Asian Journal of Chemistry; Vol. 26, No. 15 (2014), 4543-4549

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.16261

REVIEW

Extraction and Separation of Astaxanthin from Marine Products

YU RI LEE, BAOKUN TANG and KYUNG HO ROW*

Department of Chemistry and Chemical Engineering, Inha University, Incheon 402-751, Republic of Korea

*Corresponding author: Fax: +82 32 8720959; Tel: +82 32 8607470; E-mail: rowkho@inha.ac.kr

Received: 30 August 2013; *Accepted*: 19 December 2013; *Published online*: 16 July 2014; AJC-15521

Astaxanthin is a carotenoid used widely in salmonid and crustacean aquaculture to provide the pink color characteristics of that species. The ketocarotenoid astaxanthin can be found in microalgae, yeast and crustacean byproducts. Astaxanthin represents the unique properties of the structure. The astaxanthin molecule has two geometric isomers, *cis* or *trans* due to the double bond from the poliene chain. The separation of these isomers is very difficult using ordinary techniques. This paper reviews the current available evidence regarding astaxanthin chemistry and its potential beneficial effects in humans as well as the methods of the extraction and separation of astaxanthin.

Keywords: Astaxanthin, Carotenoid, Separation, Geometric isomer, Extraction.

INTRODUCTION

Astaxanthin is a carotenoid red pigment present in microalgae, yeast, salmon, trout, krill, shrimp, crayfish and *Adonis annua* flower. The compound belongs to the xanthophyll class of carotenoids and the chemical name is 3,3'-dihydroxy-β,β-carotene-4, 4'-dione (Fig. 1)[1,2]. Astaxanhin is closely related to β-carotene, lutein and zeaxanthin, sharing many of the general metabolic and physiological functions attributed to carotenoids. Astaxanthin has unique chemical properties owing to its molecular structure chemical properties and light-absorption characteristics[3,4]. The astaxanthin molecule has two asymmetric carbons atoms located at the 3 and 3' positions of the benzenoid rings on either end of the molecule and astaxanthin, unlike a pigment that belongs to the family of xanthophylls, has two carbonyl groups, two hydroxyl groups and eleven conjugated ethylenic double bonds[5]. Different enantiomers of the molecule result from the way the hydroxyl groups are attached to the carbon atoms at these centers of asymmetry. When the hydroxyl group is attached so that it projects above the plane of the molecule, it is said to be in the 'R configuration' and when the hydroxyl group is attached to project below the plane of the molecule, it is said to be in the 'S configuration'. Therefore, astaxanthin may have three configurational isomers: two enantiomers 3R, 3R'; 3S, 3S'; and a *meso* form 3R, 3S'[6]. The astaxanthin molecule has geometric isomers, *cis* or *trans*, due to the double bond of the poliene chain[7]. Only a few of these geometrical isomers are found in nature. The presence of a *cis* double bond creates greater steric hindrance between the nearby hydrogen atoms and/or methyl groups, so that *cis* isomers are generally less stable thermodynamically than the *trans* form. Therefore, most carotenoids found in nature are predominantly *trans*-isomers[8]. On the other hand, all-*trans* natural astaxanthin is isomerized easily to *cis-trans*, particularly 9-*cis* and 13-*cis* for steric reasons under certain conditions by chemical analysis. The increased temperature, exposure to light, or the presence of acids can cause the formation of *cis*-isomers[9]. Free astaxanthin is particularly sensitive to oxidation. Therefore, astaxanthin is conjugated with proteins or is esterified with one or two fatty acids to form the monoester and diester forms[10]. This unique structure of astaxanthin have higher antioxidant activity, preventative cardiovascular problems, different types of cancer and some diseases of the immunological system owing to its more polar nature than other carotenoids[11]. Many studies have reported astaxanthin to be an anticancer agent in a range of human cancer cell lines. Dietary astaxanthin exhibits antitumoral activity in the post-initiation phase of carcinogen induced oral cancer models. As a result, the cell proliferation activity is decreased in the nonlesional squamous epithelium exposed to astaxanthin[12]. Ultraviolet light has a range of effects depending on its wavelength: UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm)[13]. UV light can cause significant damage to the skin, such as DNA alterations, inflammation, oxidative alterations of collagen, melanin production and skin cancer. This skin damage is considered to be caused, in part, by reactive oxygen species (ROS), such as singlet oxygen and free radicals, which are generated in the skin by UV irradiation[14]. All carotenoids have a polyene

