# Chromatographic Separation and Purification of *trans*-Astaxanthin from the Extracts of *Haematococcus pluvialis*

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A gradient reversed-phase HPLC method was developed for the separation of astaxanthin esters and the isomers of astaxanthin from the unsaponified and saponified pigment extracts of the microalga *Haematococcus pluvialis*. Four kinds of isomers of astaxanthin and astaxanthin esters, (3*S*,3′*S*)-*trans*-astaxanthin, (3*S*,3′*S*)-9-*cis*-astaxanthin, (3*S*,3′*S*)-13-*cis*-astaxanthin, (3*R*,3′*R*)-*trans*-astaxanthin, and their esters, were separated and identified. A small amount of (3*S*,3′*S*)-15-*cis*-astaxanthin was also detected from the saponified extract. In addition, a chromatographic purification method was established for the preparation of natural *trans*-astaxanthin from the saponified extract of *H. pluvialis*. With this method, 3.7 mg of astaxanthin was isolated from 1 g of dry biomass of *H. pluvialis*. The purified astaxanthin contained approximately 97.7% *trans*-astaxanthin, 1.1% *cis*-astaxanthin, and 1.2% impurity.

**Keywords:** *Astaxanthin; astaxanthin esters; purification; carotenoids; Haematococcus pluvialis; HPLC*

## INTRODUCTION

Astaxanthin (3,3′-dihydroxy-$\beta$,$\beta$′-carotene-4,4′-dione) is a ketocarotenoid oxidized from $\beta$-carotene. Due to its attractive pink color, biological functions as a vitamin A precursor, and antioxidative activity higher than those of $\beta$-carotene and vitamin E, astaxanthin can be used as a food colorant and in medicine (Johnson and An, 1991). In the United States, astaxanthin is permitted for use as a color additive in salmonid feed by the Food and Drug Administration (Turujman et al., 1997). Recent studies (Gradelet et al., 1997) have demonstrated the preventive effects of astaxanthin against aflatoxin $B_1$ carcinogenicity.

In the alga *Haematococcus pluvialis*, astaxanthin is the major carotenoid and exists mainly as astaxanthin esters of various fatty acids (Johnson and An, 1991). Astaxanthin possesses two identical asymmetric carbon atoms at C-3 and C-3′, making possible three optical isomers with all-trans configuration of the chain: (3*S*,3′*S*)-astaxanthin, (3*R*,3′*R*)-astaxanthin, and (3*S*,3′*R*)-astaxanthin (Vecchi and Müller, 1979). All-trans natural astaxanthin is readily isomerized to cis−trans mixtures, especially the 9-cis and 13-cis unhindered isomers by the action of light or heat (Johnson and An, 1991). It is thus likely that astaxanthin esters exist also as cis isomers other than all-trans isomers in the pigment extract of *H. pluvialis*. Therefore, the composition of astaxanthin esters in the algal extract is very complex (Yuan et al., 1996, 1997; Yuan and Chen, 1997). The complexity of the pigment composition in the algal extract makes it difficult to separate all of these pigments in a single chromatographic run. It is also difficult to separate all astaxanthin esters. Although a number of HPLC methods for the separation of pigments in *H. pluvialis* have been reported (Grung et al., 1992; Hagen et al., 1993; Fan et al., 1995; Yuan et al., 1996, 1997; Yuan and Chen, 1997), few are considered ideal for the simultaneous separation of astaxanthin, isomers of astaxanthin, astaxanthin esters, other carotenoids, and chlorophylls in the alga.

The primary aim of the present work is to develop a gradient reversed-phase HPLC method for the separation of astaxanthin esters and the isomers of astaxanthin from the unsaponified and saponified pigment extracts of *H. pluvialis* and also to develop a chromatographic purification method for the preparation of natural *trans*-astaxanthin from the alga.

