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Preparative isolation and purification of lutein from the microalga *Chlorella vulgaris* by high-speed counter-current chromatography

Hua-Bin Li[a], Feng Chen[a,*], Tian-You Zhang[b], Fu-Quan Yang[b], Guo-Qing Xu[b]

[a]*Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong, Hong Kong*
[b]*Beijing Institute of New Technology Application, Beijing 100035, China*

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Abstract

High-speed counter-current chromatography (HSCCC) was applied to the isolation and purification of lutein from microalgae. Analytical HSCCC was used for the preliminary selection of a suitable solvent system composed of *n*-hexane–ethanol–water (4:3:1, v/v). Using the above solvent system, preparative HSCCC was successfully performed yielding lutein at 98% purity from 200 mg of the crude extract in a one-step separation.

Keywords: Counter-current chromatography; *Chlorella vulgaris*; Preparative chromatography; Lutein; Carotenoids; Vitamins

1. Introduction

Carotenoids have been proposed as cancer prevention agents, life extenders, and the inhibitors of ulcer, heart attack and coronary artery disease [1–3]. Lutein is not only one of the most prominent carotenoids in human serum and foods, but also the representative of α,β-carotenoids [4,5]. The chemical structure of lutein is shown in Fig. 1. Naturally occurring lutein is produced mainly in higher plants and algae. Compared with higher plants, algae have an advantage since they can be cultivated in bioreactors on a large scale and thus are a continuous and reliable source of the product [6].

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography that uses no solid support matrix. Therefore, it eliminates irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column. The method has been successfully applied to the analysis and separation of various natural products [7–10]. However, no report has been published on the use of HSCCC for the isolation and purification of lutein from microalgae. The purpose of this study, therefore, was to develop a method for the isolation and purification of lutein from the microalga by HSCCC.

2. Experimental

2.1. Apparatus

Analytical HSCCC was carried out with a Model

*Corresponding author. Tel.: +852-2299-0309; fax: +852-2858-3477.

E-mail address: sfchen@hkusua.hku.hk (F. Chen).

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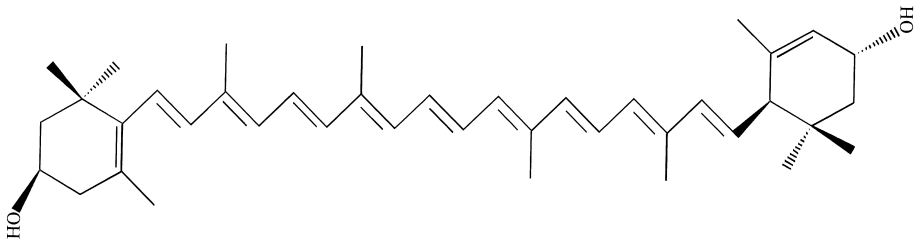

Fig. 1. Chemical structure of lutein.

GS-20 analytical high-speed counter-current chromatograph (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 polytetrafluoroethylene (PTFE) tube (50 m×0.85 mm I.D.) directly onto the holder hub forming multiple coiled layers with a total capacity of 30 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 is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The rotation speed is adjustable from 0 to 2000 rpm, and an optimum speed of 1600 rpm was used in the present study.

Preparative HSCCC was performed with a Model GS10A2 preparative high-speed counter-current chromatograph (Beijing Institute of New Technology Application). The multilayer coil separation column was similarly prepared with a PTFE tube (110 m× 1.6 mm I.D.). The total capacity is 230 ml. The β value of this preparative column ranged from 0.5 to 0.8. The rotation speed is adjustable from 0 to 1000 rpm, and an optimum speed of 800 rpm was used.

These two HSCCC systems are equipped with a constant-flow pump (Model NS-1007), a UV monitor (Model 8823A) operating at 254 nm, a recorder (Yokogawa Model 3057), and a sample inject valve with a 2-ml (for the analytical HSCCC) or 20-ml sample loop (for the preparative HSCCC).