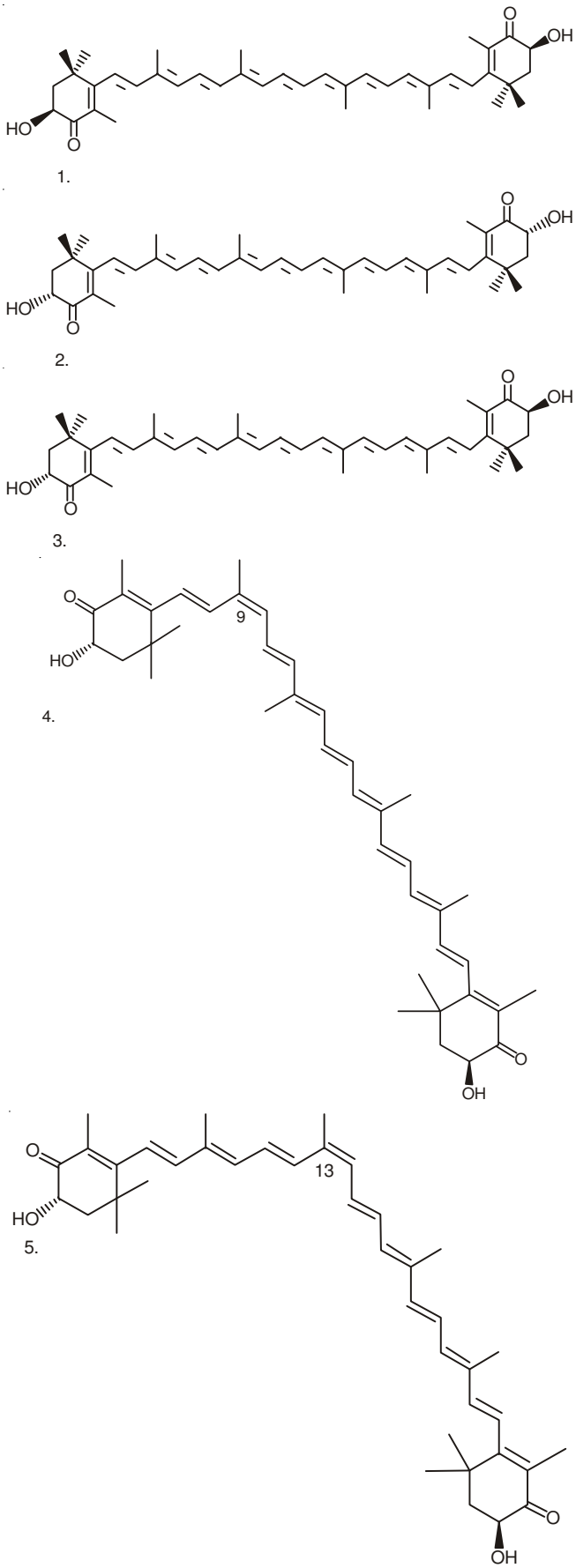

Fig. 1. Structure of astaxanthin (1: (3S, 3'S)-astaxanthin, 2: (3R, 3'S; *meso*)-astaxanthin, 3: (3R, 3'R)-astaxanthin, 4:9-*cis*-astaxanthin, 5: 13-*cis*-astaxanthin)

