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Preparative separation and purification of four cis–trans isomers of coumaroylspermidine analogs from safflower by high-speed counter-current chromatography

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ABSTRACT

High-speed counter-current chromatography (HSCCC) was successfully applied for the first time to isolate and purify four cis–trans isomers of coumaroylspermidine analogs from Safflower. HSCCC separation was achieved with a two-phase solvent system composed of chloroform–methanol–water (1:1:1, v/v/v) with the upper phase as the mobile phase. In a single run, a total of 1.3 mg of N^1, N^5, N^{10}-(E)-tri-*p*-coumaroylspermidine (EEE), 4.4 mg of N^1(E)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (EZE), 7.2 mg of N^1(Z)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (ZZE), and 11.5 mg of N^1,N^5,N^{10}-(Z)-tri-*p*-coumaroylspermidine (ZZZ) were obtained from 100 mg of crude sample. High Performance Liquid Chromatography (HPLC) analysis showed that the purities of these four components are 95.5%, 98.1%, 97.5% and 96.2%, respectively. The chemical structures were identified by ESI-MS, ^{1}H NMR and ^{13}C NMR.

1. Introduction

Safflower (*Carthamus tinctorius* L.), a member of the family Compositae, is widely cultivated in China and used as herbal medicines for the treatment of inflammatory diseases, arteriosclerosis, hyperlipemia, gynecological disorders, and osteoporosis [1]. Previous studies showed that the main bioactive compounds in Safflower are flavonoids [2], cartharmin, safflower yellow A, quinochalone, safflomin, cartorimine, alkaloids [3], lignans, fatty acids [4], triterpene alcohols [5], and serotonin derivatives [6–8]. Additionally, several coumaroylspermidine analogs were also found in safflower, such as N^1(E)-N^5-(Z)-N^{10}-(Z)-tri-*p*-coumaroylspermidine (EZZ) and its four cis–trans isomers, including N^1,N^5,N^{10}-(E)-tri-*p*-coumaroylspermidine (EEE), N^1(E)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (EZE), N^1,N^5,N^{10}-(Z)-tri-*p*-coumaroylspermidine (ZZZ) [9] and N^1(Z)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (ZZE) [10] (Fig. 1). Preliminary pharmacological study demonstrated that EEE had a moderate inhibitory activity on HIV-1 protease [11] and ZZZ was a potent serotonin transporter (SERT) inhibitor [12]. However, quality control and pharmacological research were seriously blocked due to the lack of reference compounds. Thus, it is urgent to develop an efficient method for the preparation of these compounds with high purities.

Traditionally, EEE, EZE, ZZE and ZZZ were isolated and purified from Safflower by conventional column chromatography such as silica gel and Sephadex LH-20, which usually require large amounts of organic solvents and cause degradation of target compounds. In the preliminary test, we tried to separate these four compounds by Preparative HPLC but failed due to the similar polarity. Finally, high-speed counter-current chromatography (HSCCC) was selected for the preparative separation and purification of these four compounds.

In the present study, an efficient method for the separation and purification of EEE, EZE, ZZE and ZZZ from Safflower was successfully established by HSCCC.