## EXPERIMENTAL PROCEDURES

**Alga Strain and Culture Conditions.** *H. pluvialis* (*H. lacustris*, UTEX 16), obtained from the University of Texas Culture Collection, was grown in batch culture in a 3.7-L fermentor (Bioengineering, Wald, Switzerland) containing 2.5 L of MCM medium with 1 g/L sodium acetate at 25 °C (Yuan et al., 1996).

**Chemicals and Reagents.** HPLC grade methanol, acetonitrile, and dichloromethane were obtained from BDH Laboratory Supplies (Poole, England). Astaxanthin, lutein, $\beta$-carotene, and chlorophylls *a* and *b* were obtained from Sigma Chemical Co. (St. Louis, MO).

**Pigment Extraction.** Algal cells were collected by centrifuging the culture fluid at 3000*g* for 10 min, the supernatant was discarded, and the cell pellet was rinsed with distilled water (20 mL) twice and then freeze-dried using a Heto FD3 freeze-dryer (Heto-holten, Allerod, Denmark). The dry cells (25 mg) were homogenized using a 15-mL tissue homogenizer (B. Braun, Melsungen, Germany), and the total pigments were extracted in the solvent mixture (2-mL aliquots) of dichloromethane and methanol (25:75, v/v). The mixture was then separated by centrifugation at 10000*g* for 5 min, and the supernatant containing pigments was collected. The extraction procedure was repeated at least three times until the cell debris was almost colorless, and 10 mL of pigment extract was obtained. The total pigment extracts were centrifuged again at 10000*g* for 15 min and stored at −20 °C for subsequent saponification and HPLC separation. All of the above processes were conducted in darkness.

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**Saponification of Astaxanthin Esters.** One milliliter of 0.05 M NaOH dissolved in methanol, which was freshly prepared, was added to 5 mL of the pigment extract solution under a nitrogen atmosphere. The mixture (6 mL) was evaporated and concentrated to 5 mL under nitrogen and then kept overnight or for at least 8 h under nitrogen in darkness at room temperature for complete saponification of astaxanthin esters. The resulting product was directly separated and determined by HPLC.

**Analytical HPLC Method.** HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector. The pigment extract, saponified extract, and purified astaxanthin solution were separated and analyzed (20-$\mu$L aliquots) by using a 250 × 4.6 mm Beckman Ultrasphere $C_{18}$ (5 $\mu$m) column at 25 °C. The mobile phase consisted of solvent A (dichloromethane/methanol/acetonitrile/water, 5.0:85.0:5.5:4.5, v/v) and solvent B (dichloromethane/methanol/acetonitrile/water, 22.0:28.0:45.5:4.5, v/v). For the simultaneous separation of free astaxanthin and astaxanthin esters, the following gradient procedure was used: 0% of B for 8 min; a linear gradient from 0 to 100% of B for 6 min; 100% of B for 51 min. The flow rate was 1.0 mL/min. The tridimensional chromatogram was recorded from 250 to 700 nm. Peaks were measured at a wavelength of 480 nm to facilitate the detection of astaxanthins. Chromatographic peaks were identified by comparing retention times and spectra against known standards or by comparing their spectra (by photodiode array detection) with published data (Grung et al., 1992; Yokoyama and Miki, 1995; Stradi et al., 1995; Harker and Hirschberg, 1997; Fraser et al., 1997; Yuan et al., 1997; Yuan and Chen, 1997).

**Semipreparative HPLC Method.** The saponified pigment extract was separated (0.5-mL aliquots) by using a 250 × 10 mm Beckman Ultrasphere $C_{18}$ (5 $\mu$m) semipreparative column at 25 °C. The mobile phase, which consisted of methanol (90%), water (8%), and dichloromethane (2%), was used for the separation and purification of *trans*-astaxanthin. The flow rate was set at 3.0 mL/min. The solvent mixture of dichloromethane and methanol (25:75, v/v) was used for the purge of the column before the purification of astaxanthin.

