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Purification of *trans*-astaxanthin from a high-yielding astaxanthin ester-producing strain of the microalga *Haematococcus pluvialis*

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Abstract

The purification method including extraction, saponification, and separation was established for preparing free *trans*-astaxanthin from a high-yielding astaxanthin ester-producing strain of the microalga *Haematococcus pluvialis* which contained 3.67% *trans*-astaxanthins and 1.35% *cis*-astaxanthins of the dry cells. Low temperature (5°C) was chosen to minimize the degradation of astaxanthins during saponification, and 94.4% free *trans*-astaxanthin was obtained from *trans*-astaxanthin esters after 12 h of saponification. With this method, 32.2 mg *trans*-astaxanthin was obtained from 1 g dry algal cells. In addition, a new gradient reversed-phase HPLC method, suited for the quick analysis of free astaxanthins and astaxanthin esters in the unsaponified and saponified pigment extracts from the high-yielding astaxanthin ester-producing strain of the microalga *Haematococcus pluvialis*, was developed and applied to the determination of astaxanthin contents during the processes of extraction, saponification, and purification.

1. Introduction

The ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is one of the most important and economically valuable carotenoids due to its biological functions as a vitamin A precursor (Gobantes, Choubert, Milicua & Gómez, 1998) and a more efficient antioxidant than β-carotene and vitamin E (Johnson & An, 1991). Astaxanthin can be produced by a number of microorganisms, such as the green algae *Haematococcus pluvialis* (Fan, Vonshak, Gabbay, Hirshberg, Cohen & Boussiba, 1995; Hagen, Braune, Birckner & Nuske, 1993; Harker, Tsavaloc & Young, 1996; Kobayashi, Kakizono & Nagai, 1993) and *Chlorella zofingiensis* (Bar, Rise, Vishkautsan & Arad, 1995), the red yeast *Phaffia rhodozyma* (Bon, Leathers & Jayaswal, 1997; Calo, Velazquez, Sieiro, Blanco, Longo & Villa, 1995; Parajo, Santos & Vazquez, 1998), and the marine bacterium *Agrobacterium aurantiacum* (Yokoyama and Miki, 1995; Fraser, Miura and Misawa, 1997). Gene transfer technology has also been developed to aid alternation of the carotenoid biosynthesis pathway so as to produce astaxanthin and related ketocarotenoids in the blue-green alga *Synechococcus* sp. PCC7942 where the other carotenoids such as β-carotene and zeaxanthin were already present at high concentrations in the cells (Harker & Hirschberg, 1997).

Astaxanthin exists mainly as free astaxanthin in the red yeast *Phaffia rhodozyma* (Parajo et al., 1998) and as astaxanthin esters in the green algae *H. pluvialis* (Johnson & An, 1991) and *C. zofingiensis* (Bar et al., 1995). The red yeast *Phaffia rhodozyma* is more suitable for the preparation of purified *trans*-astaxanthin, as judged by the state of astaxanthin, than the green alga, *H. pluvialis*, but the content of astaxanthin is lower than in *H. pluvialis*. In all of these microorganisms, the content of astaxanthin reported was between 0.04 and 2.7% of the dry cell weight (Bon et al, 1997; Calo et al., 1995; Chourbert & Heinrich, 1993; Grung, D'Souza, Borowitzka & Liaaen-Jensen, 1992; Harker et al., 1996; Parajo et al., 1998). For the preparation of the purified *trans*-astaxanthin from microorganisms, it is necessary to choose some high-yielding astaxanthin ester-producing strains.

During the preparation of the purified *trans*-astaxanthin from astaxanthin ester-containing strains, saponification for hydrolyzing astaxanthin esters is necessary. The concentration of methanolic NaOH solution was important for promoting the hydrolysis of

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astaxanthin esters and minimizing the degradation of astaxanthin during the saponification (Yuan & Chen, 1999).

The primary objective of the present work is to establish an optimal saponification method at a low temperature for the hydrolysis of astaxanthin esters and prepare the natural *trans*-astaxanthin from the pigment extract of the high-yielding astaxanthin ester-producing strain of the microalga *H. pluvialis* by a semipreparative chromatographic method, and also develop a novel HPLC method for rapidly analyzing astaxanthins in the unsaponified and saponified pigment extracts.

