in different solvent systems

Solvent systems (*v/v*)	*K*-value		
	Compound 1	Compound 2	Compound 3
Ethyl acetate:methanol:*n*-butanol:water (8:5.2:1:10)	0.23	0.71	0.58
Ethyl acetate:methanol:*n*-butanol:water (8:3:1:10)	0.73	2.06	1.44
Ethyl acetate:*n*- butanol: water (9:1:10)	1.02	2.39	1.57
Ethyl acetate:methanol:water (8:5.2:10)	0.87	1.59	1.37

Results and discussion

Optimisation of HPLC method

Since the analyses of the EPS, the partition coefficients and the HSCCC fractions were performed by HPLC, suitable HPLC separation conditions were required. Thus, different compositions of mobile phase and elution modes were tested. The results indicated that a good separation was achieved when the mobile phase was composed of water and methanol in a programme as follows: 0 min, 90:10 water:methanol, *v/v*; 25 min, 75:25 water:methanol, *v/v*. The flow rate was maintained at 1.0 mL/min and the column temperature was set at 30 °C. The detection wavelength was 280 nm and UV spectra were recorded from 190 to 400 nm. Under these conditions, the target compounds can reach baseline separation at a relatively short separation time. The HPLC chromatogram of the EPS from the seed coats of *I. lactea* is shown in Fig. 2; the retention times of the three target proanthocyanidins (prodelphinidin B3, procyanidin B3 and procyanidin B1) were 7.5 min, 12.1 min and 13.2 min, respectively.

Selection of the two-phase solvent system

Successful HSCCC separation largely depends on the selection of a suitable two-phase solvent system, which can provide an ideal range of the partition coefficients (*K*-values) for all the target compounds. According to the polarity of the target compounds, three sets of two-phase solvent systems including ethyl acetate:methanol:*n*-butanol:water (8:3:1:10, 8:5.2:1:10, *v/v/v/v*), ethyl acetate:*n*-butanol:water (9:1:10, 9:1:8, *v/v/v*) and ethyl

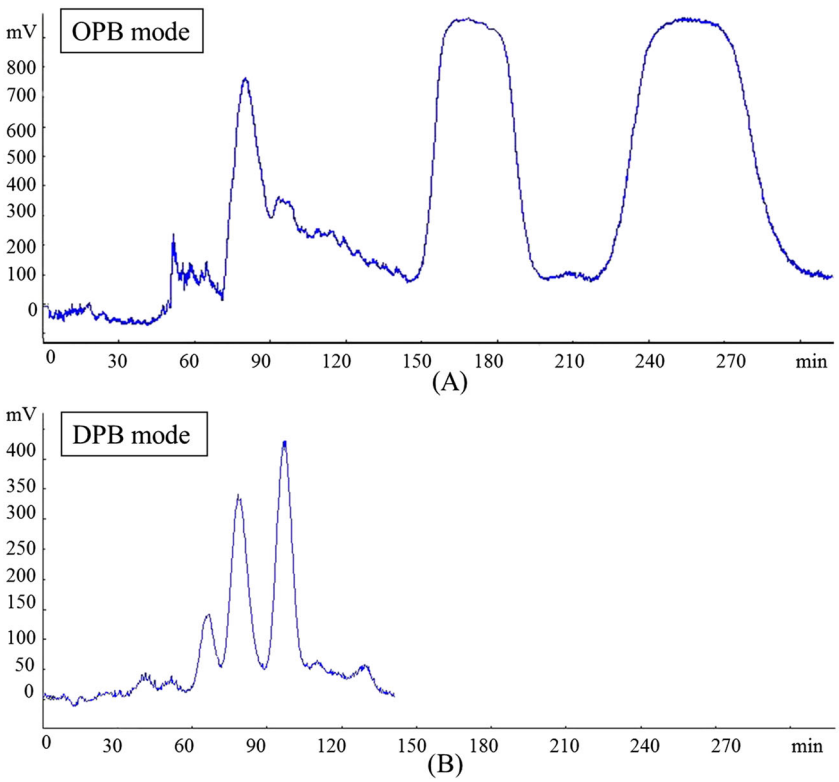

Figure 3. HSCCC chromatograms of the EPS with (A) OPB mode and (B) DPB mode. HSCCC conditions: solvent system: ethyl acetate:*n*-butanol:water (9:1:10, *v/v/v*); revolution speed: 900 rpm; separation temperature: 30 °C; flow rate: 1.8 mL/min; detection wavelength: 280 nm; sample size: (A) 200 mg of the EPS in 10 mL of the lower phase; (B) 50 mg of the EPS in 5 mL of the upper phase and 5 mL of the lower phase.