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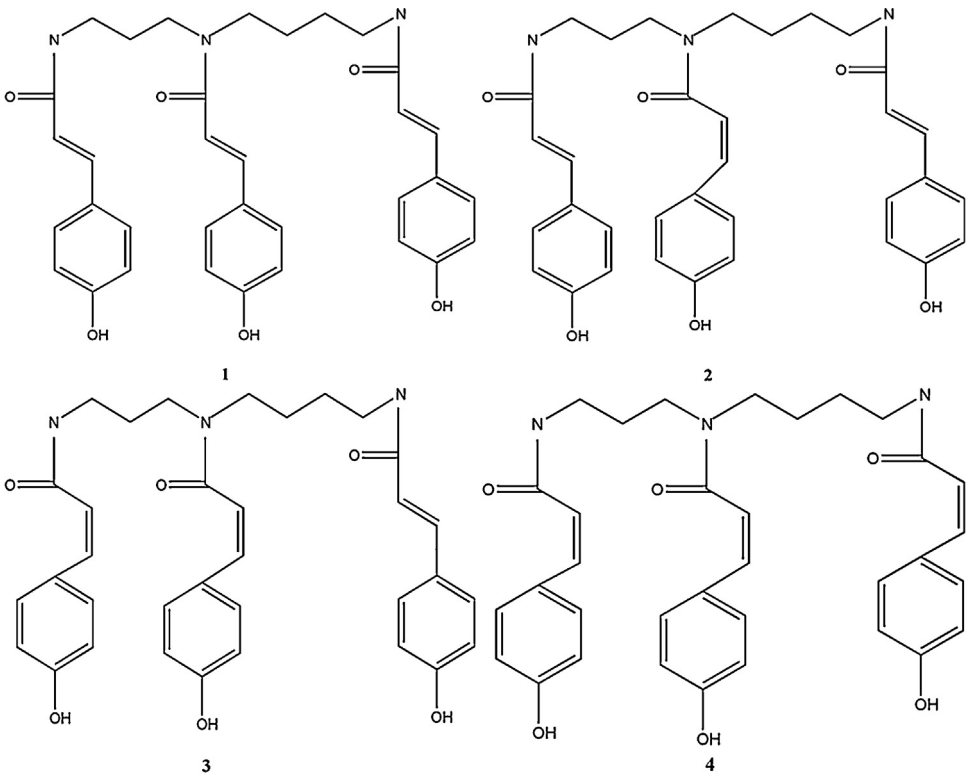

Fig. 1. The chemical structures of (1) N^1,N^5,N^{10}-(E)-tri-*p*-coumaroylspermidine (EEE), (2) N^1(E)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (EZE), (3) N^1(Z)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (ZZE) and (4) N^1,N^5,N^{10}-(Z)-tri-*p*-coumaroylspermidine (ZZZ).

2. Experimental

2.1. Apparatus

HSCCC was performed using a TBE-300A high-speed countercurrent chromatography instrument (Shanghai Tauto Biotech Co., Ltd., Shanghai, China), which equipped with three polytetrafluoroethylene (PTFE) coil separation columns (tube diameter: 1.6 mm, total volume: 305 mL), and a 20 mL sample loop. The separation temperature was controlled by a HX-1050 constant temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China). The revolution radius was 5 cm and the β-values of the multilayer coil ranged from 0.5 (at internal terminal) to 0.8 (at the external terminal, $\beta = r/R$, where r is the distance from the coil to the holder shaft). The revolution speed of the apparatus could be adjusted from 0 to 1000 rpm using a speed controller. An ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Uppsala, Sweden) was used to pump the two-phase solvent system and achieve the UV absorbance measurement. The system contained a switch valve and a mixer, which were used for gradient formation. The data were collected and analyzed by an N2000 workstation (Zhejiang University Star Information Technology Co., Ltd., Hangzhou, Zhejiang, China). An Agilent 1260 High Performance Liquid Chromatography (HPLC) equipped with a Gemini C_{18} column (5 μm, 4.6 mm × 250 mm, Phenomenex Corp. Ltd., USA) was used for HPLC analysis.

2.2. Chemicals and reagents

Safflower (*C. tinctorius* L.) was collected from Datong county, Qinghai Province, China and explicitly determined by Professor Changfan Zhou (Northwest Institute of Plateau Biology, Chinese Academy of Sciences). All organic reagents used for crude sample and HSCCC separation procedure were of analytical grade and produced from Tianjin Chemical Factory (Tianjin, China). The chromatographic grade methanol for HPLC analysis was purchased from Yuwang Chemical Ltd. (Shandong, China). The deionized water used throughout the experimen is from a Milli-Qplus purification system (Millipore Corp., Bedford, MA).

