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Determination of Carotenoids in Spear Shrimp Shells (*Parapenaeopsis hardwickii*) by Liquid Chromatography

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The objectives of this study were to develop a high-performance liquid chromatography method for analysis of carotenoids in spear shrimp shells (*Parapenaeopsis hardwickii*) and to compare the extraction efficiency of carotenoids by supercritical carbon dioxide (SCD) and solvents. Results showed that the most appropriate HPLC method was accomplished by employing a Cosmosil 5C18-AR-II column and a mobile phase of methanol−dichloromethane−acetonitrile (90:5:5, v/v/v) (A) and water (100%) (B) with the following gradient elution: 92% A and 8% B in the beginning, decreased to 4% B in 9.5 min, 1% B in 26 min, 0% B in 35 min, maintained for 25 min, and returned to 92% A and 8% B in 61 min. All-*trans*-astaxanthin and its two cis isomers, as well as five astaxanthin monoesters and 11 diesters were resolved within 60 min with a flow rate at 2 mL/min and detection at 480 nm. Astaxanthin diesters were found to contain 12 fatty acids, of which palmitic acid and stearic acid constituted a large portion, whereas astaxanthin monoesters were found to contain 10 fatty acids with arachidonic acid dominating. Solvent extraction could generate a higher content of *trans*-astaxanthin and astaxanthin esters, while SCD extraction could produce greater levels of 9-*cis*-astaxanthin and 13-*cis*-astaxanthin.

KEYWORDS: Spear shrimp shell; astaxanthin; HPLC

INTRODUCTION

Astaxanthin, a naturally occurring carotenoid, which is mainly present in seafood products, especially in shells of lobster and shrimp, represents an important biological compound. Some studies have demonstrated that astaxanthin may possess strong antioxidant activity (*1*, *2*) and exert a protective effect against chronic diseases such as cancer (*3*, *4*). Several authors reported that the antioxidant activity of astaxanthin was higher than β-carotene and α-tocopherol (*1*, *3*, *5*, *6*). However, astaxanthin is susceptible to isomerization and oxidative degradation, because of the presence of long-chain conjugated double bonds (*7*, *8*). As the formation of cis isomers of astaxanthin or its derivatives may possess different biological activity, it is important to learn about the carotenoid profile in crustacea such as shrimp shell.

The traditional method used for extraction of carotenoids is often carried out by using solvents such as methanol and dichloromethane (*7*); however, this may create a safety problem. In recent years, supercritical carbon dioxide (SCD) has been used for extraction of carotenoids instead of solvents (*9*, *10*). However, the extraction efficiency of astaxanthin and its derivatives remains uncertain. Two high-performance liquid chromatography (HPLC) methods have been developed to separate various astaxanthin and its derivatives (*11*, *12*); however, the resolution needs to be improved.

In Taiwan, more than 35000 tons of shrimp were produced in 2002, and about 20000 tons of shrimp shell wastes were obtained (*13*). Most shrimp shell wastes were used for the feed industry, the production of enzyme for meat tenderization, and the processing of chitin products (*14*, *15*). As shrimp shell waste is a rich source of astaxanthin, it would be a great advantage to the health food industry if astaxanthin could be recovered as a functional ingredient. The objectives of this study were to compare the extraction efficiency of carotenoids in spear shrimp shells as affected by solvent and SCD extraction and to develop an HPLC method for their determination.

MATERIALS AND METHODS

Materials. A total of 30 kg of spear shrimp shells (*Parapenaeopsis hardwickii*) were obtained from a fishery market located in Keelung and were cleaned and freeze-dried prior to grinding. A total amount of 7.5 kg of dried shrimp shell powder was obtained and stored at −70 °C before analysis. All-*trans*-astaxanthin standard with a purity of 98% was from Sigma Co. (St. Louis, MO), and internal standard all-*trans*-β-*apo*-8′-carotenal was from Fluka Chemical Co. (Buchs, Switzerland). Fatty acid methyl esters, including dodecanoic acid methyl ester (C12:0), tetradecanoic acid methyl ester (C14:0), pentadecanoic acid methyl ester (C15:0), hexadecanoic acid methyl ester (C16:0), hexadecenoic acid methyl ester (C16:1), heptadecanoic acid methyl ester (C17:0), eicosanoic acid methyl ester (C20:0), eicosapentaenoic acid methyl ester (C20:5), and docosahexaenoic acid methyl ester (C22:6) were from Nu-Chek-Prep. Co. (Elysian, MN), and stearic acid methyl ester (C18:0), oleic acid methyl ester (C18:1), and linoleic acid methyl ester (C18:2) were from Sigma Co. The derivatization reagent 2,2-dimethoxypro-

