Separation and Analysis of Carotenoids and Chlorophylls in *Haematococcus lacustris* by High-Performance Liquid Chromatography Photodiode Array Detection

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An improved HPLC method was developed for the separation and analysis of carotenoids and chlorophylls in a green alga, *Haematococcus lacustris*. Six astaxanthin esters, the principal carotenoids in *H. lacustris*, were separated and indicated as astaxanthin esters 1−6. The retention times of the six astaxanthin esters were 11.8, 14.3, 15.0, 18.2, 22.0, and 28.4 min, respectively. Astaxanthin ester 4 was the main astaxanthin ester, which accounted for 44−49% of the total astaxanthin esters. The six astaxanthin esters had the same absorption spectra, and their maximum absorption wavelengths in the mobile phase were all 481.3 nm. Relatively small amounts of β-carotene, canthaxanthin, and echinenone were detected from the alga extracts compared to astaxanthin esters, lutein, and chlorophylls. During the cultivation, the light intensity for illumination of the culture was increased from 90 to 190 μmol/(m^2 s) to investigate its effect on pigment formation. The content of the total astaxanthin esters was higher with enhanced light intensity after 4 days of cultivation (6.8 mg/g of dry weight) than after 8 days of cultivation (5.0 mg/g of dry weight). This HPLC method could be used to separate and determine the various algal pigments and assist in the study of biosynthesis of ketocarotenoids in algae.

Keywords: *Astaxanthin esters; carotenoids; chlorophylls; Haematococcus lacustris; HPLC*

INTRODUCTION

Haematococcus lacustris contains large amounts of astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) esters and is being considered as a potential natural source of astaxanthin (Johnson and An, 1991). In the alga, astaxanthin is present in the lipid bodies primarily as esters of various fatty acids (Johnson and An, 1991).

Although a number of HPLC methods for the separation of pigments have been employed (Grung *et al*., 1992; Hagen *et al*., 1993; Bar *et al*., 1995; Dall *et al*., 1995; Fan *et al*., 1995; Yuan *et al*., 1996), few are considered ideal for the separation of carotenoids and chlorophylls in *H. lacustris*. The complexity of pigment composition in the algal extracts makes it difficult to separate all of these pigments in a single chromatographic run. It is also difficult to separate all astaxanthin esters (Yuan *et al*., 1996).

The primary aim of the present work is to develop an improved isocratic reversed-phase HPLC method for separating and analyzing carotenoids and chlorophylls, especially astaxanthin esters from the pigment extracts of *H. lacustris*. Since the primary functions of carotenoids and chlorophylls are as light harvest accessory pigments, the effect of light intensity on the pigment formation is also investigated.

EXPERIMENTAL PROCEDURES

Alga Strain and Culture Conditions. *H. lacustris* (UTEX No. 16), obtained from the University of Texas Culture Collection, was grown in batch culture in a 3.7-L fermentor (Bioengineering, Wald, Switzerland) in 2.5 L of MCM medium (Yuan *et al*., 1996) containing 1 g/L sodium acetate at 25 °C. The medium and fermentor were sterilized *in situ* at 121 °C for 15 min before inoculation. The initial pH of the medium was adjusted to 7.0. The culture was agitated at 300 rpm, and sterile air was supplied to the culture at a flow rate of 100 L/h. Continuous illumination was provided by fluorescent lights surrounding the fermentor. The light intensity was raised from 90 to 190 μmol/(m^2 s) after 4 days of cultivation or after 8 days of cultivation.

The cell dry weight concentration was determined according to the procedure of Chen *et al*. (1996).

Chemicals and Reagents. HPLC grade methanol, acetonitrile, and dichloromethane were obtained from BDH Laboratory Supplies (Poole, England). Lutein, α-carotene, β-carotene, chlorophyll *a*, and chlorophyll *b* were obtained from Sigma Chemical Co. (St. Louis, MO).

Pigment Extraction and Sample Preparation. Cells were harvested by centrifuging 5 mL of the culture fluid at 3000*g* for 10 min, and the supernatant was discarded. The cell pellet rinsed with distilled water twice was transferred to a 15-mL homogenizer (B. Braun, Melsungen Germany) and homogenized with 2 mL of solvent consisting of 75% methanol and 25% dichloromethane. The mixture was then separated by centrifugation at 10000*g* for 15 min, and the pigment-containing supernatant was collected. The extraction procedure was repeated until the cell debris was almost colorless. The pigment extract was filtered through 0.45-μm filters and stored at −20 °C for the subsequent HPLC analysis. All of the above processes were conducted in darkness.