2.3. Preparation of crude sample

The dry florets of safflower (500 g) was extracted with 75% EtOH (10 L, reflux, 2 h × 3). The EtOH extract (90 g) was suspended in H_2O and partitioned into PetEt, EtOAc, and residual fractions. The residual fraction (20 g) was subjected to column chromatography on macroporous resin (AB-8, 400 g), eluted with H_2O, 10%, 30% and 50% EtOH–H_2O to give 4 fractions. The 50% EtOH–H_2O fraction (3 g) was then submitted to purification by HSCCC."

2.4. Selection of the two-phase solvent system

The selected two-phase solvent system was based on the partition coefficient (K) values of each target compound, as the proper K values always manifest a successful isolation. The K values were determined as follows: 2.0–3.0 mg of crude extract was dissolved in the prepared two-phase solvent and shaken violently to achieve equilibration of target compounds between lower and upper phases, and kept still for 30 min. The upper and lower phases were separated and evaporated respectively to dryness by a rotary evaporator at 60 °C, then the residues were dissolved in 2 mL of 50% ethanol fraction and analyzed by HPLC. The K value was determined as the peak area of each target compound in stationary phase divided by in mobile phase.

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

The two-phase solvent system composed of chloroform–methanol–water (1:1:1, v/v/v) was finally selected for

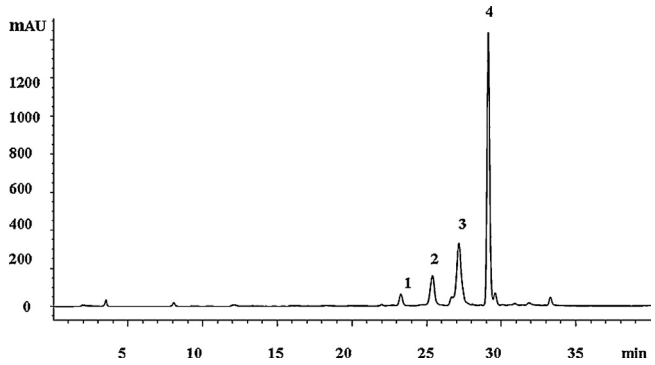

Fig. 2. The HPLC chromatography of the 50% EtOH–H_2O fraction. Column: Gemini C_{18} column (5 μm, 4.6 mm × 250 mm, Phenomenex Corp. Ltd., USA); mobile phase: methanol and water in gradient mode (methanol concentration raised from 30% to 70% in the 40 min); flow rate: 1.0 mL/min; column temperature: 25 °C; detection wavelength: 280 nm.

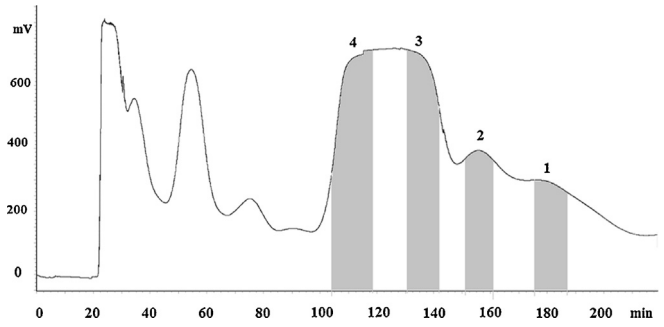

Fig. 3. HSCCC chromatogram of the crude sample from Safflower. Two-phase solvent system: chloroform–methanol–water (1:1:1, v/v/v); stationary phase: lower phase; mobile phase: the upper phase; flow-rate: 2.0 mL/min; revolution speed: 850 rpm; detection wavelength: 280 nm; sample size: 100 mg of crude sample dissolved in 10 mL of the upper phase; separation temperature: 25 °C.

HSCCC separation. The solvent was completely equilibrated in a separatory funnel according to their corresponding proportions. Then the upper phase (aqueous phase) and lower phase (organic phase) were separated and degassed in an ultrasonic bath for 30 min before using. The sample solution was prepared by dissolving 100 mg of crude extract in 10 mL of upper phase solvent for separation.