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pane was also from Sigma Co. The HPLC grade solvents, including methanol, ethanol, *n*-hexane, acetone, 2-propanol, dichloromethane, acetonitrile, and ethyl acetate were from Mallinckrodt Co. (Paris, KY) and petroleum ether was from J. T. Baker Co. (Phillipsburg, NJ). The analytical grade solvent *n*-hexane was from Grand Chemical Co. (Taipei, Taiwan). Deionized water was made using a Milli-Q water purification system (Millipore Co., Bedford, MA). Reagents, including sodium hydroxide, potassium hydroxide, and anhydrous sodium sulfate were from Riedel-de Häen Co. (Barcelona, Spain). Adsorbent silica gel 60 was from Merck Co. (Darmstadt, Germany). Two HPLC C-18 columns both containing 5 μm particles, one (250 mm $\times$ 4.6 mm i.d.) was from Cosmosil Co. (Kyoto, Japan), and the other (150 mm $\times$ 4.6 mm i.d.) was from Hypurity Co. (Runcorn, Cheshire, United Kingdom). One HPLC CN column (250 mm $\times$ 4.6 mm i.d., 5 μm particle) was from Merck Co. The GC column (DB-1, 60 m $\times$ 0.32 mm i.d., 0.25 μm film thickness) was from J & W Scientific Co. (Folsom, CA).

Instrumentation. The HPLC instrument is composed of a DP-4010 on-line degasser (Sanwa Tsusho Co., Tokyo, Japan), an injector (model 7161, Rheodyne Co., CA), two PU-980 pumps (Jasco Co., Tokyo, Japan), and a MD-915 photodiode array detector (Jasco Co.). The GC instrument (model 6890) equipped with a flame ionization detector (FID) was from Agilent Technology Co. (Palo Alto, CA). The rotary evaporator (model N-1) was from Eyela Co. (Tokyo, Japan). The freeze dryer (model FD-24) was from Chin-Ming Co. (Taipei, Taiwan). The sonicator (model 2210R-DTH) was from Branson Co. (Danbury, CT). The low temperature incubator (model TL 520R) was from Sheng-Long Co. (Taipei, Taiwan). The supercritical fluid extractor was from Applied Separation Co. (Allentown, PA), with the 10 mL extraction vessel from Thermo Co. (Bellefonte, PA).

Methods. *Extraction of Carotenoids from Shrimp Shells by Solvent.* A method similar to that described by Wu (*16*) was used to extract carotenoids from spear shrimp shells. Initially, 1 g of shrimp shell powder was mixed with 10 mL of acetone in a flask and shaken for 10 min. The filtrate was collected using a suction pipe, and the residue was extracted with 10 mL of acetone again. Likewise, the residue was extracted with 10 mL of petroleum ether twice until it became colorless. All of the extracts were combined in a flask, and 40 mL of 10% sodium sulfate solution was added and shaken for 1 min, after which the mixture was left in the dark until two layers were formed. The supernatant was collected, and the residue was repeatedly extracted with 40 mL of petroleum ether four times. All of the extracts were combined, evaporated to dryness, and dissolved in 2 mL of methanol−dicholoromethane (50:50, v/v). Then, 100 μL of internal standard β-*apo*-8′-carotenal (6 μg/mL) was added to the extract and filtered through a 0.22 μm membrane filter for HPLC analysis. To avoid oxidation and degradation of carotenoids, the extraction procedure was performed under dimmed light and inert atmosphere; that is, nitrogen gas was flushed into the flask. No antioxidant such as BHT was used during extraction because it has been reported that the incorporation of nitrogen gas could result in a higher yield of carotenoids than BHT (*17*).

Extraction of Carotenoids from Shrimp Shells by SCD. The extraction condition was similar to that used by Leticia et al. (*10*). A combination of 16 treatments, including four pressures, 250, 300, 350, and 400 bar, and four temperatures, 45, 55, 65, and 75 °C, were used for comparison of extraction efficiency. The flow rate of carbon dioxide was 3.0 mL/min with or without 10% ethanol as modifier, and the extraction time was 30 min. The extract was dissolved in 2 mL of methanol−dichloromethane (50:50, v/v). Then, 100 μL of internal standard β-*apo*-8′-carotenal (6 μg/mL) was added to the extract and filtered through a 0.22 μm membrane filter for HPLC analysis. Likewise, the extract was stored under an inert atmosphere prior to injection into HPLC.

