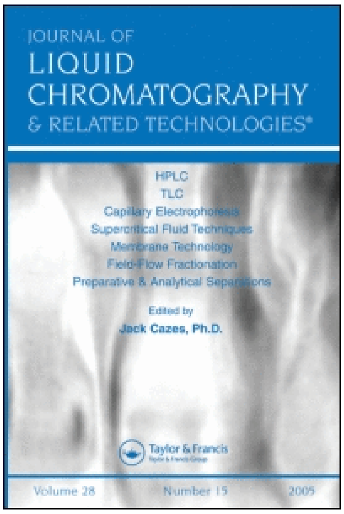

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Application of CCC for the Separation of Lutein from a Crude Extract of Marigold Flower Petals

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ABSTRACT

Analytical high-speed countercurrent chromatography (HSCCC) was used for the systematic selection and optimization of the two-phase solvent system to separate lutein from the crude extract of the marigold flower petal. Lutein was obtained by preparative HSCCC with a two-phase solvent system composed of *n*-heptane–chloroform–acetonitrile (10 : 3 : 7, v/v/v). HPLC analyses of the lutein revealed that it was over

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98.5% purity. The final identification was performed by UV–VIS absorption, ^{1}H-NMR, and ^{13}C-NMR Spectra.

Key Words: Lutein; Countercurrent chromatography; Carotenoids; Marigold flower petal.

INTRODUCTION

Carotenoids are highly conjugated polyprenoids found in a variety of natural sources including fruits, vegetables, and sea products with high nutritional and biological importance.[1] Increased interest in the isolation and measurement of carotenoids in biological matrices and natural products can be attributed to the numerous studies linking the intake of various micronutrients to cancer prevention.[2–7] The health benefits associated with high dietary intake of fruits and vegetables have motivated numerous studies of dietary consumption of carotenoids with absorption and deposition in humans.[8] Consumption of more vegetables and fruits is associated with a reduced risk of cancer. According to reports and clinical studies, carotenoids may be important in the prevention of several degenerative human heart conditions, including cancer and health disease.[9] Carotenoids are classified into two major groups, carotenes and xanthophylls. The former are polyene hydrocarbons, the latter are usually oxygenated at the end groups. This class of substances consists of numerous related compounds, including isomers that can be quite difficult to separate in natural samples. In research, the possible anticarcinogenic effects of fruits and vegetables are not only ascribed to carotenoids like β-carotene, but also to others like lutein and lycopene.[10]

Isolation and purification of lutein by column chromatography has been reported before,[11,12] but high purity lutein has not been obtained. High-speed countercurrent chromatography (HSCCC), being a support-free liquid–liquid partition method, eliminates irreversible adsorption of sample onto the solid support,[13] and has been widely used in preparative separation of natural products. The aim of the study was to develop a method for purifying natural lutein. In our laboratory, the lutein has been extracted from the microalga *Chlorella vulgaris* by HSCCC using an aqueous system.[14] But using an aqueous system, lutein cannot be obtained in a higher purity from marigold flower petal, so the organic system was used. Purification of lutein by HSCCC is superior to that by column chromatography. The purity of the lutein available was only 70% (UV–VIS), and information is lacking about the impurities. Moreover, the standard is quite expensive. In this study, the term lutein refers to all stereoisomers of lutein. The present paper, describes the successful preparative separation and purification of lutein, which is unstable in air and under light, from the crude extract of marigold petal by HSCCC.

EXPERIMENTAL

Apparatus

The analytical HSCCC instrument employed in the present study was a Model GS 20 analytical HSCCC, designed and constructed in Beijing Institute of New Technology Application, (Beijing, China). The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 50 m long, 0.85 mm I.D. PTFE (polytetrafluoroethlene) tube directly onto the holder hub, forming multiple coiled layers with a total capacity of 40 mL. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a speed controller in the range between 0 and 2000 rpm, an optimum speed of 1800 rpm was used in the present studies.

Preparative HSCCC was performed using a Model GS10A2 multilayer coil planet centrifuge (Beijing Institute of New Technology Application, Beijing, China) equipped with a PTFE multilayer coil of 110 m × 1.6 mm I.D. with a total capacity of 230 mL. The β value of the preparative column ranged from 0.5 to 0.8.

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 254 and 460 nm. A manual sample injection valve with a 1.0 mL loop (for the analytical HSCCC) or a 20 mL loop (for the preparative HSCCC) (Tianjin High-New Science and Technology Company, Tianjin, China) was used to introduce the sample into the column, respectively. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to plot the chromatogram.

