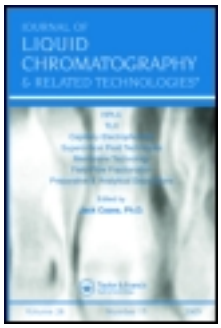

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SEPARATION OF THREE BIOACTIVE ISOMERS FROM BIDENS PILOSA BY COUNTERCURRENT CHROMATOGRAPHY

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Abstract

Under LC-TOF-MS fraction-guided analyzed the *n*-butanol extract from *Bidens pilosa*. Three bioactivity isomers compounds, including methyl 3,4-dicaffeoyl quinate (**1**), methyl 3,5-dicaffeoyl quinate (**2**) and methyl 4,5-dicaffeoyl quinate (**3**) were successfully isolated by countercurrent chromatography (CCC) with two phase solvent system composed of chloroform-methanol-water-*n*-buthanol (4:3:2:0.5, v/v) elution. Their purities were 93.7%, 82.4% and 91.8% determined by HPLC. HR-ESI-MS and NMR was used to confirm their structures. This is the first time to report the isolation of geometrical isomers of methyl dicaffeoyl quinates by CCC.

KEYWORDS: *Bidens pilosa* L., Countercurrent chromatography, LC-TOF-MS, Methyl dicaffeoyl quinate.

INTRODUCTION

Bidens pilosa L. (Asteraceae) is widely distributed in tropical and subtropical regions of the world, and has been used to treat various human health conditions, including inflammation, hepatitis, diabetes, and hypertension [1-5]. Various natural products like phenolic compounds, flavonoids, polyacetylenes, caffeoyl derivatives, phenylpropanoids, and triterpeniods have been isolated from different part of *B. pilosa* [6]. Among of them, methyl dicaffeoyl quinate (MDQ) is a caffeyolquinic acid derivative that demonstrate different biological activity with their geometrical isomers. Methyl 3, 5-dicaffeoyl quinate (**1**) has antitumor, antioxidative, cytoprotective activities and anti-melanogenesis [7-10]. Specifically, methyl 3, 5-dicaffeoyl quinate (**1**) was shown to have significant anti-proliferative effect via a cell cycle arrest and apoptotic induction in HT-29 cells, and has therapeutic potential against human colon carcinoma [9]. The other two isomers, methyl 3, 4-dicaffeoyl quinate (**2**) and methyl 4, 5-dicaffeoyl quinate (**3**) showed high hepatoprotective activity [11-12]. Various bioactive of the MQD need much pure product for further pharmacological research in order to develop and utilize *B. pilosa*. However, many MDQ compounds exist in plants as complex isomeric mixtures. It is usually difficult to large-scale separate isomeric MDQ with traditional separation methods because of their structural similarities and complexities. Therefore we developed an effective method to isolate and purify MDQ compounds.

Technique countercurrent chromatography (CCC) is a unique separation techniques with continuous liquid-liquid partition chromatographic without stationary phase material support. Many advantages of CCC including: no irreversible absorption of the samples,

different solvent systems for choice, low risk the sample denaturation, high sample recovery, and large load capacity, easy to large-scale preparation[13]. It has been successfully applied to isolate and purify bioactive compounds from natural products [14-16]. CCC method was also considered as suitable alternative for the separation of isomeric compounds [17-19]. The objective of this study is to develop a CCC method for preparative isolation of three isomeric active compounds: methyl 3,4-dicaffeoyl quinate (**1**), methyl 3,5-dicaffeoyl quinate (**2**), and methyl 4,5-dicaffeoyl quinate (**3**) (Fig. 1), from *B. pilosa*. ^{1}H-NMR, ^{13}H-NMR, HR-ESI-MS and UV were applied to confirm their chemical structure.

EXPERIMENTAL

Apparatus

Two CCC instruments (Shanghai Tauto Biotechnique, Shanghai, China, Co., Ltd., Shanghai, China) were used: the TBE-20A (analytical scale) instrument consisted of three analytical coils (I. D. of tube, 0.8 mm; total volume, 20mL) and a 0.5 mL sample loop. The β values of the multilayer coil columns ranged from 0.60 to 0.78 (β= r/R, R = 4.5 cm, where r is the distance from the coil to the holder shaft, R is the distances between the holder axis and central axis of the centrifuge). The revolution speed of the centrifuge is between 0 and 2000 rpm. A model UV500 detector was applied to perform the UV absorbance measurement.