2. Materials and methods

2.1. *Microalga* Haematococcus pluvialis

NatuRed, the dry biomass of the microalga *H. pluvialis* with high content of astaxanthin esters, was obtained from Professor Sammy Boussiba, the Microalgal Biotechnology Laboratory of Ben-Gurion University of the Negev (Israel).

2.2. Chemicals and reagents

HPLC-grade methanol, acetonitrile and dichloromethane were obtained from BDH Laboratory Supplies (Poole, England). Astaxanthin, lutein, β-carotene, chlorophyll *a* and chlorophyll *b* were obtained from Sigma Chemical Co. (St Louis, MO, USA). Canthaxanthin was kindly provided by Professor Sammy Boussiba of Ben-Gurion University of the Negev (Israel).

2.3. Analysis of pigments

HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array (PDA) detector (Waters Corporation, Milford, MA, USA). A Waters Symmetry C18 column (150×3.9 mm; 5 μm) was chosen for the separation and analysis of the pigment extracts, saponified extracts and purified *trans*-astaxanthin solutions (20-μl aliquots). 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 established by Yuan and Chen (1999) was used: 0% of B for 8 min; a linear gradient from 0 to 100% of B for 6 min; 100% of B for 24 min. The flow rate was set at 1.0 ml/min. The detection wavelength of the PDA detector was set between 250 and 700 nm and the chromatographic peaks were measured at a wavelength of 480 nm to facilitate the detection of astaxanthins.

2.4. Extraction of pigments

The alga sample (0.1 g) in a mortar was ground with a pestle for one minute and 5 ml of extraction solvent; the mixture of dichloromethane and methanol (25: 75, v/v), was added. After 1-min grinding of the algal cells, together with the extraction solvent, the mixture of the algal cells and the solvent was then separated by centrifugation at 10,000 *g* for 5 min, and the supernatant containing pigments was collected. The extraction procedure was repeated at least three times until the algal cell debris was almost colourless. Dichloromethane was used to dissolve and recover the pigments absorbed on the mortar and the pestle. The combined supernatants were centrifuged again at 10,000 *g* for 10 min and 30 ml of the pigment extracts were obtained. The pigment extracts were stored at −20°C for subsequent saponification of astaxanthin esters and purification of *trans*-astaxanthin. All the above processes were conducted in darkness.

2.5. Hydrolysis of astaxanthin esters

Six millilitres of 0.107 M NaOH dissolved in methanol, which was freshly prepared (Yuan & Chen, 1999), was added to 30 ml of the pigment extract solution under a nitrogen atmosphere. The mixture (36 ml) was evaporated and concentrated to 30 ml under nitrogen while adding NaOH, and then kept for 12 h in the water bath (5°C) of a 9105 Refrig/Heat Circulating Bath (Polyscience, Niles, IL, USA) in darkness under nitrogen for complete saponification of astaxanthin esters. The saponified pigment extract solutions were directly analyzed by HPLC and stored at −20°C for subsequent purification of *trans*-astaxanthin by semipreparative HPLC.

2.6. Purification of trans-*astaxanthin*

The saponified pigment extract (0.5-ml aliquots) was separated by using a 250×10 mm Beckman Ultrasphere ODS (5 μm) semipreparative column (Beckman Instruments, Fullerton, CA, USA). The mobile phase consisted of solvent C (methanol:water:dichloromethane; 90:8:2, v/v) for the separation and purification of *trans*-astaxanthin and solvent D (dichloromethane and methanol; 25:75, v/v) for the purge of the column after the elution of *trans*-astaxanthin. The flow rate was set at 3 ml/min. The combined fractions of *trans*-astaxanthin collected were mixed with five millilitres of dichloromethane, followed by adding about the same volume of distilled water as the combined fractions collected. The solution then separated into two layers: the aqueous phase and the nonaqueous phase. The colourless supernatant, consisting of methanol and water, was discarded. The red dichloromethane layer containing *trans*-astaxanthin was collected and then blown gently to dryness under a stream of nitrogen.