The high-performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-10AVP system including two LC-10ATVP solvent delivery units, an SPD-M10AVP UV–VIS photodiode array detector (DAD), a Model 7726 injection valve with a 20 µL loop, an SCL-10AVP system controller, a CTO-10ASVP column oven, a DGU-12A degasser, and a Class-VP-LC workstation (Shimadzu, Kyoto, Japan).

The ^{1}H-NMR and ^{13}C-NMR Spectra were recorded on a "amms 600" Varian Inova 600 (600 MHz) spectrometer, $CDCl_3$ was used as solvent with TMS as internal standard.

Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Methanol used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory, Tianjin, China.

Extraction

One hundred grams marigold flower fresh petals were homogenized with 1000 mL isopropyl alcohol. After filtration through a Buchner funnel under vacuum, the residue was extracted with a 1 : 1 solution of acetone : hexane (100 mL). The petals were further extracted with hexane/acetone/MeOH/toluene (10 : 7 : 6 : 7,v/v/v/v), EtOAc/hexane/acetone (6 : 1 : 1, v/v/v).[15,16] The filtrates obtained by vacuum filtration were combined in a separatory funnel. Water was added to hasten the phase separation of the hexane layer, which was saved. The aliquots were removed and dried under a stream of nitrogen gas. Dried sample residues, 3 g, were stored at −20°C under nitrogen. The residue consisted of 7.6% lutein based on HPLC peak area.

Preparation of Two-Phase Solvent System and Sample Solutions

The solvent system utilized in the present study was prepared by mixing *n*-heptane–chloroform–acetonitrile according to the suitable proportion, and thoroughly equilibrating the mixtures in a separatory funnel at room temperature for 1 hr, two phases being separated shortly before use.

The sample solutions were prepared by dissolving the crude sample in the upper phase at suitable concentrations according to the analytical or preparative procedure.

Separation Procedure

Analytical HSCCC was performed with a Model GS 20 HSCCC instruments as follows: the multi-ply coiled column was first entirely filled with the lower phase. The upper phase was then pumped into the head end of the inlet column at a flow-rate of 1.0 mL/min, while the apparatus was run at a reverse revolution speed of 1800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (15 mg in 1 mL of upper phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram. The retention of the stationary phase relative to the total coil capacity was computed from the volume of the stationary phase collected from the column post-run divided by the total coil volume.

Preparative HSCCC was performed with a Model GS10A2 HSCCC instrument as follows: the multi-ply coiled column was first entirely filled with the lower phase as stationary phase. Then, the upper phase of system was pumped into the head end of the inlet column at a flow-rate of 2 mL/min, while the apparatus was reverse rotated at 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (300 mg in 20 mL of upper phase) was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram.

High Performance Liquid Chromatography Analyses and Peak Fraction Purity

The crude extraction and each peak fraction from HSCCC, were analyzed by HPLC described as in Ref.[16] The analyses were performed with an Internet ODS column (150 × 4.6 mm I.D.) at a column temperature of 30°C. The mobile phase composed of A: 100% methanol, B: 100% ethyl acetate was eluted at a flow-rate of 2.0 mL/min, using linear gradient over 20 min from 60% methanol to 40% methanol, and the effluent monitored at 446 nm and 254 nm by a DAD detector. The lutein was identified based on its UV–VIS absorbance spectra, recorded with a diode array detector, and HPLC retention time. Diode array detector was used to identify the lutein peak purity by comparing the spectral data. For example, DAD was used to identify the different part of lutein peak, the UV–VIS spectra were the same, and the relative factor was 0.999.

RESULTS AND DISCUSSION

The crude extract was analyzed by HPLC. The result indicated that the crude sample contained several compounds [see Fig. 3(A)].

In order to achieve an efficient resolution of target compounds, the two-phase solvent system of *n*-heptane–chloroform–acetonitrile was examined using analytical HSCCC, by varying the mutual volume ratios. The results indicated that the volume ratio of 10 : 3 : 7 was suitable for obtaining lutein (Fig. 1).

Figure 2 shows the result obtained from 300 mg of the crude extract of marigold petal by preparative HSCCC. After this separation, the fraction containing lutein (peak 2#) was collected. The lutein [Fig. 3(B)] yielded 15.3 mg at over 98.5% purity based on HPLC peak area. The UV–VIS spectrum of lutein is shown in Fig. 4.