The fraction of *trans*-astaxanthin was collected with a 50-mL Falcon conical tube according to the semipreparative HPLC. Only the central part of the *trans*-astaxanthin fraction was collected manually. Five milliliters of dichloromethane was then added to the *trans*-astaxanthin fraction collected. After mixing, 20 mL of distilled water was added. The solution separated into two layers after the addition of distilled water. The colorless supernatant, consisting of methanol and water and containing no pigments, was discarded. The red dichloromethane layer, containing *trans*-astaxanthin, was collected and blown gently to dryness under a stream of nitrogen. The residue was freeze-dried to remove any contaminating water, and the purified natural *trans*-astaxanthin was obtained.

## RESULTS AND DISCUSSION

**Extraction and Saponification.** The extraction solvent used in the present work was a mixture of methanol (75%) and dichloromethane (25%), which was found to be an effective extractant for the extraction of astaxanthins and other carotenoids. It is reported that 1 L of dichloromethane can dissolve 30 g of astaxanthin at room temperature, and the solubility is higher than that of chloroform, acetone, and DMSO (Johnson and An, 1991). The extraction solvent was also used as the injection solvent because it had polarity and solubility properties similar to those of the mobile phase used in the present work. Using the extraction solvent as the injection solvent could lessen the time of sample preparation.

The pigment extract of *H. pluvialis* was saponified for the hydrolysis of astaxanthin esters. Because saponification might result in the destruction and structural transformation of some carotenoids (Lietz and Henry, 1997), it is thus very important to remove air in the extract by blowing nitrogen before saponification and to add newly prepared sodium hydroxide solution under a nitrogen atmosphere. In the present experiment, mild saponification of astaxanthin esters was achieved for the complete hydrolysis of astaxanthin esters without the occurrence of astaxanthin degradation.

**Analytical HPLC.** In previous studies (Yuan et al., 1996, 1997; Yuan and Chen, 1997), we used the isocratic HPLC procedure, using the mixture solvent of dichloromethane, methanol, acetonitrile, and water as the mobile phase, to chromatograph carotenoids and chlorophylls from the extract of *H. pluvialis*. The results indicated that the retention behavior and separation effect of carotenoids and chlorophylls were affected significantly by the relative proportions of dichloromethane, methanol, acetonitrile, and water in the mobile phase. The presence of water could improve the separation of carotenoids and chlorophylls by increasing their retention times, and the presence of dichloromethane could minimize the peak tailing resulting from adding water and could reduce the retention times without compromising the separation effected by adding water (Nyambaka et al., 1996). The relative content of methanol and acetonitrile in the mobile phase was also able to affect the separation of carotenoids and chlorophylls (Yuan et al., 1997).

In comparison with astaxanthin esters, astaxanthin and lutein are poorly retained on commercially available $C_{18}$ columns (Emenhiser et al., 1996). Therefore, free astaxanthin and lutein are not easily separated. The separation of astaxanthin and lutein could be improved by increasing the content of water and adjusting the proportion of methanol and acetonitrile. We (Yuan and Chen, 1997) previously used the mobile phase containing dichloromethane (6.5%), methanol (82.0%), acetonitrile (7.5%), and water (4.0%) to separate *trans*-astaxanthin, lutein, and cis isomers of astaxanthin. The result showed that further increasing the content of water and decreasing the content of dichloromethane could further improve the separation of *trans*-astaxanthin and other carotenoids, and a compound identified tentatively as adonirubin was separated from the peak of *trans*-astaxanthin, which is very important for the purification of *trans*-astaxanthin. In addition, while the contents of water and dichloromethane were kept unchanged, increasing the content of methanol or decreasing the content of acetonitrile in the mobile phase could promote the separation of *trans*-astaxanthin, adonirubin, lutein, and cis isomers of astaxanthin. Higher content of acetonitrile enabled worse separation of lutein and cis isomers to occur, but a small amount of acetonitrile was necessary for the better separation of isomers of astaxanthin. In the present work, the mobile phase (solvent A) containing dichloromethane (5.0%), methanol (85.0%), acetonitrile (5.5%), and water (4.5%) was used for the separation of *trans*-astaxanthin, adonirubin, lutein, and cis isomers of astaxanthin. Using this mobile phase, the astaxanthin esters and $\beta$-carotene could not be eluted. Therefore, a gradient elution method (see Experimental Procedures) was required for the simultaneous separation of free astaxanthin and astaxanthin esters. The mobile phase (solvent B) containing dichloromethane (22.0%), methanol (28.0%), acetonitrile (45.5%), and water (4.5%) was