HPLC Analysis of Carotenoids. Three types of columns, including two C18 columns and one CN column, were used for comparison of separation efficiency. Two concentrations of 1 mL of all-*trans*-astaxanthin standard (30 and 50 μg/mL) were added to 1 g of shrimp shell powder separately. After extraction by solvent and SCD, then 100 μL of internal standard β-*apo*-8′-carotenal (6 μg/mL) was added to the extract and *trans*-astaxanthin was quantified by HPLC. The recovery was determined based on the ratio of the concentration of *trans*-astaxanthin standard after and before HPLC. The quantification of cis isomers of *trans*-astaxanthin was carried out using the standard curve of *trans*-astaxanthin, because of similarity in extinction coefficient. The purity of each peak was automatically determined using a photodiode array detector. The identification of all-*trans*-astaxanthin was accomplished by comparing unknown peaks with reference standards and cochromatography with added standards. In addition, the cis isomers of *trans*-astaxanthin were tentatively identified using spectral characteristics and Q ratios as described in the literature (*12*). A Q ratio can be defined as the ratio of the height of the maximum absorbance peak to the cis peak. For quantification, eight concentrations (0.5, 1, 2, 3, 4, 6, 10, and 20 μg/mL) of *trans*-astaxanthin and a fixed concentration (3 μg/mL) of internal standard β-*apo*-8′-carotenal were mixed, and the standard curve was obtained by plotting the concentration ratio against the area ratio. Because of the absence of standards for individual astaxanthin ester, both astaxanthin monoesters and diesters were quantified by calculating the area ratio of each peak to the internal standard peak and multiplying the concentration of internal standard.

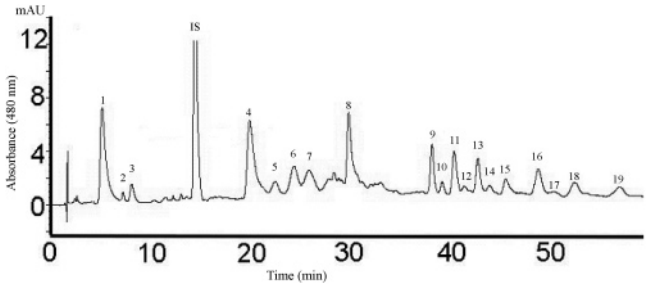

Figure 1. HPLC chromatogram of carotenoids extracted from shrimp shells. Chromatographic conditions are described in the text. See **Table 1** for peak identification.

Photoisomerization of trans-Astaxanthin Standard. trans-Astaxanthin standard (5 mg) was dissolved in 50 mL of dichloromethane and poured into 50 2 mL vials so that each contained 100 μg/mL. All of the vials were placed in an incubator at 25 °C and illuminated for a varied length of time under four fluorescent tubes (55 cm and 20 W each) with a distance of 30 cm and a light intensity of 2000−3000 lux. Three aliquots were taken at intervals, i.e., 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 11, 12, 24, 36, and 48 h, and then, each was evaporated to dryness, dissolved in methanol−dichloromethane (50:50, v/v), and filtered through a 0.22 μm membrane filter, and 20 μL was injected into HPLC. The absorption spectrum and retention time of each peak after illumination for 48 h was used to compare with those unknown peaks on the HPLC chromatogram in **Figure 1** for further identification of cis isomers of *trans*-astaxanthin.

Analysis of Astaxanthin Esters. A modified method based on Wu (*16*) was used to extract astaxanthin esters from shrimp shells. In the beginning, 1 g of shrimp shell powder was extracted following the procedure shown above, which involved extraction by shaking, twice with acetone and twice with petroleum ether. To further prove that shrimp shells contain astaxanthin esters, saponification was carried out in the dark for 16 h by using potassium hydroxide at a level of 40, 10, 5, and 1% or sodium hydroxide at 3.8, 1.9, 0.2, and 0.1%. Then, the carotenoid extracts were injected into HPLC to compare the carotenoid profile. Results showed that a complete saponification was achieved by sodium hydroxide at 0.2%, because only the peaks of *trans*-astaxanthin and its cis isomers were present in the HPLC chromatogram, implying that all of the astaxanthin esters were hydrolyzed during saponification. Thus, approximately 40 mL of carotenoid extract from shrimp shell was collected in a flask and 8 mL of 0.2% NaOH was added for saponification for 16 h in the dark, after which 40 mL of 10% sodium sulfate solution was added for partition. The supernatant was collected and evaporated to dryness under vacuum. The residue was dissolved in 2 mL of methanol−dichloromethane (50:50, v/v) and filtered through a 0.22 μm membrane filter for HPLC analysis. Both extraction and saponification procedures were also carried out under dimmed light and inert atmosphere as shown above.