HPLC Method. HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array (PDA) detector. The pigment extract filtered through 0.45-μm filters was separated by using a 250 × 4.6 mm Ultrasphere C_{18} (5 μm) column (Beckman) at 25 °C. A Resolve C_{18} column (Waters, 300 × 3.9 mm, 5 μm) was also tested. The mobile phase consisted of methanol (69.0%), dichloromethane (17.0%), acetonitrile (11.5%), and water (2.5%). The flow rate was set at 1.0 mL/min. The samples were injected with a Rheodyne 7725 valve with a 20-μL loop. A tridimensional chromatogram was recorded from 300 to 700 nm. Peaks were measured at a wavelength of 450 nm to facilitate the simultaneous detection of chlorophylls and carotenoids.

Chromatographic peaks were identified by comparing reten-

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tion times and spectra against known standards (lutein, β-carotene, chlorophyll *a*, chlorophyll *b*). Echinenone, canthaxanthin, and astaxanthin were identified using a PDA detector by comparing their spectra with published data (Grung *et al*., 1992; Yokoyama and Miki, 1995). Their concentrations were measured by area comparison with β-carotene (Calo *et al*., 1995).

RESULTS

Development of HPLC Separation Method. Two reversed-phase columns, an Ultrasphere C_{18} column (Beckman, 250 × 4.6 mm, 5 μm) and a Resolve C_{18} column (Waters, 300 × 3.9 mm, 5 μm), were chosen for the separation of carotenoids and chlorophylls. The solvent mixtures (as mobile phase) containing methanol, acetonitrile, dichloromethane, and water were tested for their ability to separate carotenoids and chlorophylls.

The chromatographic surface of Resolve C_{18} bonded phase can retain samples by a combination of hydrophobic and silanophilic retention mechanisms. It is useful when more retention and an additional interaction with silanols are needed for separation. Astaxanthin esters may have the additional interaction with silanols, rather than other pigments. The retention times of the astaxanthin esters in the column were relatively long, and so all astaxanthin esters eluted after chlorophylls *b* and *a*. β-Carotene could not be separated well from the main astaxanthin esters.

Using the Ultrasphere C_{18} column, β-carotene and the astaxanthin esters could be well separated, but the separation of chlorophyll *a* from the astaxanthin esters was difficult. Chlorophyll *a* eluted together with one of the astaxanthin esters. Although complete separation of chlorophyll *a* from astaxanthin esters was possible by increasing their retention times (Yuan *et al*., 1996), a long separation time was required.

The relative proportion of methanol, dichloromethane, acetonitrile, and water in the mobile phase was varied to effect separation. It was reported that increasing the content of water enabled better separation to occur by increasing the retention times of chlorophylls and carotenoids, but peak tailing resulted (Nyambaka *et al*., 1996). Increasing the amount of dichloromethane minimized the peak tailing and reduced the retention times without compromising the separation effected by adding water (Nyambaka *et al*., 1996). We found that the change in the relative content of methanol and acetonitrile in the mobile phase was able to alter the relative retention times of astaxanthin esters and chlorophyll *a*. Therefore, it is likely that the separation of astaxanthin esters from chlorophyll *a* will be improved by changing the relative content of methanol and acetonitrile. When the mobile phase consisted of methanol (69.0%), dichloromethane (17.0%), acetonitrile (11.5%), and water (2.5%), chlorophyll *a* (peak 13) eluted between the two peaks for astaxanthin esters 1 (peak 12) and 2 (peak 14) from the Ultrasphere C_{18} column (Figure 1). Typical chromatographic data obtained from the red extract of *H. lacustris* are shown in Figure 1 and Table 1. In the alga *H. lacustris*, astaxanthin is a group of major carotenoids and exists mainly as astaxanthin esters of various fatty acids. Six main astaxanthin esters (peaks 12, 14−17, and 21) were separated, which were indicated as astaxanthin esters 1−6, respectively. Three principal astaxanthin esters (peaks 12, 14, and 16) were previously saponified and identified (Yuan *et al*., 1996). Peaks 15, 17, and 21 were also astaxanthin esters since they had the same absorption spectra and maxima (481.3 nm) as peaks 12, 14, and 16 by PDA detection.