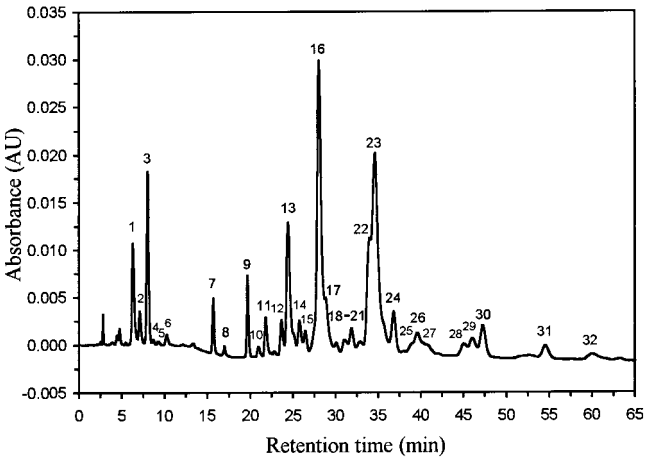


**Figure 1.** HPLC chromatogram of the pigment extract from *H. pluvialis*.

**Table 1. Identification of Carotenoids and Chlorophylls in *H. pluvialis***

| peak no. | retn time (min) | absorpn max (nm) | pigment |
|---|---|---|---|
| 1 | 6.3 | 480.0 | (3*S*,3′*S*)-*trans*-astaxanthin |
| 2 | 7.1 | 472.8 | adonirubin[a] |
| 3 | 8.0 | 443.9, 472.8 | lutein |
| 4 | 8.7 | 472.8 | (3*S*,3′*S*)-9-*cis*-astaxanthin |
| 5 | 9.2 | 371.8, 472.8 | (3*S*,3′*S*)-13-*cis*-astaxanthin |
| 9.4 | | 371.8, 472.8 | (3*S*,3′*S*)-15-*cis*-astaxanthin |
| 6 | 10.3 | 480.0 | (3*R*,3′*R*)-*trans*-astaxanthin[a] |
| 7 | 15.7 | 479.4 | canthaxanthin[a] |
| 8 | 17.0 | 472.8 | unknown |
| 9 | 19.7 | 463.2, 647.8 | chlorophyll *b* |
| 10 | 21.0 | 463.2, 647.8 | chlorophyll *b*′ |
| 11 | 21.8 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 12 | 23.7 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 13 | 24.5 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 14 | 25.8 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 15 | 26.5 | 430.6, 663.5 | echinenone[a] + chlorophyll *a* |
| 16 | 28.1 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 17 | 28.8 | 472.8 | (3*S*,3′*S*)-9-*cis*-astaxanthin ester |
| 18 | 30.1 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 19 | 31.1 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 20 | 31.9 | 472.8 | (3*S*,3′*S*)-9-*cis*-astaxanthin ester |
| 21 | 32.9 | 371.8, 472.8 | (3*S*,3′*S*)-13-*cis*-astaxanthin ester |
| 22 | 34.0 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 23 | 34.6 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 24 | 36.8 | 482.5 | (3*R*,3′*R*)-*trans*-astaxanthin ester[a] |
| 25 | 38.9 | 472.8 | (3*S*,3′*S*)-9-*cis*-astaxanthin ester |
| 26 | 39.6 | 472.8 | (3*S*,3′*S*)-9-*cis*-astaxanthin ester |
| 27 | 40.8 | 371.8, 472.8 | (3*S*,3′*S*)-13-*cis*-astaxanthin ester |
| 28 | 45.1 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |
| 29 | 46.0 | 482.5 | (3*R*,3′*R*)-*trans*-astaxanthin ester[a] |
| 30 | 47.3 | 482.5 | (3*R*,3′*R*)-*trans*-astaxanthin ester[a] |
| 31 | 54.6 | 455.9, 483.6 | *β*-carotene |
| 32 | 60.1 | 482.5 | (3*S*,3′*S*)-*trans*-astaxanthin ester |

[a] Tentatively identified.

used for the elution and separation of astaxanthin esters and *β*-carotene.