Separation of Astaxanthin Monoester and Diester by Open-Column Chromatography. A method similar to that described by Coral-Hinostroza and Bjerkeng (*18*) was used for separation. Initially, a 20 g amount of shrimp shell sample was mixed with 200 mL of acetone

in a flask and shaken for 10 min (*16*). The extract was collected, and the residue was extracted again with 200 mL of acetone. The extract was also collected, and the residue was extracted again with 200 mL of petroleum ether twice. All of the extracts were combined, and 200 mL of sodium sulfate solution (10%) was added. The mixture was subjected to shaking for 1 min for dissolving more polar impurities into the aqueous phase. Then, the solution was settled in the dark until two phases were formed. The upper phase was collected and evaporated to dryness under vacuum, after which the residue was dissolved in 10 mL of hexane and concentrated to 1 mL. Then the concentrated extract was poured into a glass column (30 cm × 20 mm i.d.) containing 25 g of silica gel (particle size 0.040−0.063 mm). Silica gel was chosen as the adsorbent because it shows much better separation when compared to the other adsorbents. Although silica gel is slightly acidic, the isomerization of *trans*-astaxanthin was not observed on the basis of HPLC analysis before and after column chromatography. Anhydrous sodium sulfate was added above the adsorbent to form a layer of 1 cm. Most triglycerides and sterol esters were first eluted with hexane (100%), followed by astaxanthin diesters with hexane−acetone (94:6, v/v), astaxanthin monoesters with hexane−acetone (92:8, v/v), and *trans*-astaxanthin as well as its cis isomers with hexane−acetone (88:12, v/v). The eluate of each fraction was evaporated to dryness, dissolved in 1 mL of hexane, and filtered through a 0.22 μm membrane filter for both GC and HPLC analyses.

For GC analysis, the crude extract before open-column chromatography plus fractions of astaxanthin monesters and diesters after open-column chromatography were each added with 2 mL of benzene, 2 mL of methanolic HCl (0.5 M), and 0.2 mL of 2,2-dimethoxypropane separately. The derivatization reaction was allowed to proceed at room temperature for 12 h, and then, 4 mL of sodium sulfite solution (6%) was added to terminate the reaction. The upper phase was extracted with hexane and evaporated to dryness under vacuum. The residue was dissolved in 1 mL of hexane and filtered through a 0.22 μm membrane filter for fatty acid analysis. The column temperature started at 150 °C, maintained for 1 min, increased to 180 °C at a rate of 3 °C/min, 215 °C at a rate of 1 °C/min, 240 °C at a rate of 5 °C/min, and maintained for 10 min. Both injector and detector temperatures were 280 °C, and the injection volume was 1 μL. Because of possible contamination of astaxanthin esters with a small amount of lipids, the tentative identification of fatty acid was carried out by comparison of retention time of unknown peaks with reference standards as well as cochromatography with added standards. The amount of each fatty acid was expressed as percentage and was based on the ratio of the area of each peak to the total peak area. For statistical analysis, all of the data were subjected to analysis of variance and Duncan's multiple range test using SAS (*19*).

RESULTS AND DISCUSSION

HPLC Analysis of Carotenoids in Shrimp Shells. Three types of columns, including two C-18 and one CN, were compared with respect to the separation efficiency of various carotenoids in shrimp shells. In addition, the various mobile phases in isocratic or gradient mode were also compared. After numerous studies, results showed that a Cosmosil 5C18-AR-II column provided better resolution than the other two columns. By calculating the polarity index and controlling the solvent strength of each solvent system, the most appropriate mobile phase was found to be composed of (A) methanol/dichloromethane/acetonitrile (90:5:5, v/v/v) and (B) water (100%) with the following gradient elution: 92% A and 8% B initially, decreased to 4% B in 9.5 min, 1% B in 26 min, 0% B in 35 min, and maintained until 60 min. A total of 19 carotenoids, including one *trans*-astaxanthin and its two cis isomers, five astaxanthin monoesters as well as 11 astaxanthin diesters, were resolved with a flow rate at 2.0 mL/min and detection at 480 nm. Several sample solvents, including methanol−dichloromethane in different proportions, were also compared, and a ratio of 50:50 (v/v) was found to be the most suitable.

Table 1. Tentative Identification Data for Carotenoids in Spear Shrimp Shells

peak no.	compound	λ (nm) (in-line)	λ (nm) (reported)[a]	Q ratio (found)[b]
1	*trans*-astaxanthin	482	480	
2	9-*cis*-astaxanthin	470 (356)[c]	470.4	5.3
3	13-*cis*-astaxanthin	470 (374)	468	2.0
4	9-*cis*-astaxanthin monoester	476	472.8	6.5
5	*trans*-astaxanthin monoester	482	480	
6	13-*cis*-astaxanthin monoester	476 (374)	472.8 (371.8)	2.8
7	9-*cis*-astaxanthin monoester	476	472.8	4.3
8	9-*cis*-astaxanthin monoester	476	472.8	5.2
9	13-*cis*-astaxanthin diester	476 (374)	472.8 (371.8)	2.7
10	*trans*-astaxanthin diester	482	480	
11	13-*cis*-astaxanthin diester	476 (374)	472.8 (371.8)	2.7
12	*trans*-astaxanthin diester	482	480	
13	*trans*-astaxanthin diester	482	480	
14	9-*cis*-astaxanthin diester	476	472.8	5.2
15	*trans*-astaxanthin diester	482	480	
16	*trans*-astaxanthin diester	482	480	
17	*trans*-astaxanthin diester	482	480	
18	*trans*-astaxanthin diester	482	480	
19	*trans*-astaxanthin diester	482	480	

[a] Based on a reference by Yuan and Chen (*7*, *12*). [b] The Q ratio can be defined as the ratio of the height of the maximum absorbance peak to the cis peak. [c] Values in parentheses represent the cis peak.

