JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation and Purification of Lutein from the Microalga *Chlorella vulgaris* by Extraction after Saponification

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A simple and efficient method for the isolation and purification of lutein from the microalga *Chlorella vulgaris* was developed. Crude lutein was obtained by extraction with dichloromethane from the microalga after saponification. Partition values of lutein in the two-phase system of ethanol−water−dichloromethane at different ratios were measured by HPLC so as to assist the determination of an appropriate condition for washing water-soluble impurities in the crude lutein. Partition values of lutein in another two-phase system of ethanol−water−hexane at different ratios were also measured by HPLC for determining the condition for removing fat-soluble impurities. The water-soluble impurities in the crude lutein were removed by washing with 30% aqueous ethanol, and the fat-soluble impurities were removed by extraction with hexane. The final purity of lutein obtained was 90−98%, and the yield was 85−91%.

KEYWORDS: Lutein; purification; *Chlorella vulgaris*; saponification; extraction

INTRODUCTION

Epidemiological study has shown that the consumption of substantial amounts of fruits and vegetables reduces the risk of cancer. One hypothesis is that carotenoids in these foods act as antioxidants through a free radical mechanism by quenching singlet oxygen and other oxidizing species, resulting in the termination of free radical chain reactions and in the prevention of cellular oxidative damage (*1*). Carotenoids have been proposed as life extenders and inhibitors of ulcer, heart attack, and coronary artery disease. Lutein is one of the most abundant carotenoids in the diet and in human blood. There has been recent evidence to suggest that lutein possesses strong antioxidant capabilities and may be useful in reducing the incidence of cancer (*2*−*5*).

Naturally occurring lutein is produced mainly in higher plants and algae. Compared with higher plants, algae have an advantage because they can be cultivated in bioreactors on a large scale and thus are a continuous and reliable source of the product (*6*−*8*). A method for the separation and purification of lutein from marigold flowers by saponification, extraction, and recrystallization has been proposed (*1*, *9*). However, the recrystallization was time-consuming, and the yield by this method was low (57−68%) (*9*). We have recently reported a high-speed counter-current chromatographic method for the isolation and purification of lutein (*10*). However, only a small sample size (e.g., 200 mg) could be treated in one run, and the consumption of organic solvents was great. Furthermore, high-speed counter-current chromatography is not easily accessible. The isomers of lutein were also isolated by extraction, saponification, and semipreparative HPLC (*11*, *12*). However, the sample size that could be handled using semipreparative HPLC was also small. The purpose of this study, therefore, was to develop a simple and efficient method for the isolation and purification of lutein from the microalga *Chlorella vulgaris* by extraction after saponification without recrystallization.

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MATERIALS AND METHODS

Chemicals and Reagents. Standard lutein and vitamin C were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium hydroxide and the HPLC grade organic solvents were obtained from BDH Laboratory Supplies (Poole, U.K.).

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

Saponification and Extraction of Lutein. Preparation of crude lutein was carried out according to the literature (*10*, *13*). In short, 250 mL of 10.0 M KOH solution containing 2.5% ascorbic acid was added to 100 g of the lyophilized algal cells, and the mixture was incubated at 60 °C for 10 min and then was cooled to room temperature. Dichloromethane (50 mL) was added to the mixture to extract lutein. The mixture was then centrifuged at 10000*g* for 15 min, and the supernatant was collected. The extraction procedure was repeated until the extract was almost colorless, and all extracts were combined.

The extract was washed with 30% aqueous ethanol (v/v) until the water phase was almost colorless and the pH was near neutral. After separation, the organic phase was dried by rotary vaporization at 40 °C.

The residue was redissolved in 85% aqueous ethanol (v/v). The fat-soluble impurities were extracted with hexane. After separation, the concentration of ethanol in the water phase was diluted to 8.5 from 85% (v/v) with distilled water to precipitate the lutein. The lutein obtained by filtration was lyophilized to dryness.

Determination of Lutein. The crude sample and purified lutein were analyzed by HPLC according to the method of Shi and Chen (*14*).