While the TBE-300B (semi-preparative scale) is large series (I. D. of tube, 1.6 mm; total volume, 280 mL; sample loop volume, 20 mL). The β values of columns range from 0.5

to 0.8 (β= r/R, R = 6.5 cm). The range speed control is 0-1000 rpm, and the instrument was equipped with a model TBD-2000 detector.

Both of CCC procedures were maintained temperature by HX-105 constant-temperature controller (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China). The chromatograms data was recorded by a N2010 workstation (Intelligence Research Institute, Zhejiang University, Hangzhou, China).

HPLC-TOF-MS consisted of a Waters 2695 analytical separations module (Milford, MA) with 2998 photodiode array detector (PDA), and a Waters LCT PremierXE TOF mass spectrometer HR-ESI-MS instrument (Waters, Miliford, USA) with an ESI interface and a Mass Lynx V4.1 software package for data collection. A Synergi C18 column (100 mm × 2.0 mm i.d. 2.5μm, Phenomenex, Torrance, USA) was used in this analysis.

The analysis HPLC (Shimadzu LC-20AVP) consisting of: two LC-20AT solvent delivery units, a SPD-10Avp detector, a CTO-10ASVP column oven (Shimadzu, Kyoto, Japan), and a LC solution workstation (Shimadzu). The column was performed on a YWC J'SPHERE ODS-H80 column (250 × 4.6 mm I.D., S-4 μm, 8 nm). NMR: ^{1}H (400MHz) and ^{13}C (100MHz) NMR spectra were recorded on a Bruker DRX-400 spectrometer using TMS as internal standard.

Reagents And Materials

All solvents used for preparation of crude sample and CCC separation were of analytical grade (Beijing Reagent Factory, Beijing, China). Methanol used for HPLC was of chromatographic grade (Beijing Chemical Factory, Beijing, China), HPLC grade MeCN and formic acid were purchased from J. T. Baker (Philipsburg, NJ, USA)，and water used was ultrapure water. The Amberlite XAD16 resin was purchased from Rohm and Hass (Rohm and Haas Co., USA).

Bidens pilosa was collected from Wanning City, Hainan Province, China, identified by Prof. Chunlin Long (Minzu University of China, Beijing, China), and a voucher specimen (LCL-1032) was deposited at the Herbarium of Minzu University of China in Beijing.

Preparation Of Crude Sample

The aerial part of dried *B. pilosa* (5 kg) was extracted three times with 95% ethanol 2 h each time, and gravity filtered. The combined filtrate was concentrated by rotary evaporation at 50° C under reduced pressure to afford the dried ethanol extract (257 g). The extract (*ca*. 240 g) was dissolved in H_2O (v/v, 500 mL) and was partitioned sequentially (3 times with each solvent) using petroleum ether, ethyl acetate, and *n*-butanol. The combined *n*-butanol fraction (70 g) was separated over Amberlite XAD16 resin column (10 cm × 50 cm), eluting with different concentrations of water and ethanol (100/30, 50/50, 30/70) to provide three subfractions(30%Fr, 50%Fr, 70%Fr.),

which were evaporated to dryness under reduced pressure and stored for HPLC-TOF-MS analysis and subsequent CCC separation.

Measurement Of Partition Coefficient

The two-phase solvent system was selected according to the partition coefficient (*K*) value of target compound determined by HPLC as follow: firstly, about 3 mg crude extract (50%Fr.) was completely dissolved in a 10 mL test tube with 3 mL upper phase of solvent system, and the solution was analyzed by HPLC-PDA. The peak area of target compound was recorded as A1. Secondly, the lower phase (3ml) was added to the test tube and shaken vigorously for several minutes, then keep it at room temperature till the equilibrium of two phases system was established. The upper phase was determined by HPLC again and the peak area of target compound was recorded as A2. The *K* value of target peak express as: $K = (A1 - A2)/A2$[20].