2.6. Procedure of HSCCC separation

First, the lower phase (organic phase) was regard as the stationary phase and pumped into the multilayer coiled column at a flow rate of 15.0 mL/min by a fluid metering pump until it is entirely full. Then, the column was rotated at 850 rpm, while the upper aqueous phase was pumped into the column at the flow rate of 2.0 mL/min in the 'head to tail' mode. The effluent was constantly monitored with a UV detector at 280 nm and the separation was conducted under 25 °C. After hydrodynamic equilibration was achieved, 10 mL of sample solution was injected into the separation column and the chromatogram was recorded and peak fractions were manually collected according to the chromatogram data.

2.7. HPLC analysis and identification of HSCCC peak fractions

The crude extract and each peak fraction obtained by HSCCC were analyzed by HPLC equipped with a Gemini C_{18} (5 μm, 250 mm × 4.6 mm) column with gradient elution at a column temperature of 25 °C (Fig. 2). The gradient elution began with 30% methanol and linearly increased to 70% methanol in 40 min at a constant flowrate of 1.0 mL/min, with the detector measuring at 280 nm. The identification of each HSCCC peak fraction was carried out by ESI-MS, ^{1}H NMR and ^{13}C NMR.

3. Result and discussion

3.1. Selection of two-phase-solvent system and other conditions of HSCCC procedure

A successful separation by HSCCC depends on the selection of a suitable two-phase solvent system, which requires the following considerations [13,14]: (1) The target compounds should be soluble and stable in the solvent system; (2) the setting time of the solvent system should be short (*i.e.* <30 s); (3) the partition of the target compounds between two-phase solvent should be appropriate (*i.e.* usually between 0.2 and 5); (4) the separation factors ($\alpha = K_1/K_2$, $K_1 > K_2$) between any two compounds all should be greater than 1.5.

In this study, a series of experiments were performed by HPLC to optimize the two-phase solvent system for HSCCC separation. The *K*-values of the four target compounds are shown in Table 1. The two-phase solvent systems with *n*-hexane–ethyl acetate–methanol–water (1:5:1:5, 6:20:5:18, 3:10:3:8, 2:5:1:4, 7:20:4:16, v/v/v/v) were tested first, the separation factors between any two compounds were too small and not appropriate for the separation of the four target compounds from the crude sample. Then, two-phase solvent systems comprised of *n*-hexane–ethyl acetate–ethanol–water (1:2:1:2, 6:12:6:11, v/v/v/v) were further investigated, the separation factors between compounds 1, 2 and 3 were too small. Finally, two-phase solvent systems comprised of chloroform–methanol–water (1:1:1, v/v/v), the *K* values of the four target compounds were between 0.2 and 5, and the separation factors were large enough. Finally, when petroleum chloroform–methanol–water (1:1:1, v/v/v) was used, good separation results could be obtained and the separation time was acceptable.

The four target compounds were separated by HSCCC using a single solvent system composed of chloroform–methanol–water (1:1:1, v/v/v) (HSCCC chromatogram shown in Fig. 2). Although high temperature would produce high retention of the stationary phase and low temperature would cause the stationary phase loss, air bubble increased along with the increasing of temperature and high temperature would lessen the life of the apparatus. The column temperature was set at 25 °C in our experiments. In addition, high rotary velocity can cause emulsification and damage the separation pipelines, and the 850 rpm speed in our isolation procedure was applied. Then the flow rate was set at 2.0 mL/min in the present separation and the retention of the stationary phase was 79.8%.

Under the optimized conditions, four fractions (1, 2, 3 and 4) were obtained in one step separation within 210 min. Component 1 (1.3 mg) elution time 176–187 min; Component 2 (4.4 mg) elution time 151–160 min; Component 3 (7.2 mg) elution time 130–140 min; Component 4 (11.5 mg) with elution time 103–115 min (Fig. 3). As shown in Fig. 3, the HPLC analysis of each HSCCC fractions revealed that the purities of these four compounds were 95.5%, 98.1%, 97.5% and 96.2%, respectively.