acetate:methanol:water (8:5.2:10, *v/v/v*) were tested. As previously reported in the literature, *K*-values of the target compounds in the range of 0.5 to 2 are a prerequisite to get effective HSCCC separation with good resolution and short elution time (Su *et al.*, 2013). As it is shown in Table 1, the solvent system ethyl acetate:methanol:*n*-butanol:water (8:5.2:1:10, *v/v/v/v*) provided small *K*-values for the three target compounds ($K_1 = 0.23$, $K_2 = 0.71$ and $K_3 = 0.58$), which would cause poor peak resolution. The solvent system ethyl acetate:methanol:*n*-butanol:water (8:3:1:10, *v/v/v/v*) provided suitable *K*-values ($K_1 = 0.73$, $K_2 = 2.06$ and $K_3 = 1.44$) for the target compounds, but the system stability was not ideal, it was accompanied with a unequal-volume elution of the two phases, and caused a higher noisy signal when employed in DPB mode. The solvent

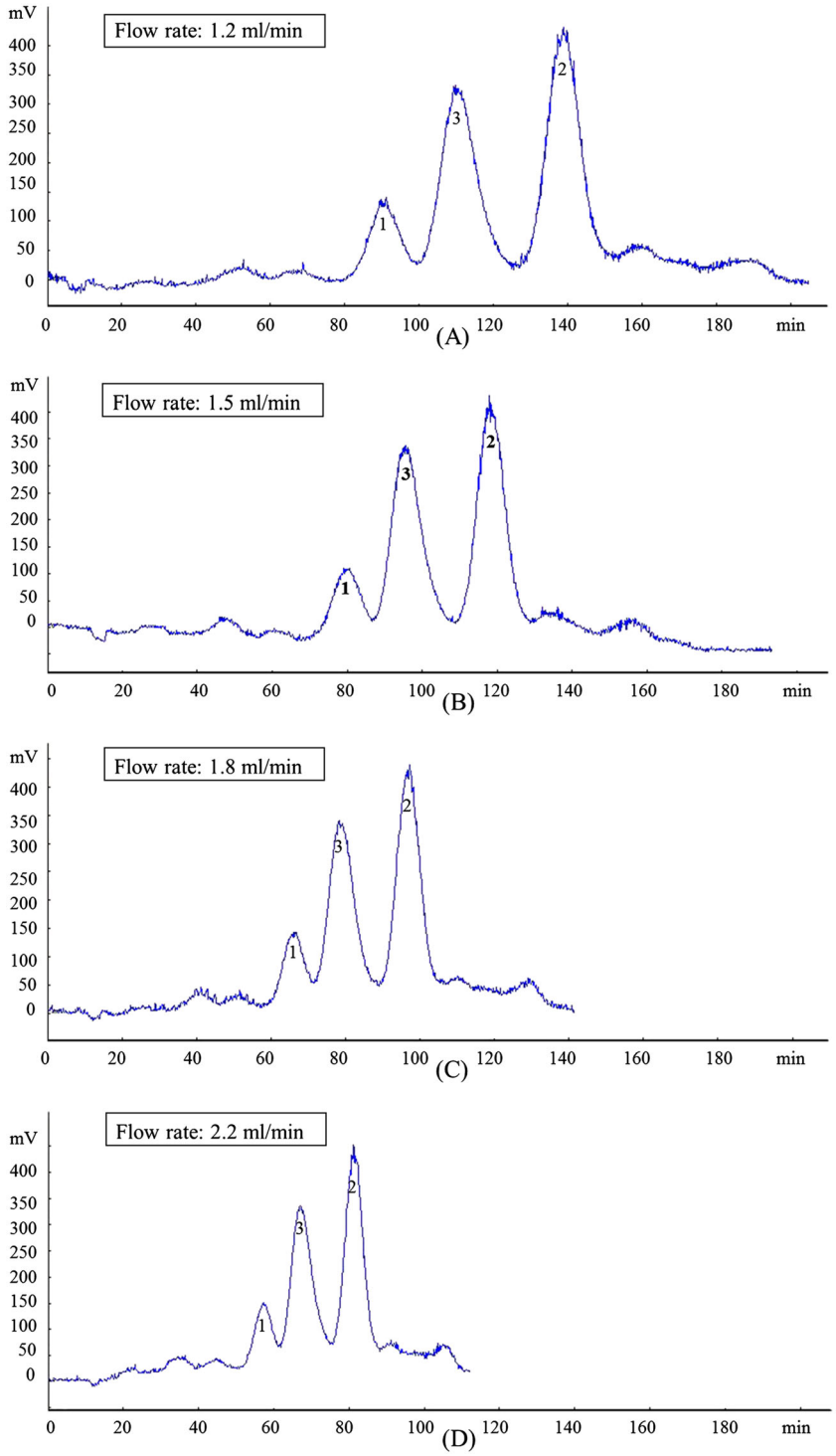

Figure 4. HSCCC chromatograms of the EPS at different flow rates. HSCCC conditions: solvent system: ethyl acetate:*n*-butanol:water (9:1:10, *v/v/v*); revolution speed: 900 rpm; separation temperature: 30 °C; sample size: 50 mg of the EPS in 5 mL of the upper phase and 5 mL of the lower phase; detection wavelength: 280 nm; flow rate: (A) 1.2 mL/min, (B) 1.5 mL/min, (C) 1.8 mL/min, (D) 2.2 mL/min.

system composed of ethyl acetate:*n*-butanol:water at the volume ratio of 9:1:10, yielding *K*-values of $K_1 = 1.02$, $K_2 = 2.39$ and $K_3 = 1.57$ ($\alpha_1 = 1.53$, $\alpha_2 = 1.52$) for the target compounds, provided a satisfactory settling time of about 12 s. Thus, this solvent system was finally chosen for the separation of the EPS in the DPB mode.

Optimized conditions of HSCCC with DPB mode

In this study, the DPB mode was employed in the whole HSCCC separation of the EPS from *I. lactea*. The characteristics of the DPB mode as compared to the one-pump balancing (OPB) mode are as follows: (1) the multi-layer coil column is firstly filled with