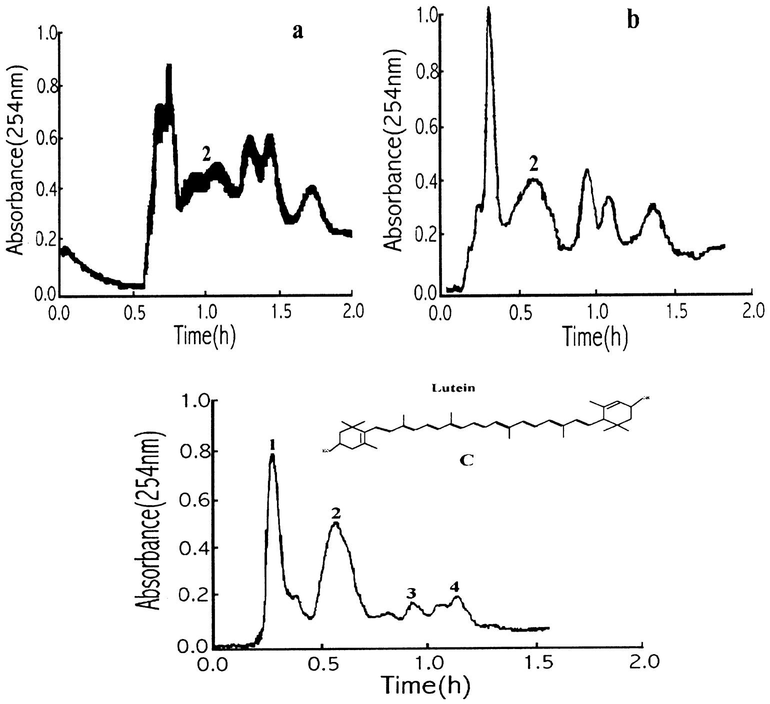

Figure 1. Chromatogram of the crude extract from marigold petal by analytical HSCCC. Solvent system: a: *n*-heptane–chloroform–acetonitrile (10 : 2 : 8, v/v/v); b: *n*-heptane–chloroform–acetonitrile (10 : 3.5 : 6.5, v/v/v); c: *n*-heptane–chloroform–acetonitrile (10 : 3 : 7, v/v/v); stationary phase: lower phase; mobile phase: upper phase; flow-rate: 1.0 mL/min; revolution speed: reverse revolution 1800 rpm; sample: 15 mg dissolved in 1 mL upper phase; retention of the stationary phase: about 50%. Peak: 2. lutein.

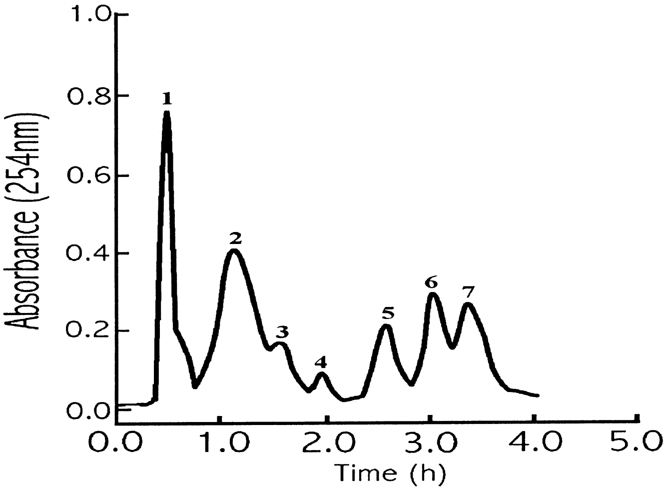

Figure 2. Chromatogram of the crude extract from marigold petal by preparative HSCCC. Solvent system: *n*-heptane–chloroform–acetonitrile (10 : 3 : 7, v/v/v); stationary phase: lower phase; mobile phase: upper phase; flow-rate: 2.0 mL/min; revolution speed: reverse revolution 800 rpm; sample: 300 mg dissolved in 20 mL upper phase; retention of the stationary phase: 58.5%. Peak 2: lutein.

The structural identification of lutein was carried out by MS, ^{1}H-NMR, and ^{13}C-NMR spectra as follows: the FAB mass spectrum showed the molecular ion at m/z 568, which is in agreement with the molecular formula $C_{40}H_{56}O_2$ of lutein.