3. Results and discussion

3.1. Analysis of pigments

The Symmetry C18 column was chosen and applied to the separation of astaxanthin isomers and esters in the pigment extract from a high-yielding astaxanthin ester-producing strain of *H. pluvialis*. Although the relative concentrations of dichloromethane, acetonitrile, methanol, and water in the mobile phase could significantly affect the retention behaviour and the separation effect of astaxanthin isomers and esters, the mobile phase compositions and gradient procedure as described previously (Yuan & Chen, 1999) were still suitable for the separation of astaxanthin isomers and esters on the Symmetry C_{18} column. A typical chromatogram obtained from the pigment extract of *H. pluvialis* is shown in Fig. 1. It showed that, in comparison with the Ultrasphere C_{18} column used in previous studies (Yuan & Chen), astaxanthin esters could be separated in a shorter time and the *trans*-astaxanthin (peak 1), lutein (peak 2), 9-*cis*-astaxanthin (peak 3), and 13-*cis*-astaxanthin (peak 4) could also be well separated. As shown in Fig. 1, since it was eluted together with one of astaxanthin esters (peak 15), β-carotene could not be quantitatively determined, but it might be determinable after astaxanthin esters are completely hydrolyzed. The content of chlorophyll *a*, the small peak between peaks 9 and 10, might be measured at the wavelength of 663 nm to eliminate the effect of astaxanthin esters on the determination of chlorophyll *a*.

Due to its low boiling point (about 40°C), dichloromethane was readily volatile at room temperature. The mobile phase containing dichloromethane should not be stored for a long time to prevent the decrease of the relative content of dichloromethane in the mobile phase, which might make the retention times of some compounds increase, especially β-carotene.

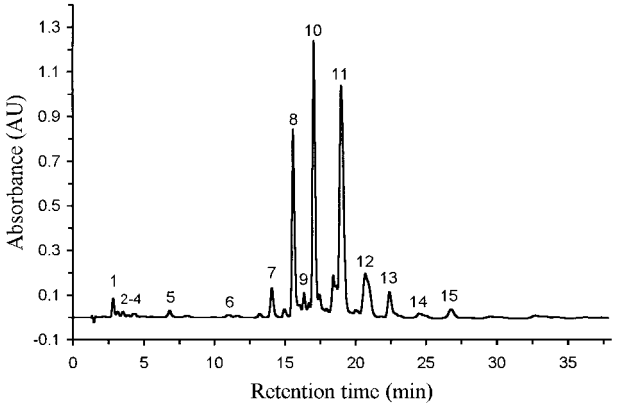

Fig. 1. HPLC chromatogram of the pigment extract from the high-yielding astaxanthin ester-producing strain of the alga *H. pluvialis*. Peaks: 1, *trans*-astaxanthin; 2, lutein; 3, 9-*cis*-astaxanthin; 4, 13-*cis*-astaxanthin; 5, canthaxanthin; 6, chlorophyll *b*; 7–14, astaxanthin esters; 15, astaxanthin ester and β-carotene.

3.2. Extraction of pigments

The breaking of algal cells was important for elevating the efficiency of pigment extraction. In some previous work, the algal cells were disrupted by using the tissue homogenizer (Harker et al., 1996), grinding with mortar and pestle (Choubert & Heinrich, 1993; Hagen et al., 1993; Kobayashi et al., 1993), heating at 60°C (Xylander & Braune, 1994) and 70°C (Bar et al., 1995; Rise, Cohen, Vishkautsan, Cojocarum, Gottlieb & Arad, 1994), or enzymatic disruption (Grung et al., 1992). Our result showed that grinding with a mortar and a pestle was simple, quick, and efficient for the extraction of pigments from the high-yielding astaxanthin ester-producing strain of the alga *H. pluvialis*, especially for a large amount of algal cells. Therefore, in the present experiment, the algal cells were set in the mortar and broken by grinding with a pestle.

According to Johnson and An (1991), dichloromethane (~30 g/l), chloroform (~10 g/l), dimethylsulfoxide (~0.5 g/l), and acetone (~0.2 g/l) could easily dissolve astaxanthin at room temperature. High solubility might facilitate the extraction of astaxanthin, especially astaxanthin esters, from the broken algal cells. Dichloromethane was a good solvent for the extraction of astaxanthins, but the cell debris and dichloromethane extract solution could not be separated completely by centrifugation and some cell debris was still suspended in the extract solution. While dichloromethane was mixed with methanol, the cell debris suspended in the extract solution would be completely precipitated by centrifugation at 10,000 *g* for 5–10 min. Therefore, in the present work, the mixture of methanol and dichloromethane was used as an effective extractant for astaxanthin esters.