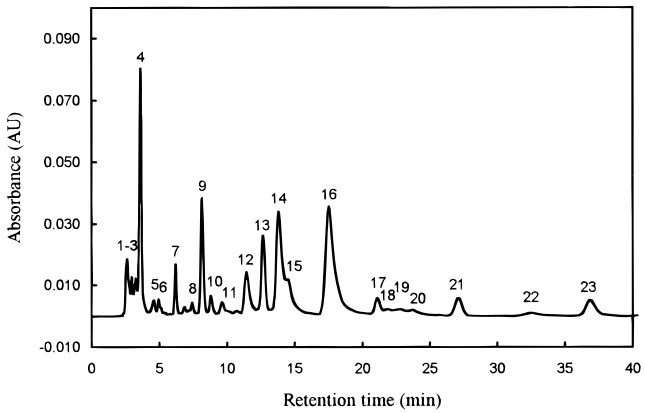

Figure 1. HPLC profile of carotenoids and chlorophylls extracted from *H. lacustris* after 13 days of cultivation (450 nm). The light intensity was raised from 90 to 190 μmol/(m^2 s) after 4 days of cultivation. For identification, see Table 1.

Table 1. Separation and Identification of Carotenoids and Chlorophylls in *H. lacustris* after 13 Days of Cultivation

peak no.	retention time (min)	absorption maxima (nm)			pigment	% of total carotenoids
1	2.6	437.9		466.8		4.1
2	3.0	437.9		466.8		1.7
3	3.3	447.5		471.6		1.7
4	3.7	447.5		476.5	lutein	14.2
5	4.7	446.8		471.6		1.4
6	5.0	334.0	437.9	466.8		0.8
7	6.3		476.5		canthaxanthin	2.7
	7.0	462.0		646.6		0.8
8	7.6		457.1			1.2
9	8.4	466.8		651.4	chlorophyll *b*	
10	9.0	466.8		651.4	chlorophyll *b′*	
11	9.9		476.5			0.9
	10.5	476.5		661.2		0.5
	11.1	476.5		661.2		0.7
12	11.8		481.3		astaxanthin ester 1	6.3
13	13.1	433.0		666.1	chlorophyll *a*	
14	14.3		481.3		astaxanthin ester 2	16.2
15	15.0		481.3		astaxanthin ester 3	6.4
16	18.2		481.3		astaxanthin ester 4	25.5
17	22.0		481.3		astaxanthin ester 5	2.8
18	22.8		471.6			1.5
19	23.6	375.4		466.8		1.3
20	24.8		476.5			1.9
21	28.4		481.3		astaxanthin ester 6	3.5
22	34.1		476.5			0.9
23	38.3	452.3		476.4	β-carotene	3.0

In the separation condition, echinenone could not be separated from astaxanthin ester 2 (peak 14). This was in agreement with the results reported by Hagen *et al*. (1993) and Fan *et al*. (1995). Fan *et al*. (1995) reported that echinenone could not be separated from astaxanthin esters by their HPLC method but could be clearly identified by silica gel thin-layer chromatography (TLC). In this work, the HPLC technique for identification of echinenone was developed. With a mobile phase consisting of methanol (16.0%), dichloromethane (17.0%), acetonitrile (64.5%), and water (2.5%), echinenone could be well separated from astaxanthin esters, but the astaxanthin ester peaks widened obviously, which made

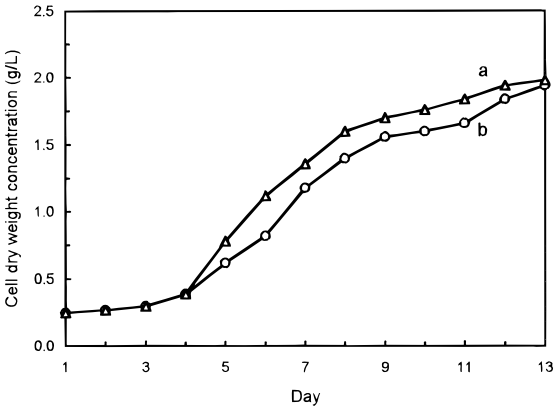

Figure 2. Growth of *H. lacustris* cells in the culture. The light intensity was raised from 90 to 190 μmol/(m^2 s) after 4 days of cultivation (a) or after 8 days of cultivation (b). (SE $\pm$ 3.1%; $n = 3$.)

the precise determination of the astaxanthin esters difficult (data not shown). In the pigment extract from *H. lacustris*, relatively small amounts of echinenone (the same level as canthaxanthin) was detected. The maximum absorption wavelength was found to be 465.6 nm for echinenone by the PDA detector.