CCC Separation Procedure

The analytical CCC separation was performed after the *K* value test. First the multilayer coil column was entirely filled with the upper phase of the solvent system at 4 mL/min. Then the apparatus was rotated at 1800 rpm, while the lower phase was pumped into the column at 0.5 mL/min. After hydrodynamic equilibrium was established in the column, 0.15 mL of sample solution (10 mg/mL) was injected. UV detector at 254 nm was recorded and the temperature maintained at 25° C. After separation time, all the solvent left in the coil was pushed out by high pressure nitrogen and estimate the retention ratio of the solvent system.

The semi-preparative CCC had a similar procedure. The coil column was entirely filled with the upper phase at a flow rate of 15 mL/min. Then CCC apparatus was rotated at 900 rpm, a flowrate of lower mobile phase was 2.0 mL/min. After reaching hydrodynamic equilibrium, the sample solution (302 mg of the crude extract dissolve in 4 mL upper phase and 4 mL lower phase) was injected into the column. The run was monitored at 254 nm. Each peak fractions was collected and further purity analysis.

HPLC Analysis And Identification Of CCC Peak Fractions

The major peak fractions collected from CCC separation were analyzed by HPLC-PDA. The analytical column was performed on a YWC J'SPHERE ODS-H80 column (250 × 4.6 mm I.D., S-4 μm, 8 nm). The mobile phase composed of water (0.1% formic acid) and acetonitrile in gradient mode as follows: 0-5 min, 15%-15% acetonitrile; 5-25 min, 15%-25% acetonitrile; 25-30 min, 25%-40% acetonitrile; 30-35 min, 40%-60% acetonitrile; 35-40 min, 60%-95% acetonitrile. All analyses were performed at 25° C with a flow rate of 1 mL/min. The sample volume injected was 10 μL and the eluent monitored by a Shimadzu SPD10Avp UV detector at 254 nm. Identification of the CCC peak fractions was conducted by HR-ESI-MS, ^{1}H NMR and ^{13}C NMR spectra.

RESULTS AND DISCUSSION

The ethanolic extraction of *B. pilosa*, was sequentially partition with solvents, and the resulting *n*-BuOH fraction was chromatographed over a Amberlite column, resulting in

three fractions: 30%, 50%, and 70% extr. For further prioritization of these fractions containing bioactive MDQ isomers, LC-TOF-MS analysis was employed.

LC-TOF-MS Analyze The Faction With Bioactive Isomers

The three resulting Amberlite fractions were analyzed by LC-TOF-MS, and the three bioactive isomers were only found in 50% Fr. with retention times of 28.74 min, 32.59 min, and 36.49 min (Fig. 2 D). For example, peak 1 (36.49 min) UV λ max (acetonitrile) as 217, 243, and 327 nm, respectively. HR-ESI-MS *m*/*z* 529.1221 $[M-H]^-$, 531.1508 $[M+H]^+$ (Fig. 2 C)was obtained and the molecular formula was determined as $C_{26}H_{26}O_{12}$. The same UV spectrum and fragmental ion exact masses suggest that the last three peaks were isomers (Fig. 2 A, B, and C). Based upon literature comparisions [12][21], compounds 1-3 were tentatively identified as MDQ derviratives, but the complete identification was not possible. Based on the LC-TOF-MS analysis, we can make sure the target compounds were in the 50% fraction.

Optimization Of CCC Conditions By HPLC-PDA And Analytical CCC

The selection of a suitable two-phase solvent system for the target compounds was the key point to successful separation in CCC. Two phase solvent system was selected according to the partition coefficient values (*K*) which were determined by HPLC-PDA. An optimum range of *K* should be from 0.5 to 2; the retention of the stationary phase should be more than 50% [13].

Considered the target compounds distributed in *n*-butanol extraction and most of constituents were high polar. At the beginning, a common solvent system chloroform-methanol-water (4/3/2，v/v/v) was evaluated and all the *K* values of the target compounds were greater than 2, as shown in Table 1. That result indicated that the target compounds distributed in aqueous phase (stationary phase) more than organic phase (mobile phase). And the elution time of three compounds was quite long. Thus, the different volume ratios of *n*-butanol were added to the solvent system to evaluate the *K* value. When chloroform-methanol-water-*n*-butanol (4/3/2/0.2, v/v/v/v) was used, the *K* value of compound **1** was acceptable, but the other two compounds were unsuitable. Likewise, chloroform-methanol-water-*n*-butanol (4/3/2/0.7, v/v/v/v) was used as the solvent system, which could achieve good *K* value for compounds **2** and **3**, but for the compounds **1**, it improved obviously and would be eluted together with other compounds with similar properties. At last we found that chloroform-methanol-water-*n*-butanol (4/3/2/0.5, v/v/v/v) as the solvent system, the *K* value of compounds **1**, **2**, and **3** were matched the optimum range, good separation result could be obtained and the separation time was acceptable.