10.1021/jf010220b CCC: $22.00
Published on Web 01/17/2002

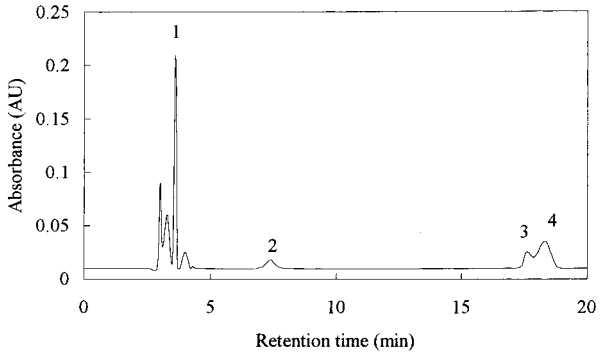

Figure 1. Chromatogram of crude lutein obtained by extraction after saponification. Peaks: 1, lutein; 2, chlorophyll *a*; 3, α-carotene; 4, *β*-carotene.

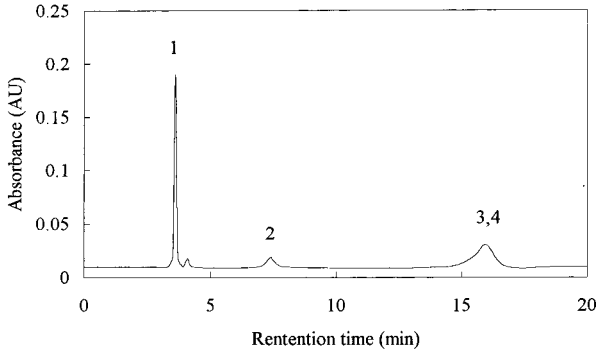

Figure 2. Chromatogram of lutein obtained after washing with 30% aqueous ethanol. Peaks: 1, lutein; 2, chlorophyll *a*; 3, α-carotene; 4, *β*-carotene.

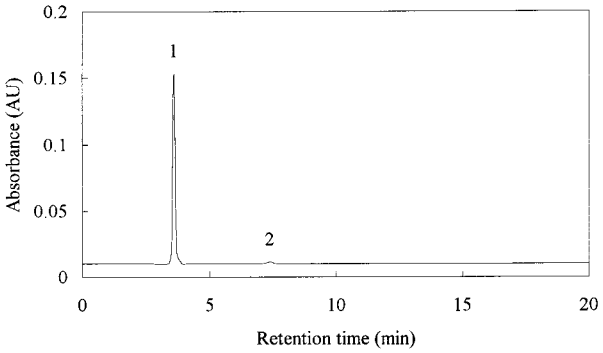

Figure 3. Chromatogram of purified lutein. Peaks: 1, lutein; 2, chlorophyll *a*.

The HPLC system used throughout this study consisted of a Waters 510 pump (Waters, Milford, MA), a sample injector (Rheodyne, Cotati, CA) with a 20 μL loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millenium chromatography data system (Waters, Milford, MA). The column used was a reversed phase ultrasphere C_{18} column (250 × 4.6 mm i.d., 5 μm, Beckman, Fullerton, CA). 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. The effluent was monitored at 450 nm. The experiments were performed in triplicate. The contents of lutein in the microalga and purified lutein (product) were calculated by comparing the peak area with that of standard lutein, and the yield was calculated by dividing the amount of lutein in the purified lutein (product) by the amount of lutein in the microalga.

RESULTS AND DISCUSSION

The crude lutein was prepared by extraction with dichloromethane after saponification under the condition of 10.0 M KOH solution containing 2.5% ascorbic acid at 60 °C (*10*, *13*). Lutein fatty acid esters were converted to lutein by saponification. Ascorbic acid was used as an antioxidant to prevent oxidation of lutein (*14*). Dichloromethane was chosen because of the excellent solubility of lutein in this solvent (*1*, *10*). However, dichloromethane is a toxic and environmentally damaging solvent. Thus, the dichloromethane should be recovered after use or disposed of properly. If saponification is carried out after lutein fatty acid esters have been extracted from the material (*1*), the algal cells should be dried and ruptured before extraction with organic solvents in order to enhance lutein extractability. Thus, the extraction after saponification was adopted in the present process. The crude extract obtained contained ∼30% lutein. The chromatogram of crude lutein is shown in **Figure 1**.