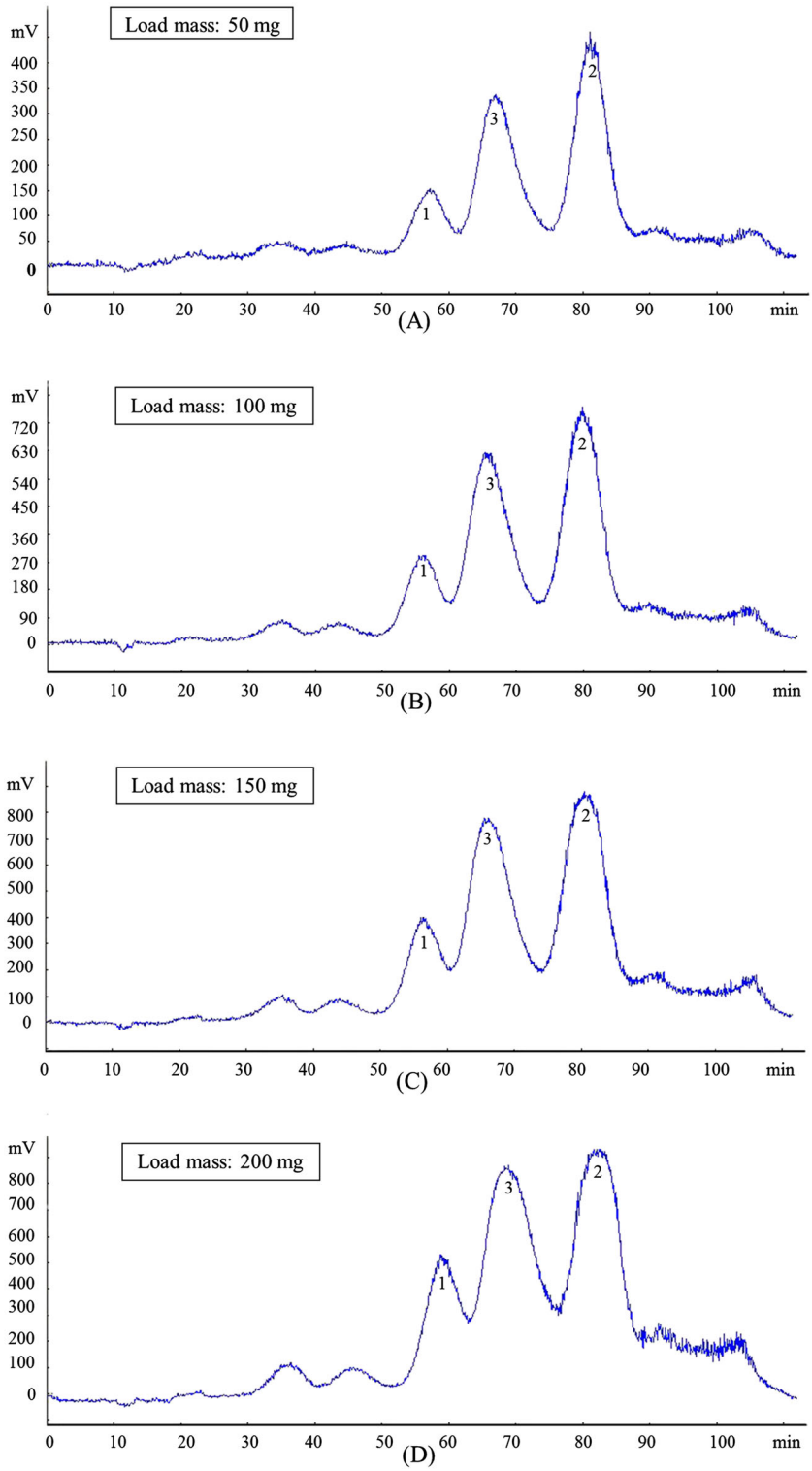

Figure 5. HSCCC chromatograms of the EPS at different load masses. HSCCC conditions: solvent system: ethyl acetate:*n*-butanol:water (9:1:10, *v/v/v*); revolution speed: 900 rpm; separation temperature: 30 °C; flow rate: 2.2 mL/min; detection wavelength: 280 nm; sample size: (A) 50 mg, (B) 100 mg, (C) 150 mg, (D) 200 mg of the EPS in 5 mL of the upper phase and 5 mL of the lower phase.

the two phases, while only the stationary phase is pumped into the column in the OPB mode; (2) then the two phases are pumped into the column at a certain flow rate to reach equilibrium during revolution, while only the mobile phase is pumped in the OPB mode; (3) in the equilibrium process, the lower phase was eluted out from the column, while the upper phase was eluted out in the OPB mode; (4) the hydrodynamic equilibrium is established in the column when the two phases elute out with the same volume, while the equilibrium is reached once the clear mobile