The response of lutein, α-carotene, β-carotene, and chlorophylls *a* and *b* was linear from 0.03 to 10 μg/mL ($r = 0.9992-0.9999$; $n = 3$). Estimated limits of detection at 450 nm are as follows: lutein, 0.005 μg/mL; α-carotene, 0.01 μg/mL; β-carotene, 0.01 μg/mL; astaxanthin esters, 0.01 μg/mL; chlorophyll *a*, 0.05 μg/mL; and chlorophyll *b*, 0.02 μg/mL. The average recoveries ($n = 5$) of these pigments were 97.4% for lutein, 92.2% for α-carotene, 93.1% for β-carotene, 98.5% for chlorophyll *a*, and 99.3% for chlorophyll *b*. The relative standard deviations ($n = 5$) of these pigments were 2.4% for lutein, 5.6% for α-carotene, 6.0% for β-carotene, 4.9% for astaxanthin esters, 4.3% for chlorophyll *a*, and 4.0% for chlorophyll *b*, respectively.

Changes in Carotenoids and Chlorophylls during Growth. The culture of *H. lacustris* was initially illuminated at a light intensity of approximately 90 μmol/(m^2 s). The light intensity was subsequently enhanced to 190 μmol/(m^2 s) after 4 or 8 days of cultivation. Growth of *H. lacustris* during the cultivation is shown in Figure 2. It was found that the cell dry weight concentration did not reveal significant differences between the two illumination cases. At the beginning of growth, only chlorophylls, lutein, and a very small amount of β-carotene were present and no other secondary carotenoids could be found. During the culture, the contents of astaxanthin esters kept increasing up to the 11th day (Figure 3a) or the 13th day (Figure 3b) and then leveled off. The content of astaxanthin esters increased because of the light intensity being raised. The contents of chlorophylls *a* and *b* increased initially, then became constant, and finally began to fall slightly along with the growth of the algal cells (Figure 4). The content of lutein increased rapidly, reaching a maximum after 8 days of cultivation (Figure 5a) or after 10 days of cultivation (Figure 5b) before decreasing. The contents of canthaxanthin and β-carotene increased slowly and slightly during the course of cultivation (Figure 5).

Light intensity was one of the most important factors affecting the accumulation of astaxanthin in *H. lacustris* (Harker *et al.*, 1996a,b). The contents of carotenoids and chlorophylls reached their maxima more quickly when