The crude extract contained water- and fat-soluble impurities and needed to be purified further. To choose conditions for washing water-soluble impurities from the extract, with a minimum loss of lutein, the partition values of lutein in the two-phase system of ethanol−water−dichloromethane with different ratios were measured by HPLC. Ethanol−water (water phase) was the upper phase, whereas dichloromethane (organic phase) was the lower phase. When the ethanol contents of the water phase were 0, 10, 20, 30, 40, 50, and 60%, the ratios of the concentration of lutein in the water phase to the concentration of lutein in organic phase were 0.3/99.7, 0.5/99.5, 1.3/98.7, 1.2/98.8, 1.4/98.6, 1.8/98.2, and 1.9/98.1, respectively. Although more impurities could be washed with water containing a greater concentration of ethanol, 30% aqueous ethanol was chosen to obtain good recovery of lutein. Thus, the dichloromethane extract was washed with 30% aqueous ethanol until the water phase was almost colorless and the pH was near neutral. After separation, the organic phase was dried by rotary vaporization at 40 °C. The lutein obtained in this stage contained ∼70% lutein (w/w), and the corresponding chromatogram is shown in **Figure 2**. Compared with **Figure 1**, the merger of α- and *β*-carotene peaks and the shift in retention time in **Figure 2** could probably be due to the denaturation of the compounds. Further investigation on this aspect was not made because α- and *β*-carotene peaks did not appear in the purified lutein (**Figure 3**).

To choose an appropriate condition for removing fat-soluble impurities from the extract, with a minimum loss of lutein, the partition values of lutein in the two-phase system of ethanol−water−hexane with different ratios were measured using HPLC. Ethanol−water (water phase) was the lower phase, whereas hexane (organic phase) was the upper phase. When the ethanol content in the water phase was 80, 85, and 90%, the ratios of the concentration of lutein in the organic phase to the concentration of lutein in water phase were 23.5/76.5, 15.5/84.5, and 13.8/86.2, respectively. Meanwhile, the proportions of chlorophyll *a* in the organic phase to that in water phase were 56.5/43.5, 55.7/44.3, and 29.0/71.0, respectively; almost all α- and *β*-carotenes were present in the organic phase. Therefore, 85% aqueous ethanol was chosen to obtain more lutein and to remove more chlorophyll *a*. The ratio of organic phase to water phase (v/v) and the number of extractions affected the purity and yield of lutein. The greater the ratio of organic phase to water phase (v/v), the greater the purity of lutein and the lower the lutein yield. The greater the number of extractions, the greater the purity of lutein, and the more lutein was lost. In general, the ratio of organic phase to water phase was kept at 1:4 (v/v), and extraction was repeated two to three times. Then, the lutein containing fat-soluble impurities was dissolved in 85% aqueous ethanol, and the fat-soluble impurities were extracted twice with hexane. After separation, the concentration of ethanol in water

Table 1. Purity and Yield of the Purified Lutein

no.	purity (%)	yield (%)	no.	purity (%)	yield (%)
1	93	88	4	95	87
2	98	85	5	90	91
3	91	90			

phase was diluted to 8.5 from 85% with distilled water in order to precipitate lutein. The lutein obtained by filtration with a 0.45 μm filter membrane (Millipore Co., Bedford, MA) was lyophilized to dryness, and its purity was determined by HPLC; the yield was calculated by comparison of the amount of lutein in purified lutein (product) to the amount of lutein in the microalga. As shown in **Table 1**, the final purity was between 90 and 98%, and the yield was between 85 and 91%. The chromatogram of purified lutein is shown in **Figure 3**.

Previously, marigold flowers were used as raw material for extracting lutein (*1*, *9*). However, marigold flowers might contain herbicide and pesticide residues, which are harmful to the health of human beings. In the present method, the alga was adopted as material for extracting lutein, which should not contain any herbicides or pesticidess or any other toxic substances because the nutrient medium for the algae could be well controlled. Recrystallization was used to remove fat-soluble impurities in the previous method (*1*, *9*), but it was time-consuming (usually overnight) and the yield of the method was low (57−68%) using a single crystallization process. In the present method, extraction with hexane was adopted to remove fat-soluble impurities. The present method was fast, and the yield was greater.

In conclusion, a simple and efficient method for the isolation and purification of lutein from the microalga *C. vulgaris* was established by extraction after saponification. The purity of lutein obtained was 90−98%, and the yield was 85−91%, which was greater than that previously reported.

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Received for review February 20, 2001. Revised manuscript received November 26, 2001. Accepted November 28, 2001. This research was supported by the Hong Kong Research Grants Council.

JF010220B