^{1}H-NMR (600 MHZ, $CDCl_3$) δ ppm:

a. 0.860 (s, 3H, 16′-CH_3), 1.028 (s, 3H, 17′-CH_3), 1.079 (s, 3H, 16-CH_3), 1.089 (s, 3H, 17-CH_3), 1.356 (m, 1H, 3-H—O), 1.367 (m, 1H, 2′-C—H), 1.494 (m, 1H, 2-C—H), 1.651 (s, 3H, 18′-CH_3), 1.773 (s, 3H, 18-CH_3), 1.787 (m, 1H, 2-C—H), 1.85 (m, 1H, 2′-C—H), 1.898 (s, 3H, 19′-CH_3), 1.965 (s, 3H, 20′-CH_3), 1.971 (s, 3H, 19-CH_3), 1.982 (s, 3H, 20-CH_3), 2.054 (m, 1H, 4-C—H), 2.403 (m, 1H, 4-C—H), 2.587 (d, 1H, 6′-C—H), 4.037 (m, 1H, 3-C—H), 4.195 (m, 1H, 3′-C—H), 5.403 (d, 1H, 7′-C—H), 5.406 (m, 1H, 4′-C—H), 6.035 (d, 1H, 10′-C—H), 6.077 (d, 1H, 10-C—H), 6.120 (d, 1H, 7-C—H), 6.252 (m, 2H, 14/14′-C—H), 6.312 (d, 2H, 12/12′-C—H), 6.636 (m, 2H, 15/15′-C—H), 6.668 (d, 2H, 8/8′-C—H), 6.735 (dd, 1H,11-C—H), 6.756 (dd, 1H, 11′-C—H).

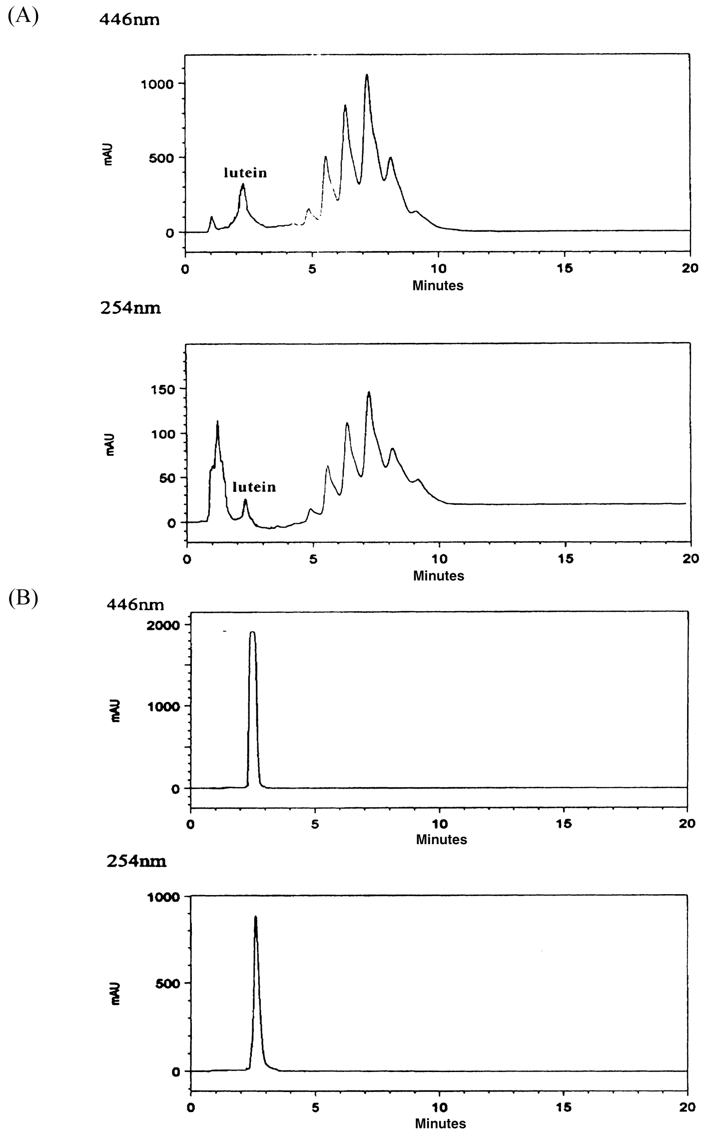

Figure 3. HPLC analysis of the crude extract from marigold petal and peak 2 (lutein) shown in Fig. 2. (A) the crude sample; (B) peak 2; Column: Inertsil ODS column (150 × 4.6 mm I.D.); Column temperature: 30°C. The mobile phase composed of A: 100% methanol, B: 100% ethyl acetate was eluted at a flow-rate of 2.0 mL/min using linear gradient over 20 min from 60% methanol to 40% methanol, and the effluent monitored at 446 nm and 254 nm by a DAD detector.