Figure 1 shows the HPLC chromatogram of carotenoids in shrimp shells. Most peaks including internal standard β-*apo*-8′-carotenal were adequately resolved within 60 min. On the basis of the criteria described in the methods section, peak 1 was identified as *trans*-astaxanthin. Three peaks (peaks 1−3 in **Figure 1**) were observed after photoisomerization of *trans*-astaxanthin. On the basis of the retention behavior and absorption spectrum, the first peak was positively identified as *trans*-astaxanthin, while peaks 2 and 3 were tentatively identified as 9-*cis*-astaxanthin and 13-*cis*-astaxanthin with a purity of 93.3 and 82.0%, respectively (**Table 1** and **Figure 2**). Peaks 4−8 were tentatively identified as 9-*cis*-astaxanthin monoester (λ_{max} = 476 nm), *trans*-astaxanthin monoester (λ_{max} = 482 nm), 13-*cis*-astaxanthin monoester (λ_{max} = 476 nm), 9-*cis*-astaxanthin monoester (λ_{max} = 476 nm), and 9-*cis*-astaxanthin monoester (λ_{max} = 476 nm), with a purity of 97.4, 95.7, 98.4, 95.7, and 81.4%, respectively. Peaks 9−19 were tentatively identified as 13-*cis*-astaxanthin diester (λ_{max} = 476 nm), *trans*-astaxanthin diester (λ_{max} = 482 nm), 13-*cis*-astaxanthin diester (λ_{max} = 476 nm), *trans*-astaxanthin diester (λ_{max} = 482 nm), *trans*-astaxanthin diester (λ_{max} = 482 nm), 9-*cis*-astaxanthin diester (λ_{max} = 476 nm), *trans*-astaxanthin diester (λ_{max} = 482 nm), *trans*-astaxanthin diester (λ_{max} = 482 nm), *trans*-astaxanthin diester (λ_{max} = 482 nm), *trans*-astaxanthin diester (λ_{max} = 482 nm), and *trans*-astaxanthin diester (λ_{max} = 482 nm), respectively, with a purity of 93.9, 99.8, 93.6, 99.9, 95.8, 99.8, 97.4, 95.9, 99.7, 98.1, and 99.5%. A difference of 6 nm between the λ_{max} of the free cis isomers (9-*cis*- and 13-*cis*-astaxanthin) and their esters (470 vs 476 nm) occurred, which may be due to the purity problem. In a study dealing with analysis of *trans*-astaxanthin in the extracts of alga *Haematococcus pluvialis*, a difference of 2.5 nm was also observed between *trans*-astaxanthin and its esters (*12*). The carotenoid composition is similar to that in some other varieties of shrimps such as Antartic krill (*20*). However, in our study, some more cis isomers and esters of astaxanthin were identified, which may be due to the difference in HPLC method employed. Yuan et al. (*11*) also developed an HPLC method for determination of astaxanthin esters in *Haematococcus locustris*; however, only 10 carotenoids were resolved. Therefore, although the difference in carotenoid composition