3.2. The structure identification of the HSCCC peak fractions

According to ESI-MS, ^{1}H NMR and ^{13}C NMR data, the chemical structures of the peak fractions separated by HSCCC were identified. Through comparison with reference data, peak 1, peak2, peak 3 and peak 4 (Fig. 3) were effectively identified as N^1,N^5,N^{10}-(E)-tri-*p*-coumaroylspermidine (EEE), N^1(E)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (EZE),

Table 1
The *K* values of the target compounds in different solvent systems.

Solvent systems	Volume ratio (v/v)	*K* values			
		1	2	3	4
n-Hexane–ethyl acetate–methanol–water	1:5:1:5	0.41	0.38	0.29	0.28
n-Hexane–ethyl acetate–methanol–water	6:20:5:18	0.39	0.37	0.28	0.24
n-Hexane–ethyl acetate–methanol–water	3:10:3:8	0.65	0.51	0.49	0.46
n-Hexane–ethyl acetate–methanol–water	2:5:1:4	0.67	0.64	0.55	0.48
n-Hexane–ethyl acetate–methanol–water	7:20:4:16	0.70	0.56	0.47	0.44
n-Hexane–ethyl acetate–ethanol–water	1:2:1:2	0.97	0.89	0.88	0.56
n-Hexane–ethyl acetate–ethanol–water	6:12:6:11	1.04	0.90	0.85	0.57
Chloroform–methanol–water	1:1:1	1.41	1.02	0.74	0.56

N^1(Z)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (ZZE) and N^1,N^5,N^{10}-(Z)-tri-*p*-coumaroylspermidine (ZZZ) (Fig. 1), respectively. Results for each peak fraction were as follows.

3.2.1. Peak fraction 1

White powder, ESI-MS: *m*/*z* 584 $[M+H]^+$; ^{1}H NMR spectrum (CD_3OD, 600 MHz) δ = 1.44/1.57 (2H, m, H-8), 1.48/1.64 (2H, m, H-7), 1.83/1.91 (2H, m, H-3), 3.03/3.21 (2H, m, H-9), 3.10/3.27 (2H, m, H-2), 3.30/3.33 (2H, m, H-6), 3.41/3.45 (2H, m, H-4), 6.36/6.41 (d, *J* = 15.5 Hz, H-8′), 6.50/6.53 (d, *J* = 15.5 Hz, H-8‴), 6.65/6.72 (m, *J* = 8.5 Hz, H-3″, 5″), 6.79 (m, *J* = 8.5 Hz, H-3′, 5′), 6.80 (m, *J* = 8.5 Hz, H-3‴, 5‴), 6.85/6.93 (d, *J* = 15.5 Hz, H-8″), 7.28/7.35 (d, *J* = 8.5 Hz, H-2′, 6′), 7.38 (d, *J* = 8.5 Hz, H-2‴, 6‴), 7.44 (d, *J* = 15.5 Hz, H-7′), 7.44/7.50 (d, *J* = 8.5 Hz, H-2″, 6″), 7.50/7.52 (d, *J* = 15.5 Hz, H-7‴), 7.52/7.57 (d, *J* = 15.5 Hz, H-7″); ^{13}C NMR spectrum (CD_3OD, 600 MHz) δ = 25.5/26.8 (C-7), 27.2/27.7 (C-8), 28.3/29.6 (C-3), 37.7/38.1 (C-2), 39.4/39.7 (C-9), 44.8/46.5 (C-4), 47.1/48.5 (C-6), 113.4 (C-8″), 116.1 (C-3′, 3″, 3‴, 5′, 5″, 5‴), 117.9/118.1 (C-8′), 118.2 (C-8‴), 127.5 (C-1′, 1‴), 128.0 (C-1″), 129.5 (C-2′, 6′, 2‴, 6‴), 130.2 (C-2″, 6″), 141.9/142.0 (C-7′, 7‴), 143.9 (C-7″), 159.7/160.0 (C-4′, 4″, 4‴), 169.2/169.3 (C-9′, C-9‴), 169.4 (C-9″). Comparing with the reported data, the ESI-MS, ^{1}H NMR and ^{13}C NMR data are in agreement with that of N^1,N^5,N^{10}-(E)-tri-*p*-coumaroylspermidine (EEE) in the literature [11].