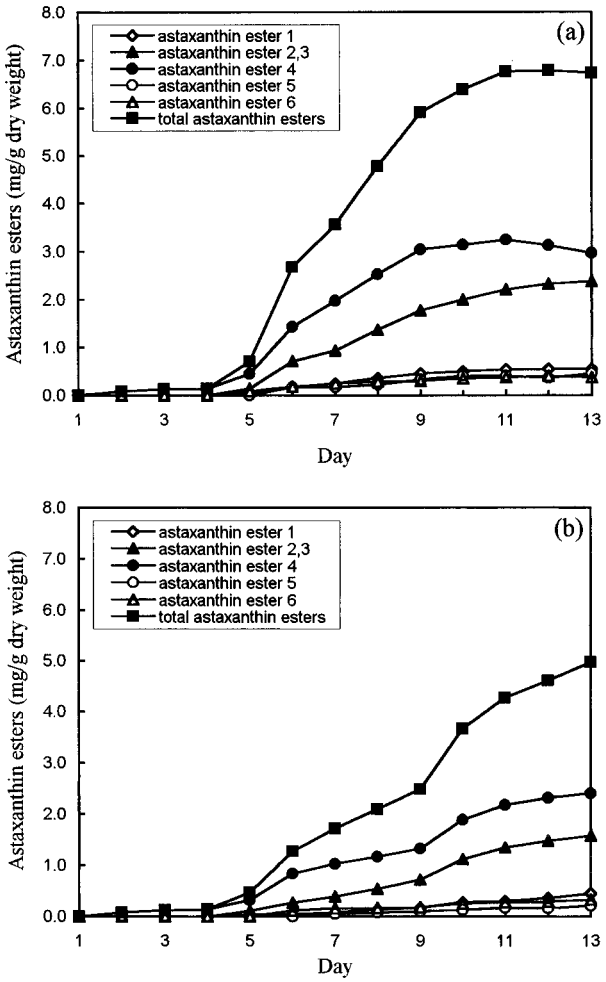

Figure 3. Formation of astaxanthin esters in *H. lacustris* during growth. The light intensity was raised from 90 to 190 μmol/(m^2 s) after 4 days of cultivation (a) or after 8 days of cultivation (b). (SE $\pm$ 4.8%; $n = 3$.)

the light intensity was raised after 4 days of cultivation than after 8 days of cultivation (Figures 3−5).

Different astaxanthin esters in *H. lacustris* formed at different times during growth. After 2 days of cultivation, astaxanthin esters 2 and 4 (peaks 14 and 16) were detected (Figure 6a). Astaxanthin esters 1, 3, and 6 (peaks 12, 15, and 21) appeared late, which were detected only after 3 days of cultivation. Astaxanthin ester 5 (peak 17) appeared last (Figure 6b,c). The HPLC chromatograms of the extracts of *H. lacustris* during growth are plotted in Figure 6.

The relative contents of astaxanthin esters changed during growth. When the light intensity was enhanced after 4 days of cultivation, the relative contents of astaxanthin esters changed from 4.5 to 8.3% for astaxanthin ester 1, from 20.5 to 35.3% for astaxanthin esters 2 and 3, and from 63.8 to 44.0% for astaxanthin ester 4 after 13 days of cultivation (Figure 3a). For astaxanthin esters 5 and 6, their content was relatively constant, being approximately 12% of the total astaxanthin esters (Figure 3a). The content of the total astaxanthin esters reached 6.8 mg/g of dry weight (Figure 3a). In the case that the light intensity was enhanced after 8 days, the relative contents of astaxanthin esters changed from 4.3 to 9.1% for astaxanthin ester 1, from 21.2 to 31.8% for astaxanthin esters 2 and 3, and from 68.1 to 48.3% for astaxanthin ester 4 (Figure 3b). For astaxanthin esters 5 and 6, the relative content did not change significantly, which was approximately

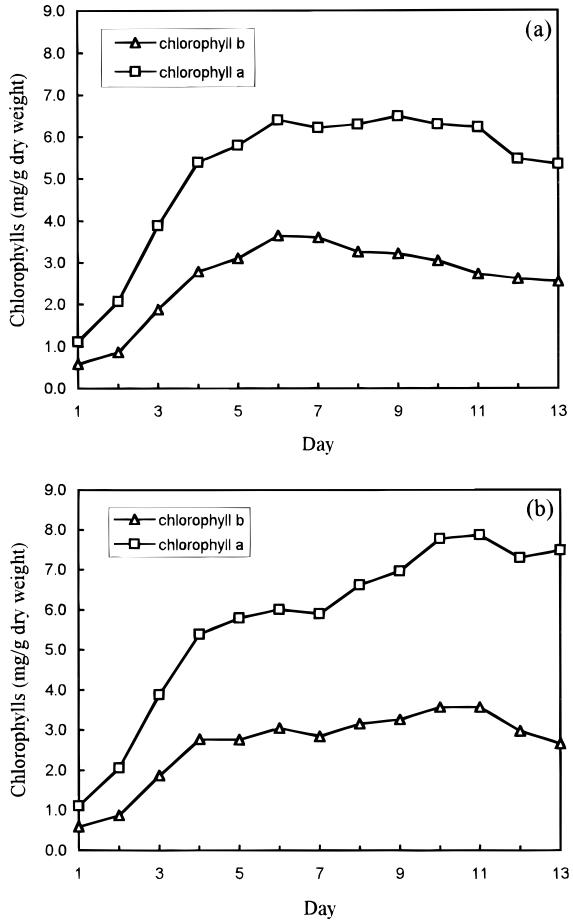

Figure 4. Changes of chlorophylls in *H. lacustris* during growth. The light intensity was raised from 90 to 190 μmol/(m^2 s) after 4 days of cultivation (a) or after 8 days of cultivation (b). (SE $\pm$ 4.1%; $n = 3$.)