After extraction at least three times, algal pigments were exhaustively extracted and 30 ml of deep red extract solution, containing 162.4 mg astaxanthin esters per litre of extract, was obtained from 0.097 g algal cells. As shown in Fig. 1, carotenoids in the pigment extract from a high-yielding astaxanthin ester-producing strain of *H. pluvialis* contained various kinds of astaxanthin esters, in addition to a very small amount of lutein (peak 2), canthaxanthin (peak 5), and β-carotene. The determination result showed that the total content of free astaxanthin and astaxanthin esters in the high-yielding astaxanthin ester-producing strain of the microalga *H. pluvialis* was 5.02% on a cell dry weight basis.

In the alga *H. pluvialis*, astaxanthins exist mainly as various astaxanthin esters formed by combining various fatty acids with different isomers of astaxanthin. For different algal strains and culture conditions, the compositions of astaxanthin esters were different. It was difficult to separate each of all astaxanthin esters in the alga *H. pluvialis*. It was also possible that some peaks were composed of at least two astaxanthin esters, for example, peaks 11 and 12 (Fig. 1).

3.3. Hydrolysis of astaxanthin esters

The addition of NaOH was necessary for the hydrolysis of astaxanthin esters, but NaOH could result in significant degradation of astaxanthins, especially when the reaction temperature was high. The results of Yuan and Chen (1999) indicated that, although high temperature favoured the hydrolysis of astaxanthin esters, the degradation of astaxanthins was also promoted markedly at the same time. Therefore, the saponification should be carried out at a low temperature to minimize the degradation of astaxanthins during the hydrolysis of astaxanthin esters. In order to study the effect of temperature on the hydrolysis of astaxanthin esters and the degradation of astaxanthins during saponification, the processes of saponification of astaxanthin esters at different temperatures were monitored. Fig. 2 shows changes in free *trans*-astaxanthin concentration during saponification with 0.021 M NaOH in the reaction mixtures at 5, 10, and 15°C. The result showed that, at a higher temperature (e.g. 15°C), the initial rate of hydrolysis reaction was higher, but the final concentration of free astaxanthin was lower than at a lower temperature (e.g. 5°C). As can be seen from the figure, while the reaction temperature was maintained at 5°C, no significant decrease in the content of *trans*-astaxanthin was found after saponification for 12 h, and on the contrary, the content of *trans*-astaxanthin reached its maximum concentration after that time. Fig. 3 shows changes in free *trans*-astaxanthin concentration in the reaction mixtures during saponification with 0.018, 0.021, 0.026 and 0.032 M NaOH in the reaction mixtures at 5°C. As shown in Fig. 3, a sodium hydroxide concentration of 0.021 M in the reaction mixtures was optimum for complete hydrolysis of astaxanthin esters after 12 h without causing the degradation of *trans*-astaxanthin at 5°C. The fact that the content of free *trans*-astaxanthin was kept unchanged indicated that the degradation reaction of *trans*-astaxanthin might occur at a very low rate, especially at a lower temperature and a lower NaOH concentration. Therefore, in the present work, the pigment extracts were saponified at 5°C for 12 h with 0.021 M NaOH in the reaction mixture, followed by purification of *trans*-astaxanthin.

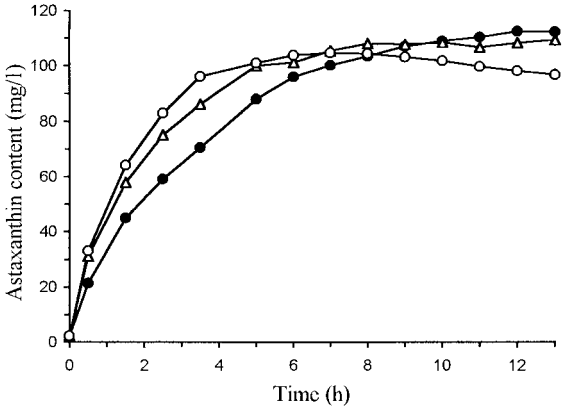

Fig. 2. Changes in free *trans*-astaxanthin content during saponification with sodium hydroxide of 0.021 M in the reaction mixtures at 5 (●), 10°C (△) and 15°C (○). (SE ± 1.8%, $n = 3$.)