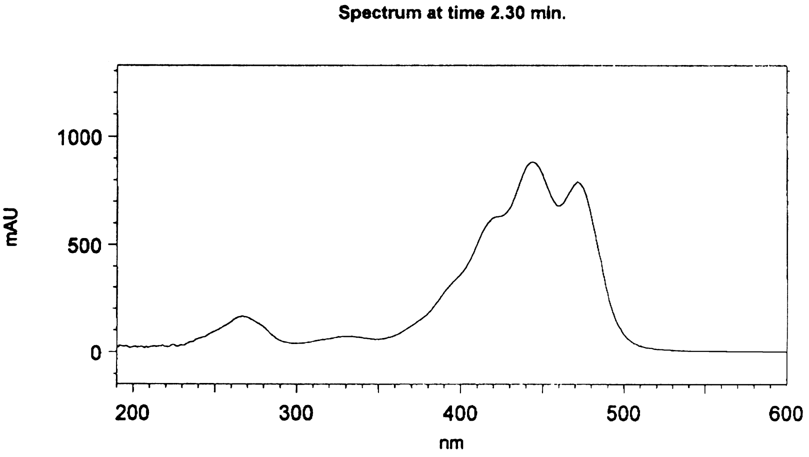

Figure 4. UV–VIS spectrum of lutein.

b. 0.853 (s, 3H, 16′-CH$_3$), 1.008 (s, 3H, 17′-CH$_3$), 1.073 (s, 3H, 17-CH$_3$), 1.079 (s, 3H, 16-CH$_3$), 1.367 (m, 1H, 2′-C—H), 1.508 (m, 1H, 2-C—H), 1.628 (s, 3H, 18′-CH$_3$), 1.773 (s, 3H, 18-CH$_3$), 1.787 (m, 1H, 2-C—H), 1.85 (m, 1H, 2′-C—H), 1.932 (s, 3H, 19-CH$_3$), 1.965 (s, 3H, 20′-CH$_3$), 1.971 (s, 3H, 20-CH$_3$), 2.065 (m, 1H, 4-C—H), 2.356 (d, 1H, 6′-C—H), 2.403 (m, 1H, 4-C—H), 4.015 (m, 1H, 3-C—H), 6.018 (d, 1H, 14′-C—H), 6.079 (d, 1H, 10-C—H), 6.088 (d, 1H, 8′-C—H), 6.102 (d, 1H, 7-C—H), 6.141 (d, 1H, 8-C—H), 6.175 (m, 1H, 14-C—H), 6.182 (d, 1H, 10′-C—H), 6.283 (d, 1H, 12-C—H), 6.485 (m, 1H, 15-C—H), 6.538 (dd, 1H, 11-C—H), 6.583 (dd, 1H, 11′-C—H), 6.715 (d, 1H, 15′-C—H), 6.813 (d, 1H, 12′-C—H).

The results were similar to those reported in Ref.[15] Lutein probably contains (9Z, 9Z′)-lutein and 13′Z-lutein.

^{13}C-NMR (600 MHz, CDCl$_3$) δppm: 11.962 (C-19), 12.030 (C-20), 12.221 (C-20′), 14.039 (C-19′), 21.194 (C-18), 25.508 (C-16′), 28.380 (C-17′), 28.998 (C-16), 29.678 (C-17), 33.313 (C-1′), 37.131 (C-1), 38.582 (C-2′), 42.285 (C-4), 47.631 (C-2), 55.175 (C-6′), 65.034 (C-3), 109.767 (C-18′), 121.680 (C-11), 121.718 (C-11′), 125.573 (C-7), 126.185 (C-5), 127.713 (C-3′), 127.896 (C-4′), 129.270 (C-7′), 130.026 (C-15), 130.187 (C-15′), 130.672 (C-10′), 131.475 (C-10), 132.415 (C-14′), 132.662 (C-14), 135.502 (C-9), 135.644 (C-9′), 136.398 (C-13), 136.517 (C-13′), 136.620 (C-8′), 137.315 (C-12′), 137.619 (C-12), 137.806 (C-6), 140.748 (C-8), 145.169 (C-5′). Some of the signals were too close to each other to be separately assigned.

The results of our studies clearly demonstrated that HSCCC is very successful for the preparative separation of lutein from the crude extract of marigold petal.

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