The typical chromatogram and chromatographic data obtained from the pigment extract of *H. pluvialis* are shown in Figure 1 and Table 1, respectively. Each of the astaxanthin ester fractions was collected by HPLC, saponified under nitrogen at room temperature, and subsequently separated by HPLC. The identification of these astaxanthin esters was achieved by analyzing the isomers of free astaxanthin in the saponified products of the astaxanthin ester fractions (Yuan and Chen, 1997). The difference in the absorption maxima of *trans*-astaxanthin (480.0 nm) and *trans*-astaxanthin

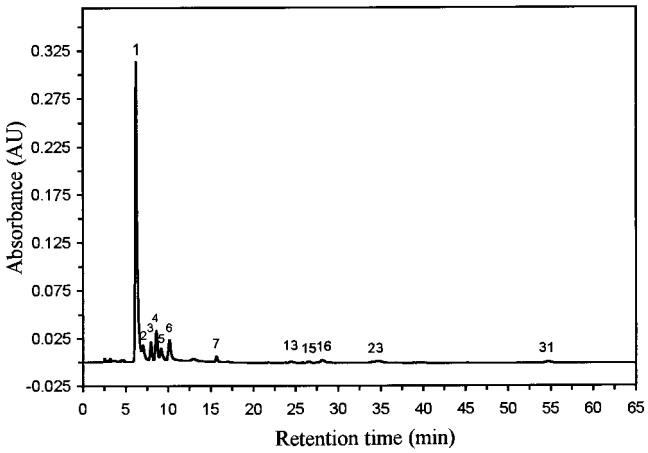


**Figure 2.** HPLC chromatogram of the saponified extract from *H. pluvialis*.

**Table 2. Results of Determination of Carotenoids and Chlorophylls in *H. pluvialis* (Milligrams per Liter)**

| pigment | unsaponified extract | saponified extract | content (mg/g of dry weight) | % of total carotenoids |
|---|---|---|---|---|
| *trans*-astaxanthin | 0.66 | 17.31 | 6.84 | 69.1 |
| 9-*cis*-astaxanthin | 0.04 | 1.83 | 0.72 | 7.3 |
| 13-*cis*-astaxanthin | 0.03 | 0.85 | 0.34 | 3.4 |
| (3*R*,3′*R*)-*trans*-astaxanthin | 0.08 | 1.66 | 0.66 | 6.6 |
| *β*-carotene | 0.25 | 0.28 | 0.10 | 1.0 |
| echinenone | | 0.11 | 0.04 | 0.4 |
| adonirubin | 0.24 | 1.27 | 0.50 | 5.1 |
| canthaxanthin | 0.27 | 0.26 | 0.10 | 1.0 |
| lutein | 1.50 | 1.54 | 0.60 | 6.1 |
| chlorophyll *a* | 2.90 | 0 | 1.14 | |
| chlorophyll *b* | 1.72 | 0 | 0.68 | |
| chlorophyll *b*′ | 0.32 | 0 | 0.13 | |

esters (482.5 nm) was perhaps due to the change of elution solvent in which they were detected.

