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Article

Preparative Separation of *N*-Feruloyl Serotonin and *N*-(*p*-Coumaroyl) Serotonin from Safflower Seed Meal Using High-Speed Counter-Current Chromatography

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High-speed counter-current chromatography (HSCCC) was successfully applied for the preparative separation and purification of *N*-feruloyl serotonin (NF) and *N*-(*p*-coumaroyl) serotonin (NP) from safflower seed meal. After the measurement of partition coefficient of the two target compounds in the two-phase solvent systems, the HSCCC was performed well with a two-phase solvent system composed of $CHCl_3$–methanol–0.1 M HCl at a volume ratio of 1 : 1 : 1, v/v. The upper phase was used as stationary phase and the lower phase was used as mobile phase. Under the optimized condition, 7.5 mg NF and 6.9 mg NP were separated from 40 mg crude sample with the purity of 98.8 and 97.3%, respectively. The structures of the isolated compounds were identified by ^{1}H NMR and ^{13}C NMR.

Introduction

Safflower is grown primarily for its oil, which may be either the common high linoleic type or the more recently developed high oleic type (1). The meal was obtained after extraction of the oil from safflower seed. In the United States, safflower seed meal is commonly classified by screening to yield a high protein fraction containing 42% protein and 16% crude fiber, and a high fiber fraction containing 20% protein and 38% fiber (2). Modern analytical chemistry and natural product chemistry researches have found that the *N*-(*p*-coumaroyl) serotonin (NP) and *N*-feruloyl serotonin (NF) (see Figure 1) in safflower seed meal possessed many pharmacological activities (3), such as anti-oxidative (4), radical-scavenging (4), melanime inhibition (5), anti-tumor (6), anti-tyrosinase (7), fibroblasts growth promoting, pain and anxiety relieving activities (8), proinflammatory cytokine inhibition (9) and cathartic effects (10). However, since safflower seed meal is bitter and mildly cathartic, the safflower seed meal is just used as cheap feedstuff (2). Considering the beneficial activities of NF and NP extracted from this herb and to control the quality of this herb, it is urgent to establish an efficient method to separate and purify the two serotonin derivatives from this plant. In the past, the method of separation of the NF and NP from safflower seed meal was carried out by column chromatography (11). However, there were some problems of these methods such as tedious, time-consuming and requiring multiple chromatographic steps resulting in low recovery and high cost.

High-speed counter-current chromatography (HSCCC), first invented by Ito (12), is a kind of continuous liquid–liquid partition chromatography technique without using solid support matrix, which has many advantages such as no irreversible adsorption, low risk of degradation, high sample recovery and low cost (13–17). To the best of our knowledge, no paper about isolation and purification of NP and NF form safflower seed meal using HSCCC has been reported. In this study, a method of preparative separation and purification of NP and NF from safflower seed meal by HSCCC has been successfully established. The economic value of safflower seed meal can be greatly increased through separation and utilization of the active compounds of NP and NF, which would not only increase the economic efficiency of safflower but also stimulate the cultivation of safflower.

Experimental

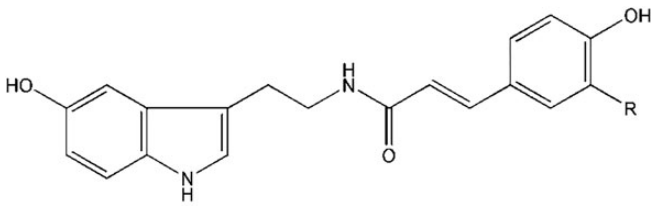
Reagents and materials

Carthamus tinctorius L. (safflower) seed meal was obtained from Xinjiang province, China, and identified by Engineer Changfan Zhou of the Northwest Institute of Plateau Biology, Chinese Academy of Science.

All organic solvents used for sample preparation and HSCCC were of analytical grade and purchased from Tianjin Chemical Factory (Tianjin, China). Methanol used for HPLC analysis was of chromatographic grade and purchased from Yuwang Chemical Ltd (Shandong, China). Deionized water was used throughout the experiment.