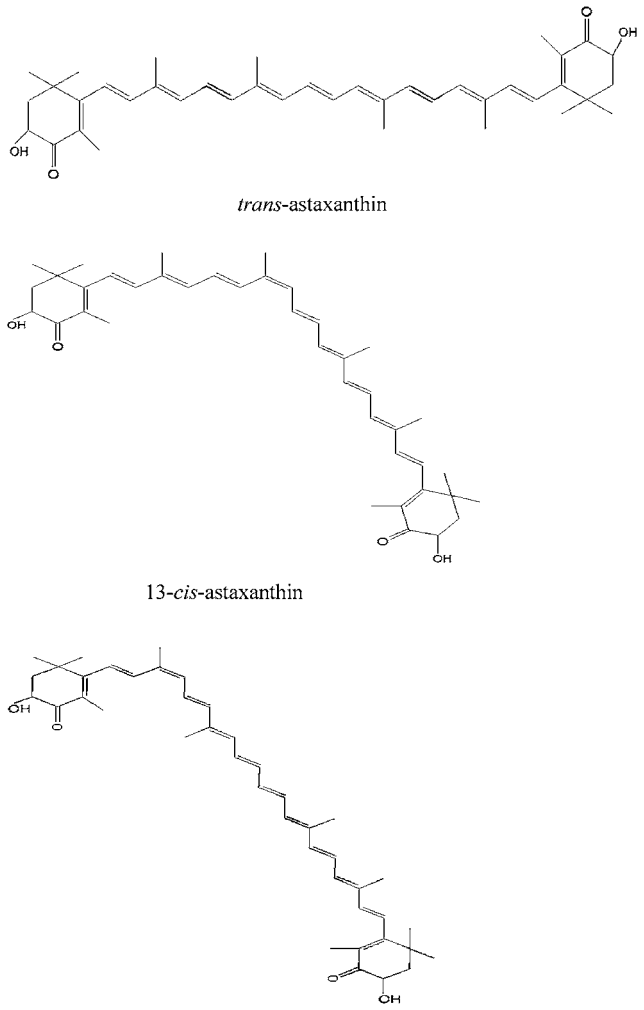

Figure 2. Structures of *trans*-astaxanthin and its cis isomers.

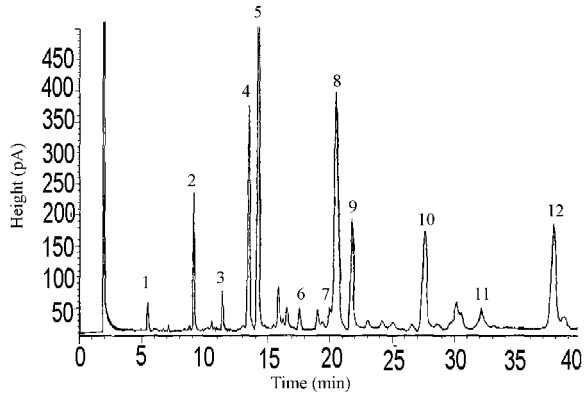

Figure 3. GC chromatogram of fatty acids in crude extract from shrimp shells. Peaks: 1, lauric acid (C12:0); 2, myristic acid (C14:0); 3, pentadecanoic acid (C15:0); 4, palmitoleic acid (C16:1); 5, palmitic acid (C16:0); 6, margaric acid (C17:0); 7, linoleic acid (C18:2); 8, oleic acid (C18:1); 9, stearic acid (C18:0); 10, eicosapentaenoic acid (C20:5); 11, arachidic acid (C20:0); and 12, docosahexaenoic acid (C22:6).

in shrimps can be affected by their species, the impact of an appropriate HPLC method on resolution of carotenoids cannot be ignored.

GC Analysis of Fatty Acids in Astaxanthin Esters. Figure 3 shows the GC chromatogram of fatty acids in crude extract from shrimp shells. A total of 12 fatty acids, including lauric acid (C12:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitoleic acid (C16:1), palmitic acid (C16:0), margaric acid (C17:0), linoleic acid (C18:2), oleic acid (C18:1), stearic acid (C18:0), eicosapentaenoic acid (C20:5), arachidic acid (C20:0), and docosahexaenoic acid (C22:6), were separated within 40 min by using a capillary column and temperature programming condition described in the methods section. **Table 2** shows the fatty acid composition in crude extract and astaxanthin esters from shrimp shells. In crude extract, oleic acid constituted the largest portion (24.9%), followed by palmitic acid (20.6%), palmitoleic acid (12.0%), eicosapentaenoic acid (11.9%), and docosahexaenoic acid (10.8%). A similar trend was found for astaxanthin diester. However, for astaxanthin monoester, a different tendency was observed, i.e., arachidic acid was present in the highest amount (36.3%), followed by lauric acid (15:9%), docosahexaenoic acid (15.0%), eicosapentaenoic acid (10.2%), oleic acid (8.0%), and palmitic acid (6.0%). In addition, astaxanthin monoester was found not to contain pentadecanoic acid and margaric acid when compared to astaxanthin diester. In a study dealing with analysis of fatty acid composition in red crab langostilla, a similar profile of the major fatty acids in astaxanthin esters was found, with the exception that eicosamonoenoic acid (C20:1), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3), eicosatetraenoic acid (C20:4), docosamonoenoic acid (C22:1), docosatetraenoic acid (C22:4), and docosapentaenoic acid (C22:5) were also present (*18*). Takaichi et al. (*21*) also reported that only five fatty acids, lauric acid, myristic acid, palmitic acid, palmitoleic acid, and oleic acid, were present in astaxanthin ester of krill. The difference in fatty acid composition of astaxanthin esters among crustacea is probably due to variety, growth environment, season of capture, and degree of maturity (*18*, *21*).