Figure 2 shows a typical chromatogram of the saponified extract of *H. pluvialis*. The identification of the peaks in Figure 2 is the same as in Figure 1 and is also shown in Table 1. The determination results of carotenoids and chlorophylls in the unsaponified and saponified pigment extracts indicated that no significant loss or isomerization of lutein, *β*-carotene, and canthaxanthin occurred during the saponification (Table 2). Echinenone in the unsaponified extract could not be determined since it coeluted with chlorophyll *a*, but it could be determined in the saponified extract, and the content of echinenone was obtained by determining the saponified extract. The content of chlorophyll *a* in the unsaponified extract was measured at the wavelength of 663 nm for eliminating the effect of echinenone and astaxanthin esters on the determination of chlorophyll *a*. In the saponified extract, no chlorophylls *a* and *b* were detected, indicating that chlorophylls were completely degraded during saponification. The contents of adonirubin in the unsaponified and saponified pigment extracts were 0.24 and 1.27 mg/L, respectively; that is, the contents of adonirubin increased during saponification, indicating that the adonirubin esters were also present in *H. pluvialis*.

The results showed that four isomers of astaxanthin (peaks 1 and 4−6 in Figures 1 and 2) were separated from the unsaponified and saponified extracts. The isomers of astaxanthin were identified with their spectra by photodiode array detection. The utilization of *Q* ratios to identify cis isomers of astaxanthin was effective

(Yuan and Chen, 1997). The first peak, which was the most abundant isomer, was inseparable from astaxanthin standard and identified as *trans*-astaxanthin. Peak 4, which had a larger spectral shift and a much lower cis peak ($Q$ ratio = 4.97), was identified as 9-*cis*-astaxanthin. Peak 5, which had a large spectral shift with a higher cis peak ($Q$ ratio = 1.93), was identified as 13-*cis*-astaxanthin. A very small peak between peaks 5 and 6, which had a large spectral shift with a higher cis peak ($Q$ ratio = 1.55) representing a central cis isomer, was identified as 15-*cis*-astaxanthin. Peak 6, which gave a spectrum with completely the same features as that obtained from the *all-trans*-astaxanthin, was tentatively identified as (3*R*,3′*R*)-*trans*-astaxanthin, which was one of the three optical isomers of astaxanthin. No (3*S*,3′*R*)-astaxanthin was found in nature (Vecchi and Müller, 1979). In *H. pluvialis*, the predominant configurational isomer was (3*S*,3′*S*)-astaxanthin (Johnson and An, 1991).

No adonirubin, canthaxanthin, and echinenone standards were available in the present work, but the lack of these standards did not hinder the identification of these carotenoids from *H. pluvialis*. Using a photodiode array detector, peaks were identified instantaneously by taking the spectrum of each peak during its elution. The peak purities were also examined by the photodiode array detector. A comparison of the spectrum with published data on the known carotenoids allows almost total identification (Ittah et al., 1993). The concentrations of canthaxanthin, echinenone, and adonirubin were measured by the comparison of the peak area with astaxanthin. The exact biosynthetic pathway of astaxanthin has not yet been completely elucidated, although it is generally agreed that $\beta$-carotene serves as a precursor of the secondary carotenoids. Our results that canthaxanthin, echinenone, and adonirubin were detected in the extract of *H. pluvialis* support the assumption that astaxanthin is synthesized from $\beta$-carotene via echinenone, canthaxanthin, and adonirubin (Fan et al., 1995; Yokoyama and Miki, 1995; Harker and Hirschberg, 1997; Fraser et al., 1997).

**Purification of *trans*-Astaxanthin.** For the purification of *trans*-astaxanthin, the content of water was increased and the content of dichloromethane was decreased for further improving the separation of *trans*-astaxanthin and adonirubin, which was necessary for the purification of natural *trans*-astaxanthin. While the elution solvent that consisted of 90% methanol, 8% water, and 2% dichloromethane was used, the chromatogram for the separation and purification of *trans*-astaxanthin obtained from the saponified pigment extract of *H. pluvialis* is shown in Figure 3. The result indicates that *trans*-astaxanthin and adonirubin were well separated using the mobile phase on the semipreparative column. Although lutein and cis isomers of astaxanthin could not be separated, it is not important for the purification of *trans*-astaxanthin. The solvent mixture of dichloromethane (25%) and methanol (75%) was used for the purge of the column before the purification of *trans*-astaxanthin and for quick elution of other compounds after *trans*-astaxanthin had been eluted and collected.