Because its many advantages of analytical CCC are less solvent, short analyze time and high repeatability to the semi-preparative CCC. It is optimization on test the selection of two phase systems after *K* value analyzed by HPLC. Under the condition of 1800 rpm revolution speed, 0.5 mL/min flow rate, 25° C apparatus temperature. The select solvent systems: chloroform–methanol-*n*–butanol–water (4:3:0.5:2, v/v/v/v) above was evaluated. The result was given in Figure 3 A. Fine peaks are obtained and 56% of

stationary phase retention was obtained for this solvent system.

Based on the analytical CCC condition, large load sample (302 mg) was separated by preparative CCC under the condition described above, 36.4 mg of peak 1, 24.3 mg of peak 2 and 16.2 mg of peak 3 were obtained (Fig. 3 B), respectively. The HPLC analysis of each compound which was collected from preparative CCC that three compounds could be obtained from the crude extract. The purities of them were 95.7%, 82.7%, and 93.6% based on HPLC area percentage (Fig. 4). They were further purified by Sephedax LH-20, and all the samples obtained were more than 96% purity.

Structural Identification

Three isomeric compounds were identified by HR-ESI-MS, ^{1}H NMR and ^{13}C NMR analysis and comparison with published data.

Peak 1: UV λ_{max} (MeOH) nm 217, 243, 327; HR-ESI-MS (positive mode) *m/z*: 531.1508 ($[M+H]^+$ call for $C_{26}H_{27}O_{12}$, 531.1503). ^{1}H NMR (400 MHz, MeOD)： δ 7.60 (1H, d, *J* = 15.9 Hz, H-3″), 7.51 (1H, d, *J* = 15.9 Hz, H-3′), 7.03 (1H, d, *J* = 2.0 Hz, H-5″), 7.01 (1H, d, *J* = 2.0 Hz, H-5’), 6.93 (1H, dd, *J* = 8.1, 2.0 Hz, H-9″), 6.92 (1H, dd, *J* = 8.1, 2.0 Hz, 9′-H), 6.76 (2H, d, *J* = 8.2 Hz, H-8″and H-8′), 6.30 (1H, d, *J* = 15.9 Hz, H-2″), 6.17 (1H, d, *J* = 15.9 Hz, H-2′), 5.54 (1H, dd, *J* = 13.0, 7.8 Hz, H-3), 5.11 (1H, dd, *J* = 8.1, 3.1 Hz, H-4), 4.35 (1H, dt, *J* = 6.5, 3.3 Hz, 5-H), 3.71 (3H, s, OCH_3), 2.33 (1H, dd, *J* = 13.9, 3.4 Hz, 6-Hax), 2.24 (2H, dd, *J* = 12.5, 8.6 Hz, 2-H), 2.09 (1H, dd, *J* = 13.9, 6.3 Hz, 6-Heq). ^{13}C NMR (100 MHz, MeOD)：175.3(s, C-7), 168.6(s, C-1″), 168.1(s, C-1′),

149.9(d, C-7′ and C-7″), 147.9(d, C-3′ and C-3″), 147.0(d, C-6′ and C-6″), 127.8(d, C-4″ and C-4′), 123.3(s, C-9′ and C-9″), 116.7(d, C-8′ and C-8″), 115.3(d, C-5′ and C-5″), 114.8(s, C-2″), 114.8(s, C-2′), 75.9(s, C-1), 74.8(d, C-4), 69.2(d, C-3), 68.7(d, C-5), 53.2(q, OCH_3), 38.6 (s, C-2), 38.5(s, C-6). These data were in good agreement with the reported compound, methyl 3, 4-dicaffeoyl quinate [11].