Apparatus

HSCCC was performed using a TBE-300A HSCCC instrument (Shanghai Tauto Biotech Co., Ltd, Shanghai, China) with PTFE (polytetrafluoroethylene), three preparative coils (tube diameter: 1.6 mm, total volume: 280 mL) and a 20-mL sample loop. The revolution radius or the distance between the holder axis and the central axis of the centrifuge (R) was 5 cm. 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 HSCCC instrument was assembled by AKTA prime (GE Co. Ltd., Fairfield, CA, USA)



R=H N-feruloyl serotonin

R=OCH_3 N-(p-coumaroyl) serotonin

Figure 1. Chemical structures of *N*-ferroalloy serotonin and *N*-(*p*-coumaroyl) serotonin.

with a UV absorbance detector to monitor the effluent, an N2000 workstation (Zhejiang University Star Information Technology Co., Hangzhou, Zhejiang, China) to collect the data, and an HX 1050 constant–temperature module (Beijing Boyikang Laboratory Instrument Company, Beijing, China) for temperature control. The revolution speed of the apparatus can be adjusted from 0 to 1000 rpm.

The HPLC system was Agilent 1200 system consisting of a G1354A solvent delivery unit, a G1315B UV–vis photodiode array detector, a G1316A column thermostat, a G1313A autosampler, an XDB-C18 (5 μm, 4.6 × 250 mm analytical column) and an Agilent HPLC workstation (Agilent Technology Co. Ltd., Palo Alto, CA, USA). The nuclear magnetic resonance (NMR) spectrometer was a Mercury-600BB NMR (Varian Co. Ltd, Palo Alto, CA, USA).

Preparation of the crude sample

The dried seed meal (0.3 kg) of the safflower was grounded into powder and extracted three times using 100% ethanol under reflux (each time for 1 h), and the solid–liquid ratio was 1:15 g/mL. All the filtrate was combined and evaporated to dryness under vacuum at 60°C. Then it was dissolved in 80% methanol (500 mL) and extracted with *n*-hexane. The methanol phase was evaporated to dryness and dissolved in ethyl acetate (300 mL). Then it was extracted with distilled water (300 mL). The ethyl acetate phase was evaporated to dryness to generate 2.1 g of crude sample for subsequent HSCCC isolation and purification.

Measurement of the partition coefficient

The partition coefficient was the concentration ratio of the target compounds distributed in the mutually equilibrated two solvent phase. A number of two-phase solvent systems were tested in order to obtain the optimum solvent system that could give suitable partition coefficient (*K*) values. The *K* values were determined according to the literature (18). In brief, a suitable amount of crude sample was added into a series of pre-equilibrated two-phase solvent systems, and the solution was shaken fully. And then, an equal volume of the upper and lower phase was separately evaporated to dryness. The residues were diluted into 2 mL methanol and then analyzed by HPLC. The *K* value was calculated as follows:

$$K = \frac{\text{HPLC peak area of solution in upper phase}}{\text{HPLC peak area of solution in lower phase}}.$$

Preparation of the two-phase solvent system and sample solution

The selected two-phase solvent system used in the present study was prepared by mixing $CHCl_3$–methanol–0.1 M HCl at a volume ratio of 1 : 1 : 1, v/v. After thoroughly equilibrated in a separated funnel at room temperature, the two phases were separated shortly before use. The sample solution was prepared by dissolving 40 mg of dried crude sample in 10 mL upper phase for isolation and purification.

HSCCC separation procedure

The HSCCC was performed as follows: the multilayer column was first completely filled with the upper phase (stationary) using the AKTA prime plus system. Then the apparatus was rotated at 850 rpm (forward direction), and the lower phase was pumped into the column at a flow rate of 1.8 mL/min when the revolution velocity was smooth. After hydrodynamic equilibrium was reached, the sample solution was injected into the column through the injection valve. The effluent from the tail end of the column was continuously monitored by a UV detector at 310 nm. Peak fractions were manually collected according to the chromatogram data, and each fraction was evaporated to dryness under reduced pressure and dissolved in methanol for subsequent purity analysis by HPLC. After the separation was completed, the apparatus was stopped and the retention of stationary phase was measured by collecting the column content into a graduate cylinder through forcing them out of the column with pressurized nitrogen gas.

HPLC analysis and identification of the HSCCC peak fractions

The crude sample and each HSCCC peak fraction were analyzed by HPLC (Figure 2). The HPLC was performed on a reversed-phase Agilent Eclipse XDB-C18 analytical column with gradient elution, and the column temperature was set at 25°C. The mobile phases were methanol and water and the gradient process was as follows: 0–15 min, 35–45% methanol; 15–30 min, 45–52% methanol. All solvents were filtered through a 0.45-μm filter prior to use. The flow-rate and detection wavelength were set at 1.0 mL/min and 310 nm, respectively. Identification of the HSCCC peak fractions was carried out by ^{1}H NMR and ^{13}C NMR spectra.