To obtain a concentrated solution containing *trans*-astaxanthin from the *trans*-astaxanthin fraction collected by the semipreparative HPLC, 5 mL of dichloromethane and 20 mL of distilled water were added in sequence to the *trans*-astaxanthin fraction collected.

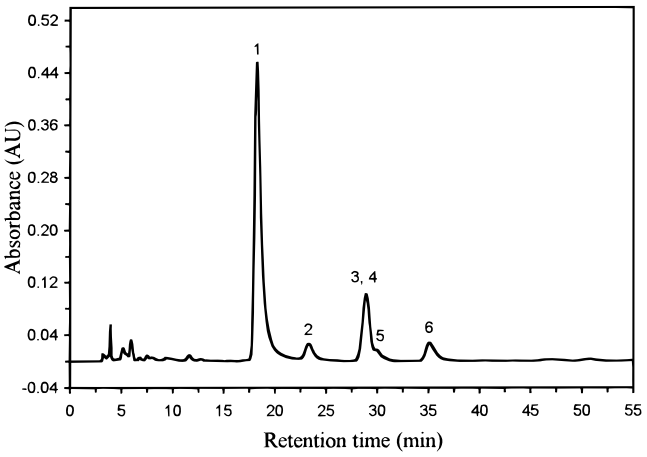


**Figure 3.** Chromatogram separated on the semipreparative column for the purification of *trans*-astaxanthin from the saponified pigment extract.

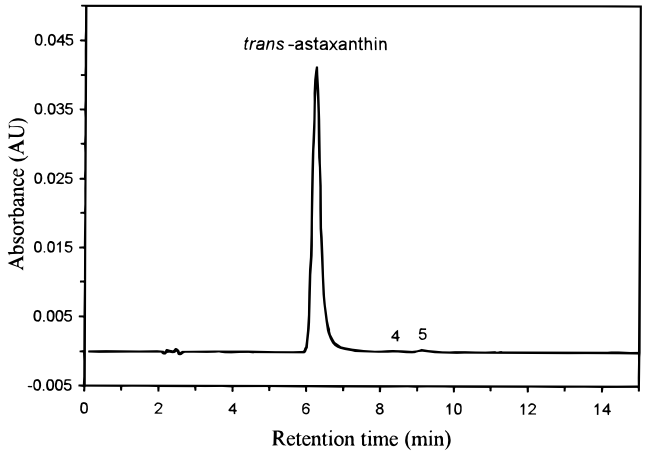


**Figure 4.** HPLC chromatogram of the purified *trans*-astaxanthin.

About 5 mL of dichloromethane solution containing *trans*-astaxanthin was obtained. After dichloromethane was removed, purified natural *trans*-astaxanthin, which has the appearance of a fine and dark violet-brown powder (Johnson and An, 1991), was obtained. The chromatogram of purified *trans*-astaxanthin is shown in Figure 4. The result indicates that, as shown in Figure 4, a very small amount of *cis*-astaxanthins was still detected from the purified *trans*-astaxanthin, indicating that *trans*-astaxanthins was easily isomerized to cis isomers. With the method developed, 3.7 mg of astaxanthin was isolated from 1 g of dry biomass of *H. pluvialis*. The purified natural astaxanthin contained about 97.7% *trans*-astaxanthin, 1.1% *cis*-astaxanthins, and 1.2% impurity.

## LITERATURE CITED

Emenhiser, C.; Simunovic, N.; Sander, L. C.; Schwartz, S. J. Separation of geometrical carotenoid isomers in biological extracts using a polymeric $C_{30}$ column in reversed-phase liquid chromatography. *J. Agric. Food Chem*. **1996**, *44*, 3887−3893.