The high-performance liquid chromatography (HPLC) system used was a Shimadzu LC-10AVP system consisting of two LC-10ATVP solvent delivery units, a UV–Vis photodiode array detector (SPD-M10AVP), an injection valve (Model 7726) with a 20 μl loop, a system controller (SCL-10AVP), a column oven (CTO-10ASVP) and a degasser (DGU-12A). Evaluation and quantification were made by a Class-VP-LC workstation (Shimadzu, Kyoto, Japan). The column used was a reversed-phase Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm; Beckman, Fullerton, CA, USA).

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Methanol, dichloromethane and acetonitrile used for HPLC analysis were of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory (Tianjin, China). The lutein stock solution was 0.100 mg ml^{-1} which was prepared by dissolving 1.00 mg of lutein (Sigma, USA) in 10.00 ml of the mobile phase and then stored in a refrigerator. The working solutions were prepared by suitable dilution of the stock solutions with the mobile phase. All other solutions were prepared by dissolving appropriate amounts of commercially available chemicals in water.

The microalga *Chlorella vulgaris* was obtained from our laboratory.

2.3. Preparation of crude lutein from the microalga Chlorella vulgaris

Preparation of crude lutein was carried out according to the literature [11]. In brief, 250 ml of 10.0 mol l^{-1} KOH solution containing 2.5% ascorbic acid was added to 100 g of the lyophilized alga, and the mixture was incubated at 60°C for 10 min before

cooling down to room temperature. Dichloromethane (50 ml) was added to the mixture for the extraction of lutein. The mixture was then separated by centrifugation, and the extract was collected. The extraction procedure was repeated until the cell debris was almost colorless. All the extracts were combined and washed with distilled water (100 ml). Then, the extract was dried by rotary vaporization at 40°C and stored in a refrigerator for the subsequent HSCCC separation.

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

In the present study, we selected a two-phase solvent system composed of *n*-hexane–ethanol–water at various volume ratios. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude sample in the mobile phase of the solvent system used for separation.

2.5. HSCCC separation procedure

Analytical HSCCC was used for selecting a suitable solvent system for the separation of lutein. The solvent system composed of *n*-hexane–ethanol–water was examined at different volume ratios such as 6:5:1, 6:4:1, 6:3:1 and 4:3:1 using analytical HSCCC.

In each analytical separation, the coiled column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 1600 rpm, while the lower phase (mobile phase) was pumped into the column at a flow-rate of 1.0 ml min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 1 ml of the sample solution containing 10 mg of the crude lutein was injected through the injection value. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the elution profile.

The preparative separation was similarly performed using a 20-ml sample volume (200 mg crude extract) at a flow-rate of 2.0 ml min^{-1} at 800 rpm.

2.6. HPLC analysis

The crude sample and each peak fraction obtained by HSCCC were analyzed by HPLC according to Shi and Chen [6]. The mobile phase was methanol–dichloromethane–acetonitrile–water (67.5:22.5:9.5:-0.5, v/v) and the flow-rate was 1.0 ml min^{-1}. The effluent was monitored at 450 nm.

3. Results and discussion

Fig. 2 shows the HPLC analysis of the lutein crude from the microalga *Chlorella vulgaris*. Peak A corresponds to lutein.

Analytical HSCCC with its speedy separation and minimum solvent consumption offers a very promising way to undertake method development for preparative HSCCC separations. Preliminary HSCCC studies were carried out on analytical HSCCC system in the normal-phase mode. The two-phase solvent systems were carbon tetrachloride–methanol–water (5:4:1), carbon tetrachloride–*n*-hexane–methanol–water (4:1:4:1), carbon tetrachloride–