phase elutes from the outlet of the column in the OPB mode; (5) after the sample solution is loaded, the two phases are both used to elute the sample, while only the mobile phase is used to elute the sample in the OPB mode. Compared to the OPB mode, the DPB mode can reduce the total separation time a great deal. The time-saving attributes are mainly demonstrated by three aspects: saving time for filling the coil column, for establishing hydrodynamic equilibrium, and for the separation process. About 300 min are needed to separate the three target proanthocyanidins with the lower phase as the

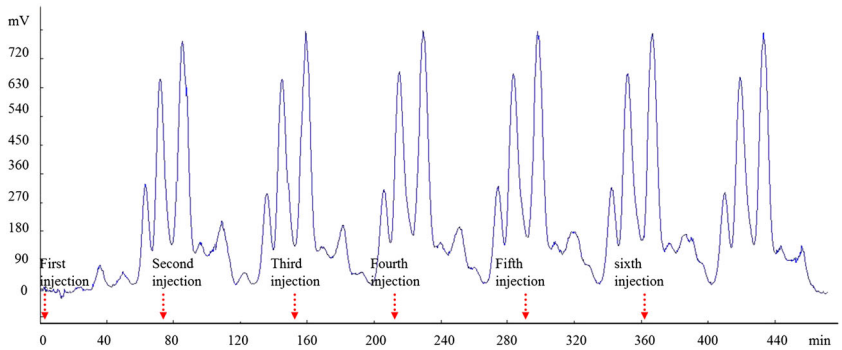

Figure 6. HSCCC chromatogram of the EPS using CLS and DPB mode. HSCCC conditions: solvent system: ethyl acetate:*n*-butanol:water (9:1:10, *v/v/v*); revolution speed: 900 rpm; flow rate: 2.2 mL/min; separation temperature: 30 °C; sample size: 100 mg of the EPS in 5 mL of the upper phase and 5 mL of the lower phase; detection wavelength: 280 nm.