3.2.2. Peak fraction 2

White powder, ESI-MS: *m*/*z* 584 $[M+H]^+$; ^{1}H NMR spectrum (CD_3OD, 600 MHz) δ = 1.39/1.53 (2H, m, H-8), 1.46/1.64 (2H, m, H-7), 1.66/1.82 (2H, m, H-3), 3.12/3.26 (2H, m, H-2), 3.15/3.26 (2H, m, H-9), 3.19/3.27 (2H, m, H-6), 3.34/3.39 (2H, m, H-4), 5.88/5.93 (d, *J* = 12.5 Hz, H-8″), 6.32/6.35 (m, *J* = 15.5 Hz, H-8‴), 6.39/6.44 (m, *J* = 15.5 Hz, H-8′), 6.49/6.54 (m, *J* = 12.5 Hz, H-7″), 6.73 (m, *J* = 8.5 Hz, H-3″, 5″), 6.77 (m, *J* = 8.5 Hz, H-3′, 3‴, 5′, 5‴), 7.19 (d, *J* = 8.5 Hz, H-2″, 6″), 7.30 (d, *J* = 8.5 Hz, H-2′, 6′), 7.38 (d, *J* = 8.5 Hz, H-2‴, 6‴), 7.42/7.46 (d, *J* = 15.5 Hz, H-7′, 7‴); ^{13}C NMR spectrum (CD_3OD, 600 MHz) δ = 25.3/26.7 (C-7), 27.3/27.7 (C-8), 28.0/29.5 (C-3), 37.5/37.9 (C-2), 39.4/39.7 (C-9), 44.9/46.6(C-4), 47.2/48.7 (C-6), 115.7 (C-3″, 5″), 115.9 (C-3′, 3‴, 5′, 5‴), 117.9/118.0 (C-8′, 8‴), 120.3/120.4 (C-8″), 127.2 (C-1′, 1‴), 127.9 (C-1″), 129.7 (C-2′, 6′, 2‴, 6‴), 130.5 (C-2″, 6″), 134.5/134.8 (C-7″), 141.9/142.0 (C-7′, 7‴), 158.2 (C-4″), 159.7/159.8 (C-4′, 4‴), 169.4/169.5 (C-9′, 9‴), 171.9/172.0 (C-9″). Comparing with the reported data, the ESI-MS, ^{1}H NMR and ^{13}C NMR data are in agreement with that of N^1(E)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (EZE) in the literature [9].

3.2.3. Peak fraction 3

White powder, ESI-MS: *m*/*z* 584 $[M+H]^+$; ^{1}H NMR spectrum (CD_3OD, 600 MHz) δ = 1.34/1.43 (2H, m, H-8), 1.38/1.50 (2H, m, H-7), 1.61/1.74 (2H, m, H-3), 3.11/3.28 (4H, m, H-2, 9), 3.31/3.33 (2H, m, H-6), 3.36/3.37 (2H, m, H-4), 5.72/5.75 (d, *J* = 12.5 Hz, H-8′), 5.88/5.91 (d, *J* = 12.5 Hz, H-8″), 6.33/5.35 (m, *J* = 15.5 Hz, H-8‴), 6.41/6.42 (m, *J* = 12.5 Hz, H-7″), 6.45/6.46 (m, *J* = 12.5 Hz, H-7′), 6.68 (m, *J* = 8.5 Hz, H-3′, 3″, 5′, 5″), 6.73 (m, *J* = 8.5 Hz, H-3‴, 5‴), 7.18 (d, *J* = 8.5 Hz, H-2″, 6″), 7.26/7.29 (m, *J* = 15.5 Hz, H-7‴), 7.35 (d, *J* = 8.5 Hz, H-2‴, 6‴), 7.53 (d, *J* = 8.5 Hz, H-2′, 6′); ^{13}C NMR spectrum (CD_3OD, 600 MHz) δ = 25.4/26.8 (C-7), 27.5/27.8 (C-8), 28.1/29.5 (C-3), 37.4/37.7 (C-2), 39.2/39.5 (C-9), 44.8/46.6 (C-4), 47.5/48.8 (C-6), 115.9 (C-3″, 5″), 116.2 (C-3′, 5′), 116.7 (C-3‴, 5‴), 117.9/118.1 (C-8‴), 120.5 (C-8″), 121.2 (C-8′), 127.2 (C-1‴), 127.7 (C-1′, 1″), 129.7 (C-2‴, 6‴), 130.4/130.5 (C-2″, 6″), 131.5/131.6 (C-2′, 6′), 134.5/134.8 (C-7‴), 137.4/137.5 (C-7′), 141.0/142.1 (C-7‴), 158.3/158.4 (C-4′, 4″), 159.8/159.9 (C-4‴), 169.4/169.5 (C-9‴), 170.6/170.7 (C-9′), 171.7/171.8 (C-9″). Comparing with the reported data, the ESI-MS, ^{1}H NMR and ^{13}C NMR data are in agreement with that of N^1(Z)-N^5-(Z)-N^{10}-(E)-tri-*p*-coumaroylspermidine (ZZE) in the literature [10].