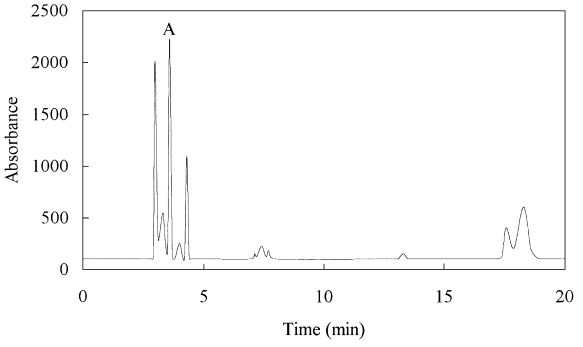

Fig. 2. Chromatogram of crude lutein from the microalga *Chlorella vulgaris* by HPLC analysis, A=lutein. Conditions: column: reversed-phase Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm); mobile phase: methanol–dichloromethane–acetonitrile–water (67.5:22.5:9.5:0.5, v/v); flow-rate: 1.0 ml min^{-1}; detection at 450 nm.

dichloromethane–methanol–water (4:1:4:1), and carbon tetrachloride–ethanol–water (10:7.5:2.5; 10:7:3; 5:3:2). It was very difficult to separate lutein from other substances by normal-phase HSCCC (data not shown). In subsequent studies, HSCCC was tested in the reversed-phase mode.

Performance of the two-phase solvent system composed of *n*-hexane–ethanol–water at various volume ratios (6:5:1, 6:4:1, 6:3:1 and 4:3:1) was evaluated by analytical HSCCC in terms of peak resolution. The results indicated that 6:3:1 and 4:3:1 could be used; the result using the solvent system of ratio 4:3:1 is shown in Fig. 3.

Fig. 4 shows the preparative HSCCC separation of 200 mg of the crude sample using the solvent system composed of *n*-hexane–ethanol–water (4:3:1, v/v). Although the sample size was 20-times as large as that used in the analytical HSCCC, an equivalent or even better separation was achieved. In order to save solvents and time, the slow eluting compounds after the target substance were removed by pumping out the stationary phase instead of eluting them with the mobile phase because the stationary phase was used only once. HPLC analysis of each peak fraction of this preparative HSCCC revealed that lutein corre-

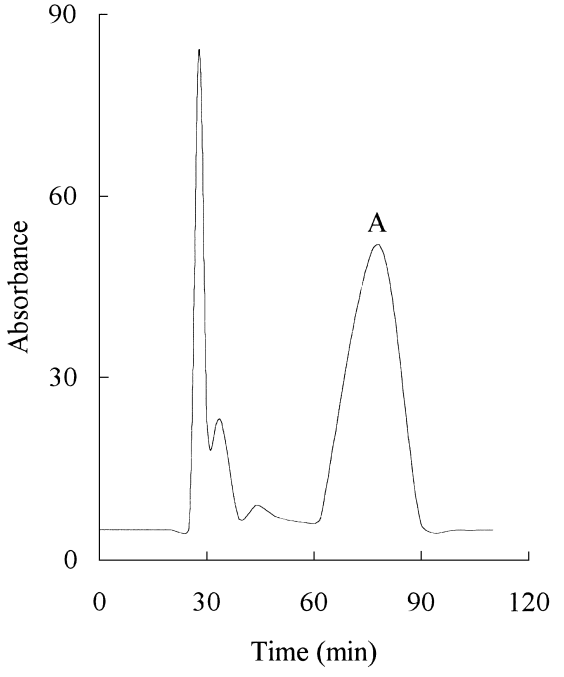

Fig. 3. Chromatogram of crude lutein from the microalga *Chlorella vulgaris* by analytical HSCCC separation, A=lutein. Conditions: column: multilayer coil of 1.6 mm I.D. PTFE tube with a total capacity of 30 ml; rotary speed: 1600 rpm; solvent system: *n*-hexane–ethanol–water (4:3:1, v/v); mobile phase: lower phase (ethanol–water); flow-rate: 1 ml min^{-1}; detection at 254 nm; sample size: 10 mg; retention of the stationary phase: 55%.