Peak 2: UV λ_{max} (MeOH) nm 218, 241, 327; HR-TOF-MS *m/z*: 531.1568 $([M+H]^+$ call for $C_{26}H_{27}O_{12}$, 531.1503). ^{1}H NMR (400 MHz, MeOD): δ 7.62 (1H, d, *J* = 15.9 Hz, H-3″), 7.55 (1H, d, *J* = 15.9 Hz, H-3″), 7.06 (1H, t, *J* = 2.2 Hz, H-5′), 7.05 (1H, t, *J* = 2.2 Hz, H-5″), 6.97 (2H, dt, *J* = 8.2, 2.0 Hz, H-9' and H-9″), 6.78 (1H, d, *J* = 8.2Hz, H-8′), 6.76 (1H, d, *J* = 8.2Hz, H-8″), 6.34 (1H, d, *J* = 15.9 Hz, H-2″), 6.22 (1H, d, *J* = 15.9 Hz, H-2′), 5.40 (1H, dt, *J* = 7.6, 3.8 Hz, H-5), 5.31 (1H, m, H-3), 3.98 (1H, dd, *J* = 6.5, 3.2 Hz, H-4), 3.68 (3H, s, OCH_3), 2.32 (1H, br d, *J* = 13.4, 5.2Hz, H-2eq), 2.28(1H, br d, *J* = 13.4Hz, H-2ax), 2.22 (2H, br m, H-6). These data were in good agreement with the reported compound, methyl 3, 5-dicaffeoyl quinate [11, 22].

Peak 3: UV λ_{max} (MeOH) nm 217, 243, 327; HR-TOF-MS *m/z*: 531.1506 $([M+H]^+$ call for $C_{26}H_{27}O_{12}$, 531.1503). ^{1}H NMR (300 MHz, MeOD) δ 7.57 (1H, d, *J* = 15.9Hz, H-3′), 7.56 (1H, d, *J* = 15.9Hz, H-3″), 7.04 (1H, d, *J* = 2.2 Hz, H-5′), 7.03 (1H, d, *J* = 2.2 Hz, H-5″), 6.92 (1H, dd, *J* = 8.4, 2.0 Hz, H-9′), 6.90 (1H, dd, *J* = 8.4, 2.0 Hz, H-9″), 6.76 (1H, d, *J* = 8.1 Hz, H-8′), 6.75 (1H, d, *J* = 8.1 Hz, H-8″), 6.28 (1H, d, *J* = 15.9Hz, H-2′), 6.28 (1H, d, *J* = 15.9Hz, H-2″), 5.64 (1H, dt, *J* = 7.3, 3.7 Hz, H-5), 5.05 (1H, dd, *J* = 8.3, 3.3 Hz, H-4), 4.33 (1H, td, *J* = 8.3, 4.8 Hz, H-3), 3.78 (3H, s, OCH_3), 2.37 (1H, dd, *J* = 14.4,

3.9 Hz, H-6_{eq}), 2.21(2H, m, H-2), 2.15 (1H, dd, J = 14.4, 4.6Hz, H-6_{ax}). These data were in good agreement with the reported compound, methyl 4, 5-dicaffeoyl quinate [11].

CONCLUSIONS

Based on LC-TOF-MS analyze target compounds in the *n*-butanol subfractions from *B. pilosa*. CCC has been successful applied to separate three geometrical isomers compounds with a two-phase solvent system composed of chloroform-methanol-water-*n*-butanol at the volume ratio of 4-3-2-0.5 (v/v). Compounds including 36.4 mg methyl 3, 4-dicaffeoyl quinate, 24.3 mg methyl 3, 5-dicaffeoyl quinate, 16.2 mg methyl 4, 5-dicaffeoyl quinate were obtained from the 302 mg crude fraction. Combining LC-TOF-MS guided CCC to isolation these bioactive isomers is a targeted strategy to separate isomeric components or other bioactive natural products. With method described here three bioactive compounds can be isolated on a large scale and preparations for botanical drugs from traditional medicinal *B. pilosa* extract.