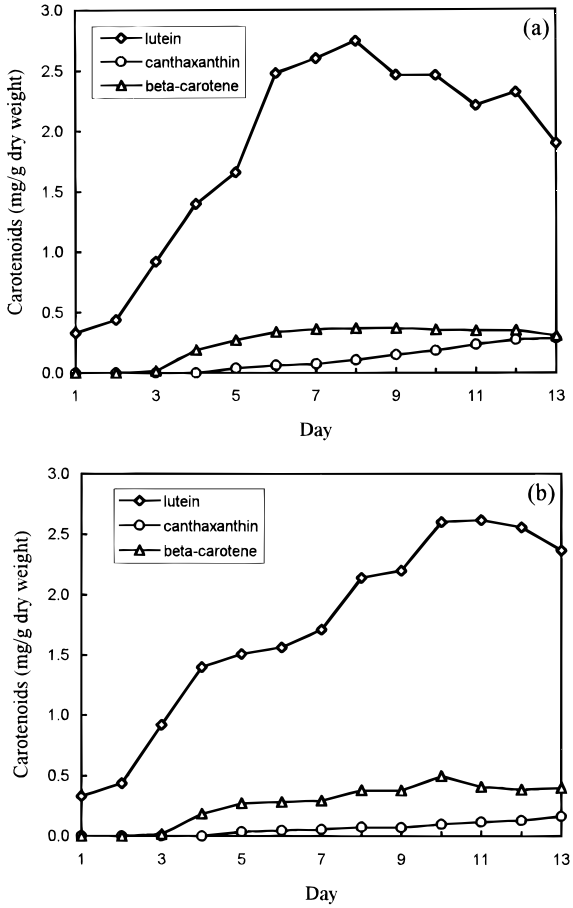

Figure 5. Changes of main carotenoids in *H. lacustris* during growth. The light intensity was raised from 90 to 190 μmol/(m^2 s) after 4 days of cultivation (a) or after 8 days of cultivation (b). (SE $\pm$ 4.4%; $n = 3$.)

11% of the total astaxanthin esters throughout (Figure 3b). The content of total astaxanthin esters reached only 5.0 mg/g of dry weight after 13 days of cultivation (Figure 3b).

DISCUSSION

No astaxanthin, canthaxanthin, echinenone, and astaxanthin ester standards were available in the present work, but the lack of these standards did not hinder the identification of these carotenoids from *H. lacustris*. Using a PDA detector, peaks were identified instantaneously by taking the spectrum of each peak during its elution. The peak purities were also examined by the PDA detector. A comparison of the spectrum with published data on the known carotenoids allows almost total identification (Ittah *et al*., 1993). The concentrations of these carotenoids were measured by area comparison with β-carotene (Calo *et al*., 1995).

Carotenoids are sensitive to light, heat, oxygen in air, and active surfaces. Their separation may be accompanied by degradation, structural rearrangement, formation of stereoisomers, and other physicochemical reactions (Hart *et al*., 1995). The addition of appropriate substances to the mobile phase may improve the recovery of carotenoids and reduce the degradation on the column. Hart *et al*. (1995) reported that the addition of triethylamine (TEA) to the mobile phase could increase the recovery of carotenoids. The exact action of the solvent modifier is still unclear (Hart *et al*., 1995). It was inferred that the improvement in the recovery was probably caused by buffering of the acidity of the free silanols in the stationary phase or by preventing reactions with free metal ions (Hart *et al*., 1995). Although carotenoids might degrade on the separation column, the degree of degradation might be different for different carotenoids (Hart *et al*., 1995). In this work, however, the stability of astaxanthin esters on the reversed-phase column and the effect of the solvent modifiers on the recovery of the carotenoids were not investigated.

Our results showed that light intensity had a significant effect on the level of astaxanthin esters accumulated in the cells of *H. lacustris*. Higher light intensities resulted in the accumulation of more astaxanthin esters in the cells. In the early stage of the culture, green and vegetative cells required a relatively low light intensity for their growth. The requirement of light increased as the cell concentration increased, due to cell mutual shading at the later stage. The light intensity was hence raised after 4 or 8 days of cultivation to facilitate the cell growth and accumulation of astaxanthin.