Table 2. Composition of Major Fatty Acids in Shrimp Shell

fatty acid	astaxanthin diester (%)	astaxanthin monoester (%)	crude extract (%)
lauric acid (C12:0)	4.0 ± 1.1[a]	15.9 ± 0.5	0.9 ± 0.0
myristic acid (C14:0)	4.6 ± 0.8	1.3 ± 0.0	4.3 ± 0.1
pentadecanoic acid (C15:0)	1.4 ± 0.1	ND[b]	1.4 ± 0.0
palmitoleic acid (C16:1)	11.4 ± 0.2	3.8 ± 0.1	12.0 ± 0.0
palmitic acid (C16:0)	23.0 ± 0.3	6.0 ± 0.2	20.6 ± 0.5
margaric acid (C17:0)	1.3 ± 0.0	ND	1.2 ± 0.0
linoleic acid (C18:2)	0.8 ± 0.0	0.7 ± 0.0	1.3 ± 0.0
oleic acid (C18:1)	25.5 ± 1.3	8.0 ± 0.1	24.9 ± 0.3
stearic acid (C18:0)	10.1 ± 0.8	2.4 ± 0.4	7.8 ± 0.0
eicosapentaenoic acid (C20:5)	6.9 ± 1.4	10.2 ± 0.0	11.9 ± 0.4
arachidic acid (C20:0)	4.4 ± 1.6	36.3 ± 0.7	2.4 ± 0.1
docosahexaenoic acid (C22:6)	6.0 ± 0.7	15.0 ± 0.4	10.8 ± 0.7

[a] Average of duplicate analyses ± standard deviation. [b] ND, not detected.

Comparison of Extraction Efficiency by Solvent and SCD. The average recoveries of solvent and SCD extraction based on triplicate analyses were 83.3 and 62.9%, respectively. This result indicated that SCD may not be appropriate for extraction of polar astaxanthin because of its nonpolar nature. The recovery of 83.3% by solvent extraction is probably due to the presence of complex matrix in shrimp shell; however, this value is much higher than that in some other reports (*10*). An extraction efficiency of 57% was shown for blue crab shell waste by SCD, as reported by Leticia et al. (*10*). **Table 3** shows the effect of SCD on the yield of *trans*-astaxanthin and its cis isomers as well as esters in shrimp shells with 10% ethanol as cosolvent. With the temperature at 45 °C and the pressure at 250 bar, a level of 3.66 μg/g *trans*-astaxanthin was found, which then further increased to 4.25 μg/g at 400 bar. However, under four

Table 3. Contents (μg/g) of Astaxanthin and Its Isomers as Well as Esters in Shrimp Shells as Extracted by SCD[a]

compound	temp (°C)	250 bar	300 bar	350 bar	400 bar
trans-astaxanthin	45	3.66 cw	3.90 bw	4.20 aw	4.25 aw
	55	3.41 bx	3.77 bw	3.98 awx	4.06 aw
	65	3.40 bx	3.60 bw	3.96 awx	3.63 bx
	75	3.17 cx	3.74 bw	3.88 ax	3.29 cy
9-*cis*-astaxanthin	45	0.83 aw	0.78 ay	0.77 ax	0.77 ax
	55	0.79 aw	0.80 ax	0.83 aw	0.82 aw
	65	0.68 bx	0.72 az	0.73 ax	0.75 ax
	75	0.80 cw	0.82 bw	0.73 dx	0.85 aw
13-*cis*-astaxanthin	45	1.12 bx	1.16a bx	1.18 ax	1.22 ax
	55	1.09 cx	1.19 bw	1.31 aw	1.25 awx
	65	1.13 bx	1.19 bw	1.17 bx	1.27 awx
	75	1.14 bw	1.13 bx	1.25 aw	1.32 aw
astaxanthin monoesters	45	8.81 cw	8.80 cw	10.07 bw	10.96 aw
	55	8.02 bx	8.38 bx	9.50 ax	9.52 ax
	65	6.92 by	7.48 by	8.70 ay	8.75 ay
	75	5.24 cz	5.65 bz	6.09 az	5.99 az
astaxanthin diesters	45	19.36 cw	20.09 bw	21.37 aw	22.61 aw
	55	18.61 dx	19.67 cx	20.92 bx	21.76 ax
	65	13.79 dy	15.44 cy	17.31 by	19.51 ay
	75	13.23 dz	14.31 cz	15.01 bz	17.49 az

[a] The same letters (a–d) represent no significant difference at the 95% confidence level among different pressure treatments. The same letters (w–z) represent no significant difference at the 95% confidence level among different temperature treatments.

pressures, 250, 300, 350, and 400 bar at 55 °C, the highest content (4.06 μg/g) of *trans*-astaxanthin was found at 400 bar. Similarly, under the same pressure at 65 °C, the largest amount (3.96 μg/g) was observed at 350 bar. However, a different result was shown at 75 °C and 350 bar, i.e., a lower yield of 3.88 μg/g *trans*-astaxanthin was generated. Apparently the high temperature treatment may facilitate degradation of *trans*-astaxanthin. This result clearly revealed that a combination of low temperature (45 °C) and high pressure (400 bar) could produce the highest level of *trans*-astaxanthin. A similar phenomenon was also observed by Charest et al. (*9*).