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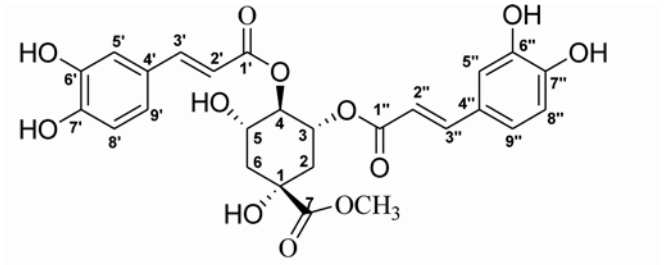
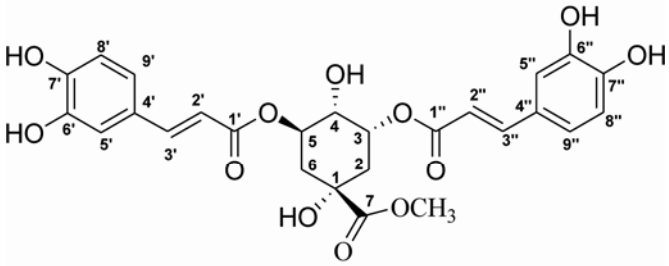
TABLE 1. The *K*-value (partition coefficient) of three target compounds in different two-phase solvent systems used in CCC

Solvent systems	V/V	Compound **1**	Compound **2**	Compound **3**
chloroform-methanol-water	4: 3: 2	2.44	2.85	3.32
chloroform-methanol-water-*n*-butanol	4: 3: 2: 0.2	0.79	2.04	2.68
chloroform-methanol-water-*n*-butanol	4: 3: 2: 0.5	0.54	1.21	1.77
chloroform-methanol-water-*n*-butanol	4: 3: 2: 0.7	0.33	0.78	1.32

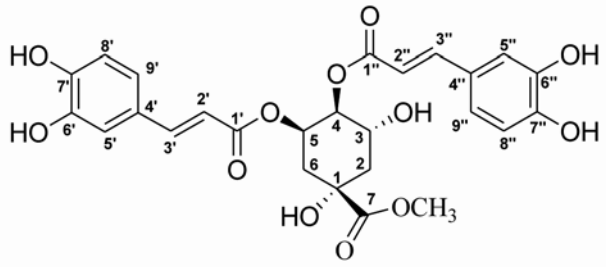
FIGURE 1. Chemical structures of compounds **1-3**



Methyl 3, 4-dicaffeoyl quinate (**1**)



Methyl 3,5-dicaffeoyl quinate (**2**)



Methyl 4,5-dicaffeoyl quinate (**3**)

FIGURE 2. LC-TOF-MS chromatograms of 50% Fr. and the target compounds; D the HPLC chromatogram (254 nm) and MS TIC negative; A, B, C correspond to the peaks 1, 2, and 3, respectively, presenting UV absorption and mass spectra. The LC mobile phase: a gradient mode of acetonitrile-water (0.1% formic acid): 0-35 min, 12%-25% acetonitrile; 35-40 min, 25%-95% acetonitrile; 40-48min, 95%-95% acetonitrile.