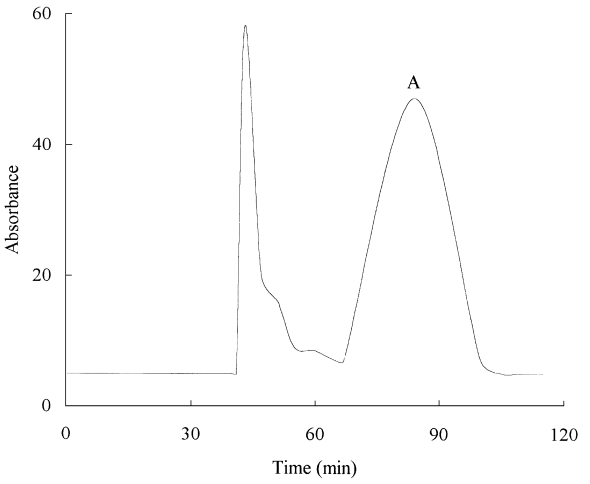

Fig. 4. Chromatogram of crude lutein from the microalga *Chlorella vulgaris* by preparative HSCCC separation, A=lutein. Conditions: column: multilayer coil of 1.6 mm I.D. PTFE tube with a total capacity of 230 ml; rotary speed: 800 rpm; solvent system: *n*-hexane–ethanol–water (4:3:1, v/v); mobile phase: lower phase (ethanol–water); flow-rate: 1 ml min^{-1}; detection at 254 nm; sample size: 200 mg; retention of the stationary phase: 58%.

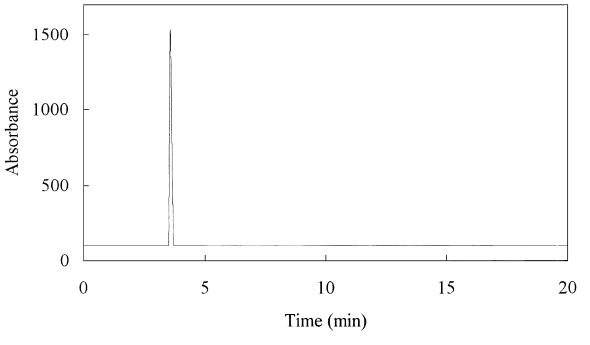

Fig. 5. HPLC chromatogram of lutein purified from the microalga *Chlorella vulgaris*. Conditions: column: reversed-phase Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm): mobile phase: methanol–dichloromethane–acetonitrile–water (67.5:22.5:9.5:0.5, v/v); flow-rate: 1.0 ml min^{-1}; detection at 450 nm.

Table 1
The *K* (partition coefficient) values of lutein in several solvent systems

Solvent system	*K* value
Carbon tetrachloride–methanol–water (5:4:1)	0.102
Carbon tetrachloride–*n*-hexane–methanol–water (4:1:4:1)	0.121
Carbon tetrachloride–dichloromethane–methanol–water (4:1:4:1)	0.138
Carbon tetrachloride–ethanol–water (10:7.5:2.5)	0.140
Carbon tetrachloride–ethanol–water (10:7:3)	0.128
Carbon tetrachloride–ethanol–water (5:3:2)	0.105
n-Hexane–ethanol–water (6:5:1)	0.264
n-Hexane–ethanol–water (6:4:1)	0.384
n-Hexane–ethanol–water (6:3:1)	0.397
n-Hexane–ethanol–water (4:3:1)	0.538

sponding to peak A was over 98% pure. The HPLC chromatogram of lutein as purified from the preparative HSCCC is shown in Fig. 5.

The *K* (partition coefficient) values of lutein in several solvent systems were measured according to the literature [12], and are given in Table 1.

In conclusion, HPCCC was successfully used for isolation and purification of lutein from the microalga *Chlorella vulgaris*. The present study indicates that HSCCC is powerful tool for the separation and purification of biologically active substances in algae and has potential applications in other biotechnological downstream processes.

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