Table 2. ^{13}C NMR and ^{1}H NMR spectral data of compound **1** (prodelphinidin B3) in CD_3OD

	Major isomer		Minor isomer	
Position	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
2	84.2	4.22 (d, *J* = 9.7 Hz)	84.3	4.28 (d, *J* = 9.7 Hz)
3	73.4	4.36 (dd, *J* = 9.7, 8.0 Hz)	73.6	4.50 (dd, *J* = 9.7, 8.0 Hz)
4	38.6	4.43 (d, *J* = 8.0 Hz)	38.5	4.52(d, *J* = 8.0 Hz)
5	157.1		157.4	
6	97.4	5.89 (d, *J* = 2.3 Hz)	97.53	5.84 (d, *J* = 2.3 Hz)
7	157.1		157.3	
8	96.9	5.74 (d, *J* = 2.3 Hz)	97.57	5.80 (d, *J* = 2.3 Hz)
4a	158.6		158.5	
8a	107.06		107.11	
1′	131.63		131.57	
2′, 6′	108.3	6.36 (s)	108.3	6.52 (s)
3′, 5′	146.5		146.7	
4′	134.1		134.2	
2″	82.0	4.65 (d, *J* = 6.7 Hz)	83.0	4.74 (d, *J* = 6.7 Hz)
3″	68.6	3.84 (ddd, *J* = 7.4, 6.7, 5.2 Hz)	68.5	4.07 (ddd, *J* = 7.4, 6.7, 5.2 Hz)
4″	27.8	2.50 (dd, *J* = 16.2, 7.4 Hz)	28.4	2.58 (dd, *J* = 16.2, 7.4 Hz)
		2.67 (dd, *J* = 16.2, 5.2 Hz)		2.81 (dd, *J* = 16.2, 5.2 Hz)
5″	155.7		155.7	
6″	96.0	6.06 (s)	96.2	5.94 (s)
7″	155.8		155.8	
8″	107.9		107.8	
4a″	154.6		154.9	
8a″	101.7		100.5	
1‴	131.9		132.1	
2‴	115.1	6.52 (d, *J* = 1.8 Hz)	115.2	6.95 (d, *J* = 1.8 Hz)
3‴	145.3		146.1	
4‴	145.6		146.1	
5‴	116.3	6.61 (d, *J* = 8.2 Hz)	116.1	6.76 (d, *J* = 8.2 Hz)
6‴	119.5	6.10 (dd, *J* = 8.2, 1.8 Hz)	120.2	6.83 (dd, *J* = 8.2, 1.8 Hz)

mobile phase at a flow rate of 1.8 mL/min in the OPB mode. However, when the DPB mode was employed, the total separation time can be largely reduced to 100 min using the same flow rate (Fig. 3). What is more, the two phases can be rationally used in the DPB mode, while most HSCCC separations usually require more mobile phase, resulting in an over-preparation of the stationary phase.

Although the selection of the two-phase solvent system is more critical, the influences of the flow rate and sample load mass on the separation efficiency should not be ignored. In this study, different flow rates and different sample load masses were investigated. The experimental design of the flow rate was conducted at four levels (1.2 mL/min, 1.5 mL/min, 1.8 mL/min and 2.2 mL/min). From Fig. 4 it can be seen that there was not much significant difference on the peak resolution; in all cases, the purities of the three compounds were all above 95%, as determined by HPLC. From the viewpoint of reducing the total separation time, the flow rate of 2.2 mL/min was eventually employed in the continuous HSCCC separation. Meanwhile, changing the sample load mass (50 mg, 100 mg, 150 mg and 200 mg) did significantly affect the peak resolution. As it can be seen from Fig. 5, with the increase of the sample load mass, the HSCCC chromatograms displayed a higher noise signal. Therefore, a sample load mass of 100 mg was finally selected for each injection in the continuous HSCCC separation.