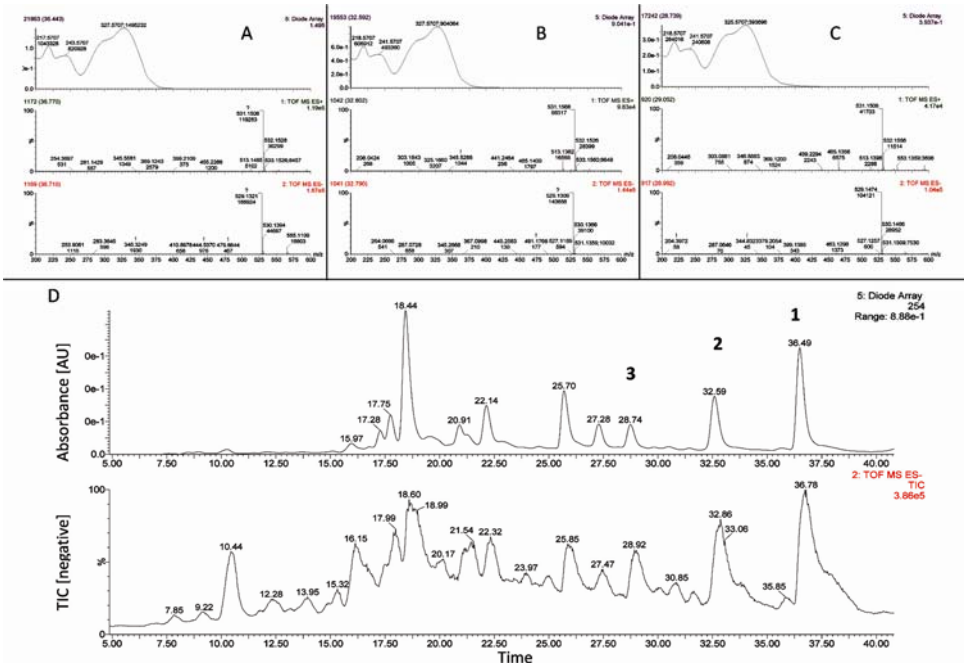


FIGURE 3. CCC chromatogram of the 50% Fr. Solvent system: chloroform–methanol–water–*n*–butanol (4:3:2:0.5, v/v/v/v); mobile phase: lower phase; A is chromatogram of analytical CCC; flow-rate: 0.5 mL/min; detection wavelength: 254 nm; sample size: 0.15 mL (concentraction: 10 mg/mL); separation temperature: 25° C; revolution speed: 1800 rpm. B is chromatogram of semi-preparative CCC; flow rate: 2.0 mL/min; revolution speed: 900 rpm; detection wavelength: 254 nm; separation temperature: 25° C; sample size: 302 mg of crude sample dissolved in 5 mL of the upper phase and 5mL of lower phase; the retention percentage of the stationary phase: 56%; peak 1: methyl-3, 4-dicaffeoyl qunate (B: collected during 95-113min); peak 2: methyl 3, 5-dicaffeoyl quinate (B: collected during 158-170min); peak 3: methyl 4, 5-dicaffeoyl quinate (B: collected during 197-216min).

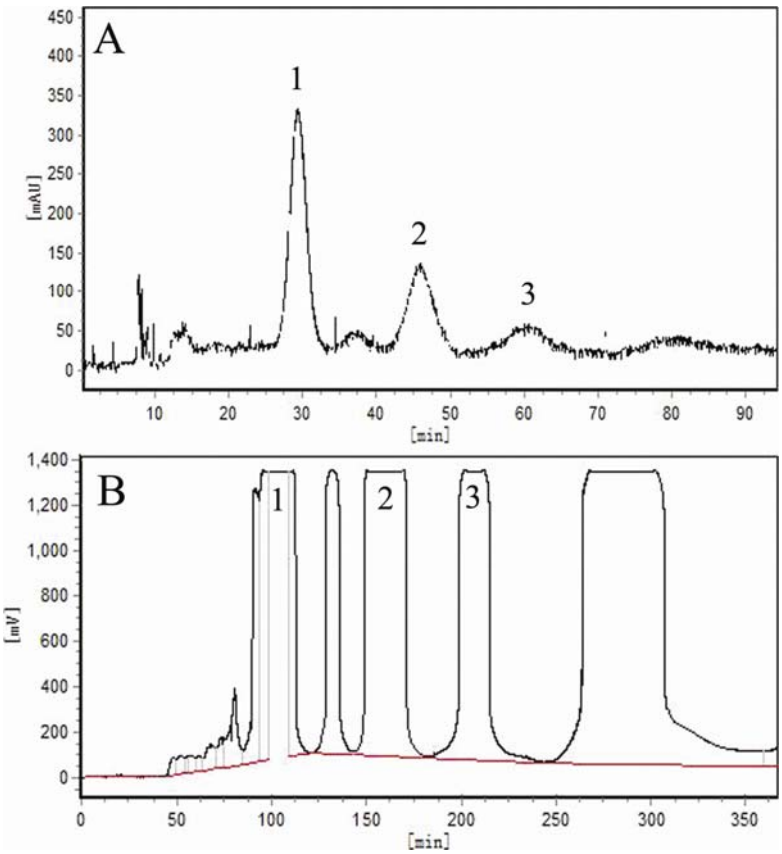


FIGURE 4. HPLC-PDA chromatogram of the 50%Fr. (**a)** and CCC peak fractions. The mobile phase composed of water (0.1% formic acid) and acetonitrile in gradient mode as follows: 0-5 min, 15%-15% acetonitrile; 5-25 min, 15%-25% acetonitrile; 25-30 min, 25%-40% acetonitrile; 30-35 min, 40%-60% acetonitrile; 35-40 min, 60%-95% acetonitrile. All analyses were performed at 25° C with a flow rate of 1 mL/min; detection wavelength: 254nm; (**b)** CCC peak fraction 1, (**c)** CCC peak fraction 2, (**d)** CCC peak fraction 3.