Results

The separation of NP and NF from safflower seed meal by HSCCC

After the measurement of partition coefficient of the two target compounds in the two-phase solvent systems, the two-phase solvent system composed of CHC_{13}–methanol–0.1 M HCl at a volume ratio of 1 : 1 : 1, v/v was selected in this study. Under the optimized condition, two fractions (7.5 mg component 1 and 6.9 mg component 2) were separated from 40 mg crude sample within 180 min, and the HSCCC chromatogram was shown in Figure 3. The HPLC analysis of each HSCCC fraction revealed that the purity of the two compounds was 97.3 and 98.8%, respectively (Figure 2).

Identification of the HSCCC fractions

According to ^{1}H NMR, ^{13}C NMR data and comparison with the literature data, the two target compounds were identified as *N*-feruloyl serotonin (compound **1**) and *N*-(*p*-coumaroyl)

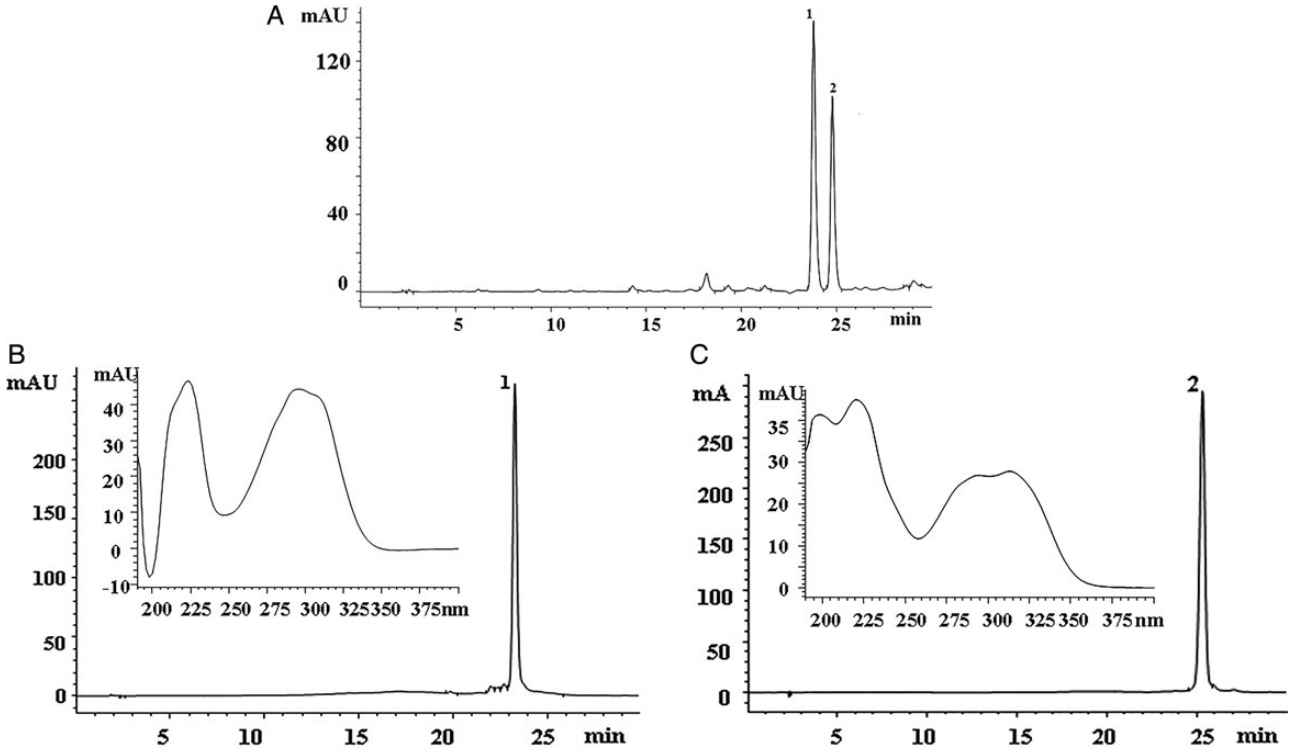


Figure 2. The HPLC chromatography of the crude sample (A) and the two target compounds (B and C) separated and purified by HSCCC. Conditions: column Agilent Eclipse XDB-C18 (5 μm, 4.6 × 250 mm); column temperature 25°C; mobile phase: methanol and water (methanol: 0–15 min, 35–45%; 15–30 min, 45–52%); flow rate 1.0 mL/min; detection wavelength 310 nm.