Separation of the EPS by HSCCC with CSL

The HSCCC chromatograms corresponding to six consecutive sample injections are shown in Fig. 6. The times of the six injections were 0 min, 73 min, 146 min, 219 min, 292 min and 365 min, respectively. The whole HSCCC separation procedure yielded 57 mg of compound **1**, 198 mg of **2**, and 162 mg of **3**, from 600 mg of the EPS, in about 460 min. The purities of the three isolated compounds were 97.2%, 98.1% and 97.3%, respectively, as determined by HPLC. Therefore, HSCCC separation with CSL has some advantages compared with the single injection: firstly, it can reduce the workload of repeatedly preparing the solvent system; secondly, it can significantly decrease the total separation time; thirdly, it can reduce the solvent consumption.

Structural identification

The structure identification of the HSCCC peak fractions was performed by ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz). ^{1}H NMR and ^{13}C NMR data of compounds **1**, **2** and **3** are shown in Tables 2–4, respectively. Compound **1** was identified as prodelphinidin B3 (gallocatechin-(4α → 8)-catechin) by comparison with ^{1}H NMR and ^{13}C NMR data given in the literature (Sun *et al.*, 1987; Esatbeyoglu *et al.*, 2014); the assignments were

Table 3. ^{13}C NMR and ^{1}H NMR spectral data of compound **2** (procyanidin B3) in CD_3OD

	Major isomer		Minor isomer	
Position	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
2	84.0	4.25 (d, *J* = 9.8 Hz)	84.1	4.25 (d, *J* = 9.8 Hz)
3	73.7	4.35 (m)	73.6	4.35 (m)
4	38.6	4.41 (d, *J* = 7.8 Hz)	38.5	4.40 (d, *J* = 7.8 Hz)
5	155.9		155.9	
6	96.1	5.88 (d, *J* = 2.2 Hz)	96.2	5.83 (d, *J* = 2.2 Hz)
7	155.6		155.8	
8	108.2	5.78 (d, *J* = 2.2 Hz)	108.3	5.81 (d, *J* = 2.2 Hz)
4a	154.9		155.0	
8a	102.2		100.5	
1′	131.8		132.2	
2′	115.5	6.73 (d, *J* = 2.0 Hz)	115.2	6.89 (d, *J* = 2.0 Hz)
3′	145.5	6.66 (d, *J* = 8.0 Hz)	145.46	6.75 (d, *J* = 8.0 Hz)
4′	145.8		145.6	
5′	116.1		115.9	
6′	116.2	6.47 (dd, *J* = 8.3, 2.2 Hz)	116.15	6.82 (dd, *J* = 8.0, 2.0 Hz)
2″	82.5	4.53 (d, *J* = 7.4 Hz)	82.9	4.74 (d, *J* = 7.4 Hz)
3″	68.9	3.79 (ddd, *J* = 8.0, 7.6, 5.6 Hz)	68.8	4.07 (ddd, *J* = 8.0, 7.6, 5.6 Hz)
4″	28.8	2.76 (dd, *J* = 16.3, 5.6 Hz)	28.5	2.81 (dd, *J* = 16.3, 5.6 Hz)
		2.48 (dd, *J* = 16.3, 8.1 Hz)		2.58 (dd, *J* = 16.3, 8.1 Hz)
5″	157.1		157.4	
6″	97.3	6.07 (s)	97.6	5.94 (s)
7″	157.1		157.3	
8″	96.9		97.5	
4a″	158.61		159.94	
8a″	107.2		107.1	
1‴	132.6		132.4	
2‴	116.4	6.58 (d, *J* = 2.0 Hz)	116.15	6.95 (d, *J* = 2.0 Hz)
3‴	146.06	6.68 (d, *J* = 8.3 Hz)	146.11	6.76 (d, *J* = 8.3 Hz)
4‴	146.11		146.4	
5‴	119.9		120.2	
6‴	120.6	6.25 (dd, *J* = 8.3, 2.0 Hz)	121.0	6.83 (dd, *J* = 8.3, 2.0 Hz)

Table 4. ^{13}C NMR and ^{1}H NMR spectral data of compound **3** (procyanidin B1) in CD_3OD