Fan, L.; Vonshak, A.; Gabbay, R.; Hirshberg, J.; Cohen, Z.; Boussiba, S. The biosynthetic pathway of astaxanthin in a green alga *Haematococcus pluvialis* as indicated by inhibition with diphenylamine. *Plant Cell Physiol*. **1995**, *36*, 1519−1524.

Fraser, P. D.; Miura, Y.; Misawa, N. *In Vitro* Characterization of astaxanthin biosynthetic enzymes. *J. Biol. Chem*. **1997**, *272*, 6128−6135.

Gradelet, S.; Astorg, P.; Bon, A.; Berges, R.; Suschetet, M. Modulation of aflatoxin B1 carcinogenicity, genotoxicity and metabolism in rat liver by dietary carotenoids: evidence for a protective effect of CYP1A inducers. *Cancer Lett*. **1997**, *114*, 221−223.

Grung, M.; D'Souza, F. M. L.; Borowitzka, M.; Liaaen-Jensen, S. Algal carotenoids 51. Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S,3′S)-astaxanthin esters. *J. Appl. Phycol*. **1992**, *4*, 165−171.

Hagen, C.; Braune, W.; Birckner, E.; Nuske, J. Functional aspects of secondary carotenoids in *Haematococcus lacustris* (Girod) Rostafinski (Volvocales). *New Phytol*. **1993**, *125*, 625−633.

Harker, M.; Hirschberg, J. Biosynthesis of ketocarotenoids in transgenic cyanobacteria expressing the algal gene for $\beta$-C-4-oxygenase, *crtO*. *FEBS Lett*. **1997**, *404*, 129−134.

Ittah, Y.; Kanner, J.; Granit, R. Hydrolysis study of carotenoid pigments of paprika by HPLC/photodiode array detection. *J. Agric. Food Chem*. **1993**, *41*, 899−901.

Johnson, E. A.; An, G. H. Astaxanthin from microbial sources. *Crit. Rev. Biotechnol*. **1991**, *11*, 297−326.

Lietz, G.; Henry, C. J. K. A modified method to minimise losses of carotenoids and tocopherol during HPLC analysis of red palm oil. *Food Chem*. **1997**, *60*, 109−117.

Nyambaka, H.; Ryley, J. An isocratic reversed-phase HPLC separation of the stereoisomers of the provitamin A carotenoids in dark green vegetables. *Food Chem*. **1996**, *55*, 63−72.

Stradi, R.; Celentano, G.; Nava, D. Separation and identification of carotenoids in bird's plumage by high-performance liquid chromatography diode-array detection. *J. Chromatogr. B* **1995**, *670*, 337−348.

Turujman, S. A.; Wamer, W. G.; Wei, R. R.; Albert, R. H. Rapid liquid chromatographic method to distinguish wild salmon from aquacultured salmon fed synthetic astaxanthin. *J. AOAC Int*. **1997**, *80*, 622−632.

Vecchi, M.; Müller, R. K. Separation of (3S,3S′)-, (3R,3R′)- and (3S,3′R)- astaxanthin via (−)-camphanic acid esters. *J. High Resolut. Chromatogr. Chromatogr. Commun*. **1979**, *4*, 195−196.

Yokoyama, A.; Miki, W. Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum*. *FEMS Microbiol. Lett*. **1995**, *128*, 139−144.

Yuan, J. P.; Chen, F. Identification of astaxanthin isomers in *Haematococcus lacustris* by HPLC-photodiode array detection. *Biotechnol. Tech*. **1997**, *11*, 455−459.

Yuan, J. P.; Gong, X. D.; Chen, F. Separation and identification of astaxanthin esters and chlorophylls in *Haematococcus lacustris* by HPLC. *Biotechnol. Tech*. **1996**, *10*, 655−660.

Yuan, J. P.; Gong, X. D.; Chen, F. Separation and analysis of carotenoids and chlorophylls in *Haematococcus lacustris* by high-performance liquid chromatography photodiode array detection. *J. Agric. Food Chem*. **1997**, *45*, 1952−1956.

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