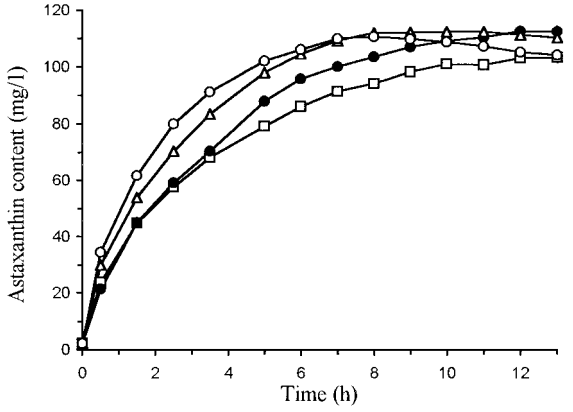

Fig. 3. Changes in free *trans*-astaxanthin content during saponification with sodium hydroxide of 0.018 M (□), 0.021 M (●), 0.026 M (△), 0.032 M (○) in the reaction mixtures at 5°C. (SE ± 1.9%, $n = 3$.)

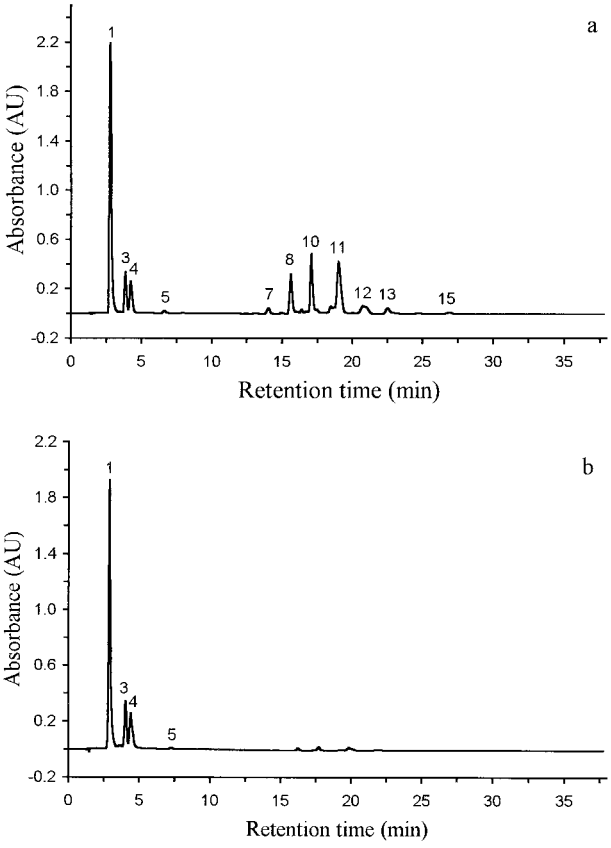

Fig. 4. HPLC chromatograms of the pigment extracts during saponification for 2.5 h (a) and 12 h (b). Peaks: 1, *trans*-astaxanthin; 3, 9-*cis*-astaxanthin; 4, 13-*cis*-astaxanthin; 5, canthaxanthin; 7–13, astaxanthin esters; 15, astaxanthin ester and β-carotene.

Fig. 4 shows the typical chromatograms of pigment extracts after saponification for 2.5 and 12 h, respectively. As shown in Fig. 4b, the carotenoid compositions in the saponified pigment extract consisted almost completely of *trans*-astaxanthin, 9-*cis*-astaxanthin, and 13-*cis*-astaxanthin. During the course of saponification, the concentrations of *trans*-astaxanthin in pigment extract increased quickly along with the continuous hydrolysis of astaxanthin esters, especially at the initial stage of saponification (Figs. 2 and 3). Since the concentration of astaxanthin esters in the pigment extract was so high that the upper limit (2.5 AU) of detection was exceeded after saponification for 6 h, 10 μl of samples were injected for the HPLC analysis (Fig. 4b).

The determination of free *trans*-astaxanthin and its isomers showed that the relative content of *trans*-astaxanthin in total astaxanthins was kept unchanged during the course of saponification and the value was 73.2% of total astaxanthins (Table 1), indicating that no isomerization of *trans*-astaxanthin occurred during saponification. The results also showed that, in the high-yielding astaxanthin ester-producing strain of *H. pluvialis*, the contents of the *trans*-astaxanthin and *cis*-astaxanthins were 3.67 and 1.35%, respectively, on a dry algal cell basis. After saponification for 12 h, the concentration of free *trans*-astaxanthin in saponified extract had reached the maximum and then the saponification reaction was stopped by storing the saponified pigment extract at −20°C. With the method for saponification, *trans*-astaxanthin esters in the pigment extract were almost completely hydrolyzed and 94.4% of free *trans*-astaxanthin on total *trans*-astaxanthin esters was produced (Table 1). That is, the percentage of both the un-hydrolyzed *trans*-astaxanthin esters and the degraded astaxanthins was 5.6% of total *trans*-astaxanthin esters in the pigment extract.