chain, which is a long conjugated double-bond that serves as the backbone of the molecule (Fig. 2)[15]. The electron-rich conjugated system of the polyene is responsible for the antioxidant activities of the carotenoids, both by quenching singlet oxygen and scavenging radicals to terminate the chain reactions[16]. The antioxidant action of carotenoids is caused by the different structure of the end groups and the number and position of methyl groups[17]. Low density lipoprotein (LDL) and C-reactive protein (CRP) are important cardiovascular risk factors[18]. Many studies have reported that high levels of LDL are related to the prevalence of cardiovascular diseases, such as angina pectoris, myocardial infarction and brain thrombosis[19]. Low density lipoprotein oxidation by macrophages is believed to be important in the progression of atherosclerotic lesions and might be inhibited by carotenoids. Many studies have reported that astaxanthin inhibits LDL oxidation in cardiovascular diseases[20]. *Haematococcus pluvialis* is used with the aim of optimizing the astaxanthin production processes. The main focus of these efforts has been an assessment of various factors and conditions that affect algal growth and the production of astaxanthin[7]. Astaxanthin is found in microalgae, such as *Haematococcus pluvialis, Neochloris wimmeri, Chorella zofingiensis* and *Scenedesmus vacuolatus*. Table-1 lists the content of astaxanthin, which is included in several microalgae. *Haematococcus pluvialis* contains the highest concentration of astaxanthin in natural sources[21]. *Phaffia rhodozyma*, which is fast-growing with excellent availability, was reported to be a good nutrient source for fish without the need to extract the pigment from the cell itself and has attracted commercial interest. The yeast can be propagated by fermentation using glucose and other sugars and it produces astaxanthin during growth[22]. Natural astaxanthin is present in abundance in the marine environment. Most crustaceans generate high levels of discarded solid waste (from 50 to 70 % of total wet weight) in the form of head and abdominal exoskeleton[23]. Table-2 lists the content of astaxanthin in several crustacean byproducts. The carotenoid content in shrimp and crab byproducts varies

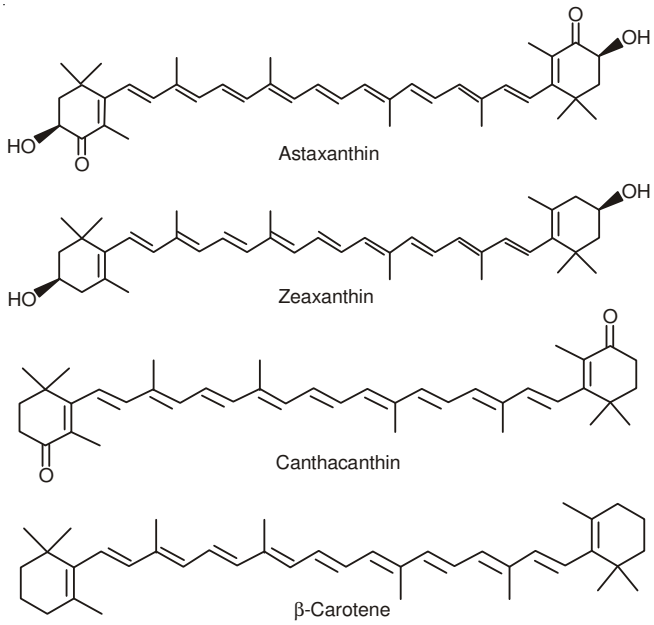

Fig. 2. Chemical structure of some carotenoids

TABLE-1
CONTENTS OF ASTAXANTHIN IN NATURAL SOURCES

Source	Contents (μg g^{-1})	Reference
Shrimp (*Litopenaeus setiferus*)	62.3	56
Snow crab (*Chinoecetes opilio*)	119.6	56
Shrimp (*Panaeus monodon*)	131.7	59
Haematococcus pluvialis	22,700	67
Neochloris wimmeri	19,200	67
Protosiphon botryoides	14,300	67
Scotiellopsis oocystiformis	10,900	67
Chorella zofingiensis	6,800	67
Scenedesmus vacuolatus	2,700	67
Crawfish waste	153.0	68
Brazilian shrimp (*Xiphopenaeus kroyeri*)	50.3	69

between 50.3 and 153 μg g^{-1}. Crustacean byproducts commonly contain less than 1000 μg g^{-1} of astaxanthin. Natural astaxanthin obtained from crab and shrimp shells of crustaceans using the traditional method to extract the product has an advantage in terms of recycling. This has attracted considerable interest in many studies examining the extraction and separation of astaxanthin from natural resources[24].

Astaxanthin has considerable potential in human health and nutrition and has been attributed with the extraordinary potential for protecting the organism against a wide range of diseases. Therefore, this is review paper provides an updated overview of the most important extraction, separation and purification methods from different natural sources.

Extraction of astaxanthin: Astaxanthin has many bioactive properties such as antioxidant, anticancer and anti-inflammation. For these reasons, there has been considerable interest in the development of extraction methods for astaxanthin from nature sources. Ruen-ngam *et al.*[25] compared of various extraction methods such as solvent extraction, ultrasound assisted extraction and microwave assisted extraction for recovery of astaxanthin from *Haematococcus pluvialis*. Many studies were extraction of astaxanthin by various methods. Table-2 lists some of the methods found in the literature for the extraction of astaxanthin.