The exact biosynthetic pathway of astaxanthin has not yet been fully elucidated, although it is generally agreed that β-carotene serves as a precursor of the secondary carotenoids. The formation of canthaxanthin and astaxanthin was considered through oxygenation of the β-ionone ring and both oxygenation and hydroxylation of the β-ionone ring, respectively (Rise *et al*., 1994). In *Chlorella zofingiensis* (Rise *et al*., 1994), the secondary carotenoids produced were identified as canthaxanthin (about 30%) and astaxanthin (about 70%). It seems possible that canthaxanthin is the last stage

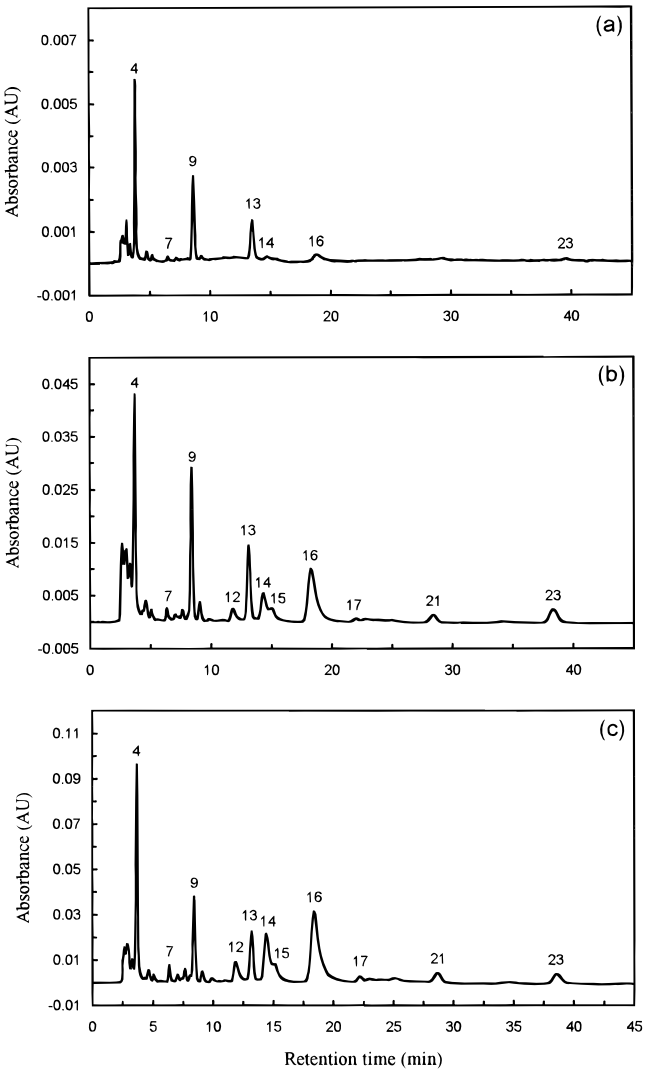

Figure 6. HPLC chromatogram of extract of *H. lacustris* during growth (450 nm): (a) after 2 days of cultivation; (b) after 6 days of cultivation; (c) after 9 days of cultivation. The light intensity was raised from 90 to 190 μmol/(m^2 s) after 4 days of cultivation.

of the oxygenation of β-carotene, while astaxanthin is produced via the oxygenation of zeaxanthin. The findings of Rise *et al.* (1994) supported that the hydroxylation step in astaxanthin biosynthesis might take place before oxygenation. Grung *et al.* (1992) suggested that astaxanthin was produced via the hydroxylation of canthaxanthin, in which a stereospecific introduction of the 3-hydroxy function after the introduction of the 4,4′-keto groups was assumed. Fan *et al.* (1995) revealed the biosynthetic pathway of astaxanthin in *Haematococcus pluvialis* by inhibition with diphenylamine, in which only echinenone and canthaxanthin, but not zeaxanthin, were the intermediates found in the conversion of β-carotene to astaxanthin. Our findings that echinenone and canthaxanthin are present during the synthesis of astaxanthin in *H. lacustris* support the conclusion that astaxanthin is synthesized from β-carotene via echinenone and canthaxanthin.

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