	Procyanidin B1	
Position	^{13}C NMR	^{1}H NMR
2	77.1	5.08 (br s)
3	73.1	3.39 (br s)
4	37.2	4.64 (br s)
5	155.7	
6	96.3	5.96 (br s)
7	158.4	
8	95.8	6.06 (br s)
4a	101.7	
8a	158.8	
1′	132.8	
2′	115.3	7.02 (br s)
3′	145.8	
4′	145.2	
5′	115.9	
6′	119.4	6.71 (d, J = 7.2 Hz)
2″	82.1	4.93 (br s)
3″	68.5	4.11 (br s)
4″	27.1	2.69 (d, J = 16.2 Hz)
		2.60 (d, J = 16.2 Hz)
5″	157.8	
6″	97.1	5.86 (brs)
7″	156.3	
8″	107.8	
4a″	100.9	
8a″	153.8	
1‴	132.5	
2‴	114.6	6.95 (br s)
3‴	145.9	673 (d, J = 7.2 Hz)
4‴	145.8	
5‴	116.1	
6‴	119.4	6.89 (d, J = 7.2 Hz)

confirmed by heteronuclear single quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC). Comparison with the data given by Saito *et al.* (2002) allowed the identification of compound **2** as procyanidin B3 (catechin-(4α → 8)-catechin), whereas, compound **3** was identified as procyanidin B1 (epicatechin-(4α → 8)-catechin) in agreement with the data reported by Shoji *et al.* (2003).

Acknowledgements

This work was supported by the National Science Foundation of China (31470426, 31300292), Open Topic of Key Laboratory of Tibetan Medicine Research, CAS (2014-TMR-03), Youth Innovation Promotion Association, CAS, the Key Programme of "The Dawn of West China" Talent Foundation of CAS.

References

DalBó S, Jürgensen S, Horst H, Soethe DN, Santos AR, Pizzolatti MG, Ribeiro-do-Valle RM. 2006. Analysis of the antinociceptive effect of the proanthocyanidin-rich fraction obtained from *Croton celtidifolius* barks: evidence for a role of the dopaminergic system. *Pharmacol Biochem Behav* **85**: 317–323.

Dauer A, Hensel A, Lhoste E, Knasmüller S, Mersch-Sundermann V. 2003. Genotoxic and antigenotoxic effects of catechin and tannins from the bark of *Hamamelis virginiana* L. in metabolically competent, human hepatoma cells (Hep G2) using single cell gel electrophoresis. *Phytochemistry* **63**: 199–207.

Dong XQ, Zou B, Zhang Y, Ge ZZ, Du J, Li CM. 2013. Preparation of A-type proanthocyanidin dimers from peanut skins and persimmon pulp and comparison of the antioxidant activity of A-type and B-type dimers. *Fitoterapia* **91**: 128–139.

Esatbeyoglu T, Juadjur A, Wray V, Winterhalter P. 2014. Semisynthetic preparation and isolation of dimeric procyanidins B1–B8 from roasted hazelnut skins (*Corylus avellana* L.) on a large scale using countercurrent chromatography. *J Agric Food Chem* **62**: 7101−7110.

Fan JL, Ding XL, Gu WY. 2007. Radical-scavenging proanthocyanidins from sea buckthorn seed. *Food Chem* **102**: 168–177.

Grace MH, Warlick CW, Neff SA, Lila MA. 2014. Efficient preparative isolation and identification of walnut bioactive components using high-speed counter-current chromatography and LC-ESI-IT-TOF-MS. *Food Chem* **158**: 229–238.

Gürbüz O, Göçmen D, Dağdelen F, Gürsoy M, Aydin S, Şahin İ, Büyükuysal L, Usta M. 2007. Determination of flavan-3-ols and trans-resveratrol in grapes and wine using HPLC with fluorescence detection. *Food Chem* **100**: 518–525.

Han C, Xu J, Wang X, Xu X, Luo J, Kong L. 2014. Enantioseparation of racemic trans-.eop;-viniferin using high speed counter-current chromatography based on induced circular dichroism technology. *J Chromatogr A* **1324**: 164–170.

Jin J, Li Y, Kipletting Tanui E, Han L, Jia Y, Zhang L, Wang Y, Zhang X, Zhang Y. 2013. Fishing and knockout of bioactive compounds using a combination of high-speed counter-current chromatography (HSCCC) and preparative HPLC for evaluating the holistic efficacy and interaction of the components of Herba Epimedii. *J Ethnopharmacol* **147**: 357–365.

Kim Y, Choi Y, Ham H, Jeong HS, Lee J. 2013. Protective effects of oligomeric and polymeric procyanidin fractions from defatted grape seeds on tert-butyl hydroperoxide-induced oxidative damage in HepG2 cells. *Food Chem* **137**: 136–141.