Under pressures of 250 and 400 bar at 45 °C, the levels of 9-*cis*-astaxanthin were 0.83 and 0.77 μg/g, respectively. Likewise, under 250 and 350 bar at 55 °C, the contents were 0.79 and 0.83 μg/g, respectively (**Table 3**). However, under 400 bar, a highest yield of 9-*cis*-astaxanthin (0.85 μg/g) occurred at 75 °C. This result indicated that a combination of high temperature and high pressure may facilitate isomerization of *trans*-astaxanthin and resulted in more formation of 9-*cis*-astaxanthin. Yuan and Chen (*8*) also demonstrated that the higher the temperature, the greater the amount of cis isomers of *trans*-astaxanthin was produced.

For 13-*cis*-astaxanthin, the contents were 1.12, 1.16, 1.18, and 1.22 μg/g, respectively, under four pressures of 250, 300, 350, and 400 bar at 45 °C (**Table 3**). This result showed that a high pressure treatment may promote the formation of 13-*cis*-astaxanthin from *trans*-astaxanthin. However, a minor increase was found for pressures 350 and 400 bar at 55 °C. Similar to 9-*cis*-astaxanthin, the highest yield of 13-*cis*-astaxanthin was observed at 400 bar and 75 °C.

By comparing various temperatures, the highest level of astaxanthin monoester was found at 45 °C, followed by 55, 65, and 75 °C. In contrast, a reverse trend was shown for pressure, i.e., the level of astaxanthin monoester showed an increased trend for the increase of pressure under all temperatures. This result can also be applied to astaxanthin diester; the higher the temperature, the lower the amount of astaxanthin diester was formed (**Table 3**). However, when the pressure for all temperatures was increased, a higher formation of astaxanthin diester was observed.

Table 4. Contents (μg/g) of Carotenoids Extracted from Shrimp Shells by SCD with or without Cosolvent Ethanol[a]

	with cosolvent		without cosolvent	
compound	45 °C 350 bar	45 °C 400 bar	45 °C 350 bar	45 °C 400 bar
trans-astaxanthin	4.20 a	4.25 a	2.35 b	2.23 b
9-*cis*-astaxanthin	0.77 a	0.77 a	ND	ND
13-*cis*-astaxanthin	1.18 a	1.22 a	0.75 b	0.71 b
astaxanthin monoesters	10.07 b	10.96 a	7.23 d	7.86 c
astaxanthin diesters	21.37 ab	22.61 a	20.89 b	22.69 a

[a] Symbols bearing different letters (a–d) in the same row are significantly different ($p < 0.05$). ND, not detected.

Table 4 shows the contents of carotenoids extracted from shrimp shells by SCD with or without cosolvent ethanol. This experiment was performed under 350 and 400 bar at 45 °C, because both showed the best extraction efficiency. Without ethanol, the content of *trans*-astaxanthin was lowered by 1.85 and 2.02 μg/g, respectively, under the pressures of 350 and 400 bar at 45 °C. For cis isomers, no 9-*cis*-astaxanthin was detected in the absence of cosolvent, and a decrease of 0.43 and 0.51 μg/g was shown for 13-*cis*-astaxanthin under the same pressure and temperature. Likewise, without ethanol, the levels of astaxanthin monesters also declined by 2.84 and 3.10 μg/g under the same condition. Surprisingly, only a minor difference was found in the yield of astaxanthin diester with and without ethanol, which may be attributed to its lower polarity when compared to astaxanthin monoester. This result further proved that with cosolvent ethanol, the yields of *trans*-astaxanthin and its cis isomers, as well as astaxanthin monoester, can be substantially enhanced. Nevertheless, in comparison with SCD extraction, solvent extraction was found to produce a higher level of *trans*-astaxanthin (4.95 μg/g), astaxanthin monoesters (14.38 μg/g), and astaxanthin diesters (30.04 μg/g), whereas no significant difference was shown for 9-*cis*-astaxanthin (0.74 μg/g) and 13-*cis*-astaxanthin (1.21 μg/g). In conclusion, the HPLC method developed in this study was able to resolve all-*trans*-astaxanthin and its two *cis*-isomers, as well as 16 astaxanthin esters in spear shrimp shells. On the basis of GC analysis, astaxanthin monoesters were found to contain 10 fatty acids while astaxanthin diesters contained 12. The SCD extraction could generate higher levels of 9-*cis*-astaxanthin and 13-*cis*-astaxanthin, whereas the solvent extraction could produce more *trans*-astaxanthin and astaxanthin esters.

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