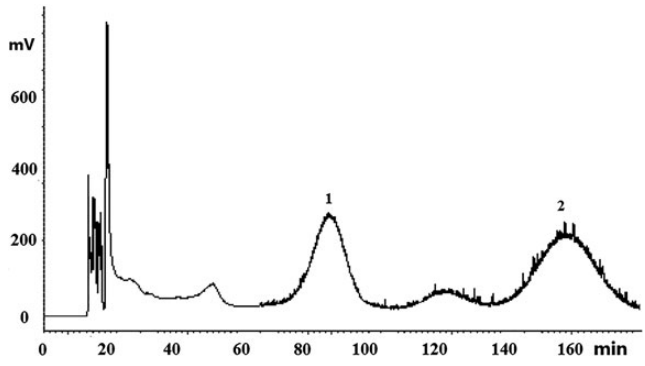


Figure 3. HSCCC chromatogram of the safflower seed meal crude sample. Two-phase solvent system: chloroform–methanol–0.1 M HCl; stationary phase: the upper phase; mobile phase: lower phase; flow rate: 1.8 mL/min; revolution speed: 850 rpm; detection wavelength: 310 nm; sample size: 80 mg of the crude sample dissolved in 10 mL of the upper phase; separation temperature: 25°C.

serotonin (compound **2**), respectively. The spectroscopic data of the two compounds were listed below.

Compound **1**. ^{1}H NMR (DMSO-d_6, 600 MHz) δ = 2.97 (2H, t), 3.55 (2H, t), 6.37 (1H, d, J = 15.5 Hz), 6.70 (1H, d, J = 7.5 Hz), 6.81 (1H, d, J = 8.5 Hz), 6.82 (1H, d, J = 8.5 Hz), 6.95 (1H, d, J = 2.5 Hz), 7.07 (1H, S), 7.18 (1H, d, J = 8.7 Hz), 7.40 (2H, d, J = 8.5 Hz), 7.43 (1H, d, J = 15.5 Hz); (DMSO-d_6, 600 MHz) δ = 25.071 (C-10), 40.03 (C-11), 102.11 (C-4), 111.08 (C-6), 111.39 (C-7), 111.51 (C-3), 115.24 (C-3′, 5′), 117.38 (C-8′), 122.77 (C-2), 126.71 (C-1′), 128.17 (C-9), 129.35 (C-2′, 6′), 131.86 (C-8), 140.47 (C-7′), 149.82 (C-5), 159.32 (C-4′), 167.98 (CO).

Compound **2**. ^{1}H NMR ((DMSO-d_6, 600 MHz) δ = 2.97 (2H, t), 3.56 (2H, t), 3.86 (3H), 6.38 (1H, d, J = 15.5 Hz), 6.71 (1H, d, J = 2.5 Hz), 6.82 (1H, d, J = 8.5 Hz), 6.95 (1H, d, J = 2.5 Hz), 7.05 (1H, d, J = 2.2 Hz), 7.07 (1H, S), 7.15 (1H, d, J = 1.5 Hz), 7.18 (1H, d, J = 8.7 Hz), 7.42 (1H, d, J = 15.5 Hz); ^{13}C NMR (DMSO-d_6, 600 MHz) δ = 25.07 (C-10), 40.09 (C-11), 54.99 (OCH3), 102.13 (C-4), 110.51 (C-6′), 111.10 (C-6), 111.41 (C-7), 111.55 (C-3), 115.24 (C-3′), 117.68 (C-8′), 121.97 (C-2′), 122.98 (C-2), 127.11 (C-1′), 128.25 (C-9), 131.92 (C-8), 140.69 (C-7′), 147.95 (C-5′), 148.60 (C-4′), 149.87 (C-5), 167.95 (CO).

Compared with the reported data, the ^{1}H and ^{13}C NMR were in agreement with that of *N*-feruloyl serotonin (compound **1**) and *N*-(*p*-coumaroyl) serotonin (compound **2**) in the literatures (19, 20).