3.2.4. Peak fraction 4

White powder, ESI-MS: *m*/*z* 584 $[M+H]^+$; ^{1}H NMR spectrum (CD_3OD, 600 MHz) δ = 1.40/1.52 (2H, m, H-8), 1.41/1.55 (2H, m, H-7), 1.65/1.78 (2H, m, H-3), 3.12/3.29 (4H, m, H-2, 9), 3.32/3.35 (2H, m, H-6), 3.35/3.36 (2H, m, H-4), 5.77/5.80 (d, *J* = 12.5 Hz, H-8‴), 5.83/5.86 (d, *J* = 12.5 Hz, H-8′), 5.89/5.93 (d, *J* = 12.5 Hz, H-8″), 6.50/6.52 (m, *J* = 12.5 Hz, H-7‴), 6.56/6.59 (m, *J* = 12.5 Hz, H-7′), 6.61/6.64 (m, *J* = 12.5 Hz, H-7‴), 6.72 (m, *J* = 8.5 Hz, H-3′, 3″, 3‴, 5′, 5″, 5‴), 7.18 (d, *J* = 8.5 Hz, H-2″, 6″), 7.39 (d, *J* = 8.5 Hz, H-2′, 2‴, 6′, 6‴); ^{13}C NMR spectrum (CD_3OD, 600 MHz) δ = 25.3/26.7 (C-7), 27.1/27.6 (C-8), 28.3/29.7 (C-3), 37.6/38.1 (C-2), 39.5/39.9 (C-9), 44.5/46.2 (C-4), 47.3/48.6 (C-6), 116.3/116.4 (C-3′, 3″, 5′, 5″), 116.7 (C-3″, 5″), 120.5 (C-8″), 121.2/121.3 (C-8′, 8‴), 127.6/127.7 (C-1′, 1‴), 127.8/127.9 (C-1″), 130.4 (C-2′, 6′, 2″, 6″), 131.5/131.6 (C-2‴, 6‴), 134.5/134.8 (C-7″), 137.2/137.3 (C-7‴), 137.8/138.1 (C-7′), 158.5/158.6 (C-4′, 4″, 4‴), 169.9/170.1 (C-9′, 9‴), 17 (C-9″). Comparing with the reported data, the ESI-MS, ^{1}H NMR and ^{13}C NMR data are in agreement with that of N^1,N^5,N^{10}-(Z)-tri-*p*-coumaroylspermidine (ZZZ) in the literature [9].

4. Conclusions

In this study, four cis–trans isomers of coumaroylspermidine analogs were successfully separated from safflower by HSCCC for the first time. Four compounds were isolated and purified using stepwise elution with the two-phase solvent system composed of chloroform–methanol–water (1:1:1, v/v/v). The results demonstrated that this method was efficient and rapid for the separation of coumaroylspermidine analogs from safflower.

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