Kohler N, Wray V, Winterhalter P. 2008. Preparative isolation of procyanidins from grape seed extracts by high-speed counter-current chromatography. *J Chromatogr A* **1177**: 114–125.

Kothe L, Zimmermann BF, Galensa R. 2013. Temperature influences epimerization and composition of flavanol monomers, dimers and trimers during cocoa bean roasting. *Food Chem* **141**: 3656–3663.

Ma XC, Sun C, Huang SS, Wang JK, Zhang BJ, Li FY, Wang G, Deng S, Cui J. 2010. Preparative isolation and purification of four prenylflavanones from microbial biotransformation of kurarinone by high-speed counter-current chromatography. *Sep Purif Technol* **76**: 140–145.

Molan AL, Sivakumaran S, Spencer PA, Meagher LP. 2004. Green tea flavan-3-ols and oligomeric proanthocyanidins inhibit the motility of infective larvae of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* in vitro. *Res Vet Sci* **77**: 239–243.

Saito A, Nakajima N, Tanaka A, Ubukata M. 2002. Synthetic studies of proanthocyanidins. Part 2: stereoselective gram-scale synthesis of procyanidin-B3. *Tetrahedron* **58**: 7829–7837.

Savitri Kumar N, Maduwantha BWWM, Kumar V, Nimal Punyasiri PA, Sarath BAI. 2009. Separation of proanthocyanidins isolated from tea leaves using high-speed counter-current chromatography. *J Chromatogr A* **1216**: 4295–4302.

Shen WJ, Qin MJ, Shu P, Zhang CF. 2008. Two new C-glycosylflavones from the leaves of *Iris lactea* var. chinensis. *Chinese Chem Lett* **19**: 821–824.

Shoji T, Mutsuga M, Nakamura T, Kanda T, Akiyama H, Goda Y. 2003. Isolation and structural elucidation of some procyanidins from apple by low-temperature nuclear magnetic resonance. *J Agric Food Chem* **51**: 3806−3813.

Su W, Liu Q, Yang Q, Yu JG, Chen XQ. 2013. Separation and purification of four compounds from *Desmodium styracifolium* using off-line two-dimensional high-speed counter-current chromatography. *J Sep Sci* **36**: 3338–3344.

Sun D, Wong H, Foo LY. 1987. Proanthocyanidin dimers and polymers from *Quercus dentate*. *Phytochemistry* **26**: 1825–1829.

Tong X, Xiao XH, Li GK. 2011. On-line coupling of dynamic microwave-assisted extraction with high-speed counter-current chromatography for continuous isolation of nevadensin from *Lyeicnotus pauciflorus* Maxim. *J Chromatogr B* **879**: 2397–2402.

Wang C, Pan YJ, Fan GR, Chai YF, Wu YT. 2010. Application of an efficient strategy based on MAE, HPLC-DAD-MS/MS and HSCCC for the rapid extraction, identification, separation and purification of flavonoids from Fructus Aurantii Immaturus. *Biomed Chromatogr* **24**: 235–244.

Wu H, Feng R, Guan S, Yu W, Man W, Guo J, Liu X, Yang M, Jiang B, Wu W, Zhang L, Guan S, Guo D. 2012. Rapid preparative isolation of a new phenylpropanoid glycoside and four minor compounds from *Sparganium stoloniferum* using high-speed counter-current chromatography as a fractionation tool. *J Sep Sci* **35**: 1160–1166.
Wu SJ, Yang QZ. 1980. Chemical studies on *Iris pallasii* Fisch. *var. chinensis* Fisch. *Acta Chim Sinica* **38**: 156–161.
Wu SJ, Yang QZ. 1981. Chemical studies on *Iris pallasii* Fisch. *var. chinensis* Fisch. II. The isolation and structure studies of pallasone B and pallasone C. *Acta Chim Sinica* **39**: 767–770.
Xia GB, Hong S, Liu SB. 2014. Simultaneous preparation of naturally abundant and rare catechins by tannase-mediated biotransformation combining high speed counter current chromatography. *Food Chem* **151**: 380–384.
Yousef MI, Saad AA, El-Shennawy LK. 2009. Protective effect of grape seed proanthocyanidin extract against oxidative stress induced by cisplatin in rats. *Food Chem Toxicol* **47**: 1176–1183.