3.4. *Purification of* trans-*astaxanthin*

In the purification of *trans*-astaxanthin, a semipreparative chromatographic purification method was used to prepare the purified *trans*-astaxanthin from the high-yielding astaxanthin ester-producing strain of *H. pluvialis*. Fig. 5 shows the semipreparative chromatogram of the separation of free astaxanthins in 0.5 ml of saponified pigment extract. The result indicated that *trans*-astaxanthin and *cis*-astaxanthins were well separated on the semipreparative reversed phase column. The peaks after the peak of 13-*cis*-astaxanthin (peak 3) were un-hydrolyzed astaxanthin esters (small amounts) and other carotenoids such as canthaxanthin, echinenone, and β-carotene, which were quickly eluted by solvent D, the solvent mixture of dichloromethane (25%) and methanol (75%). About 5 ml of *trans*-astaxanthin fraction was collected every time from 0.5 ml of saponified pigment extract. The combined *trans*-astaxanthin fractions dissolved in the elution solvent consisting of methanol (90%), water (8%), and dichloromethane (2%) were mixed with about 5 ml (or more than 5 ml according to the volume of fractions) of dichloromethane, followed by mixing with distilled water, to obtain a concentrated *trans*-astaxanthin solution in dichloromethane. The results showed that, if the *trans*-astaxanthin fractions were first mixed with distilled water and then dichloromethane, *trans*-astaxanthin was also present in the supernatant consisting of methanol and water, in addition to being in the dichloromethane solution. The red dichloromethane fraction should be promptly dried with a stream of nitrogen to prevent the isomerization of *trans*-astaxanthin in dichloromethane. With the separation and purification method, 92.8% of *trans*-astaxanthin, a fine and dark violet-brown powder (Johnson & An, 1991), was obtained from the saponified pigment extract (Table 1).

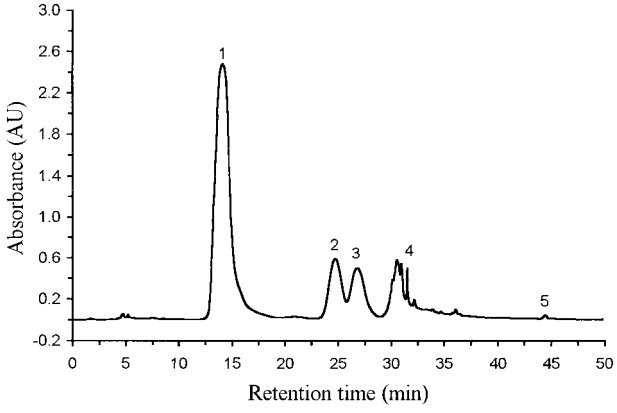

Fig. 5. The chromatogram showing the separation of free astaxanthins in 0.5 ml of saponified pigment extract from the high-yielding astaxanthin ester-producing strain of the alga *Haematococcus pluvialis*. Peaks: 1, *trans*-astaxanthin; 2, 9-*cis*-astaxanthin; 3, 13-*cis*-astaxanthin; 4, canthaxanthin and astaxanthin esters; 5, β-carotene.

Table 1
Concentrations of astaxanthin esters and free *trans*-astaxanthin in the pigment extracts and dry algal cells after extraction, saponification and purification

	Extracted total astaxanthin esters	Extracted *trans*-astaxanthin esters	Hydrolyzed free *trans*-astaxanthin	Purified *trans*-astaxanthin
Percentage (%)	99.9	73.2	94.4	92.8
Content on extracts (mg/l)	162.4	118.9	112.3	104.2
Content on a dry algal cell (%)	5.02	3.67	3.47	3.22

With the methods developed for extraction, saponification, separation, and purification, 32.2 mg of the purified *trans*-astaxanthin was obtained from 1 g of the high-yielding astaxanthin ester-producing strain of the alga *H. pluvialis* containing 36.7 mg of *trans*-astaxanthins and 13.5 mg of *cis*-astaxanthins (Table 1). The efficiency of purification of *trans*-astaxanthin from the pigment extract was 64.1% on total astaxanthins or 87.6% on *trans*-astaxanthins. Since the saponification at low temperature (5°C) was applied, the efficiency of purification was higher than the saponification at room temperature. Therefore, the saponification at low temperature was important for minimizing the loss of astaxanthins during the process of purification.

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