Discussions

Selection of solvent system and other HSCCC conditions

In HSCCC separation, a suitable two-phase solvent system is critical, which requires the following considerations (18, 21): (i) the target compounds should be soluble and stable in the solvent system; (ii) the retention of the stationary phase should be satisfactory; (iii) the setting time of the solvent system should be <30 s; (iv) the partition coefficient (K) of the target compounds should be appropriate (usually between 0.5 and 5); (v) the separation factor (α) between the two components ($\alpha = K_1/K_2$, $K_1 > K_2$)

Table I
The *K* Values of the Target Components in Several Solvent Systems

Solvent system	Ratio (v/v)	K_1	K_2
n-Hexane–ethyl acetate–methanol–water	1 : 1 : 1 : 1	15.05	15.72
n-Hexane–ethyl acetate–methanol–water	3 : 7 : 5 : 5	2.56	2.88
n-Hexane–ethyl acetate–methanol–water	2 : 8 : 6 : 4	1.91	2.06
n-Hexane–ethyl acetate–methanol–water	3 : 6 : 5 : 5	15.05	13.18
n-Hexane–ethyl acetate–methanol–water	3 : 7 : 6 : 5	14.91	14.85
n-Hexane–ethyl acetate–methanol–water	2 : 8 : 5 : 4	9.09	9.22
n-Hexane–ethyl acetate–methanol–water	4 : 8 : 6 : 5	5.49	5.56
Chloroform–methanol–water	1 : 1 : 1	2.62	1.11
Chloroform–methanol–0.1 M HCl	1 : 1 : 1	3.69	1.15

should be >1.5 in the semi-preparative multilayer separation column of a commercial HSCCC unit.

Therefore, some two-phase solvent systems including *n*-hexane–ethyl acetate–methanol–water (1 : 1 : 1 : 1, 3 : 6 : 5 : 5, 3 : 7 : 6 : 5, 2 : 8 : 5 : 4, 4 : 8 : 6 : 5, 3 : 7 : 5 : 5, 2 : 8 : 6 : 4, v/v), chloroform–methanol–water (1 : 1 : 1, v/v), and chloroform–methanol–0.1 M HCl (1 : 1 : 1, v/v) were tested in this paper. The *K* and α values of the two target compounds were listed in Table 1. The two-phase solvent systems with *n*-hexane–ethyl acetate–methanol–water (1 : 1 : 1 : 1, 3 : 6 : 5 : 5, 3 : 7 : 6 : 5, 2 : 8 : 5 : 4, 4 : 8 : 6 : 5, v/v) were firstly tested, and the *K* values of the target compounds did not satisfy the conditions mentioned above. Then, two-phase solvent systems constituted of *n*-hexane–ethyl acetate–methanol–water (2 : 8 : 6 : 4, 3 : 7 : 5 : 5, v/v) were further investigated, but the separation factors between the compounds were too small and not suitable for the separation of the two target compounds from the crude sample. Finally, the two-phase solvent system composed of chloroform–methanol–water (1 : 1 : 1, v/v) and chloroform–methanol–0.1 M HCl (1 : 1 : 1, v/v) were tested. The results showed that the *K* values of the two target compounds were both between 0.2 and 5, and the separation factor was >1.5. The two target compounds could be separated well by both of the two solvent systems. However, the separation effect of the solvent system (chloroform–methanol–0.1 M HCl) was better than that of the solvent system (chloroform–methanol–water), so the solvent system (chloroform–methanol–0.1 M HCl) was finally selected for the preparative separation of NF and NP from safflower seed meal by HSCCC. In addition to the *K* values and the separation factors of the target compounds in the two-phase solvent system, other important factors including flow rate (1.6, 1.8 and 2.0 mL/min) of the mobile phase and the revolution speed (800, 850 and 900 rpm) of the apparatus were also studied. The results showed that the low flow rate of the mobile phase led to a long separation time, but it could improve the peak resolution and increased stationary phase retention. Considering the elucidated separation time and the peak resolution, a flow rate of 1.8 mL/min was used in subsequent HSCCC separation procedures. Additionally, the high revolution speed could increase the retention of the stationary phase, but it could also cause emulsification and damage the separation pipelines. Ultimately, the speed of 850 rpm was selected.

Conclusions

Presently, the safflower seed meal was just used as cheap animal feeds, because the high-value active compounds in it was neglected. This study has established an efficient method to separate and purify *N*-feruloyl serotonin and *N*-(*p*-coumaroyl) serotonin from safflower seed meal. The economic value of safflower seed meal can be greatly increased through extracting the active compounds of NP and NF from the meal using HSCCC, which would not only increase economic efficiency of safflower but also stimulate the cultivation of safflower.

Acknowledgments

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