Solvent extraction: Solvent extraction is a common sample preparation method in the analytical process for a long time. Paviani *et al.*[26] used organic solvents, such as *n*-hexane: isopropyl alcohol (6:4 v/v), acetone and *n*-hexane: isopropyl alcohol (1:1 v/v) to extract astaxanthin from shrimp waste and amount astaxanthin extracted using these solvents was 53 μg g^{-1}, 40.6 μg g^{-1} and 49.9 μg g^{-1}, respectively. Gimeno *et al.*[27] used only one-solvent extraction of astaxanthin from shrimp wastes. Acetone gave the highest extraction yields of free astaxanthin with up to 115 μg g^{-1} of material. Astaxanthin has relatively high solubility in organic solvents, such as acetone and acetic acid but is almost insoluble in water. The effect of pH on the solubility of astaxanhin was reviewed. The solubility increased 10-20 times under acidic condition (pH 2) compared to that under neutral or alkaline conditions. Astaxanthin oxidation and light stability were observed at a very low pH (pH 3), indicating a decrease in stability Regarding the effect temperature, astaxanthin was easily decomposed even when stored at room temperature. The rate of degradation was particularly high at elevated temperatures. In particular, more than 90 % astaxanthin was decomposed when heated to 100 °C for 5 s[28]. The solvent extraction method has some problems, such as time consuming, expensive and requiring large amounts of toxic organic solvent. On the other hand, solvent extraction

TABLE-2
CONTENTS OF ASTAXANTHIN ACCORDING TO THE SOURCE AND EXTRACTION METHOD

Source	Method	Condition	Content	Reference
Shrimp waste (*Panaeus monodon*)	Oil extraction	Palm oil	131.7 (μg g^{-1})	24
Shrimp waste (*Farfantepenaeus paulensis*)	Solvent extraction	*n*-hexane: isopropyl alcohol (6:4 v/v)	53.0 (μg g^{-1})	26
Shrimp waste (*Penaeus indicus*)	Solvent extraction	Acetone	40.6 (μg g^{-1})	26
Shrimp waste (*Penaeus indicus*)	Solvent extraction	*n*-hexane: isopropyl alcohol (1:1 v/v)	43.9 (μg g^{-1})	26
Shrimp waste (*Farfantepenaeus paulensis*)	Supercritical extraction	CO_2, 49 °C, 37 MPa,	20.7 (μg g^{-1})	26
Haematococcus pluvialis	Supercritical extraction	CO_2, 65 °C, 43.5 MPa,	87.42%	34
Shrimp waste (*Brazilian redspotted*)	Supercritical extraction	CO_2 + ethanol (5%) mixtures, 50 °C, 30 MPa,	1325 (μg g^{-1})	37
Haematococcus pluvialis	Oil extraction	Soybean oil	91.7 (%)	38
Haematococcus pluvialis	Oil extraction	Corn oil	89.3 (%)	38
Haematococcus pluvialis	Oil extraction	Grapeseed oil	87.5 (%)	38
Haematococcus pluvialis	Oil extraction	Olive oil	93.9 (%)	38
Saccharina japonica	Solvent extraction	Dichloromethane	29.3 (μg g^{-1})	46
Shrimp waste (*Penaeus indicus*)	Oil extraction	Coconut oil	24.7 (μg g^{-1})	65
Shrimp waste (*Penaeus indicus*)	Oil extraction	Rece bran oil	24.3 (μg g^{-1})	65
Shrimp waste (*Penaeus indicus*)	Oil extraction	Sunflower oil	26.3 (μg g^{-1})	67
Shrimp waste (*Penaeus indicus*)	Oil extraction	Groundnut oil	23.1 (μg g^{-1})	67
Shrimp waste (*Penaeus indicus*)	Oil extraction	Gingelly oil	23.9 (μg g^{-1})	67
Shrimp waste (*Penaeus indicus*)	Oil extraction	Mustard oil	16.1 (μg g^{-1})	67
Shrimp waste (*Penaeus indicus*)	Oil extraction	Soya oil	24.8 (μg g^{-1})	67
Shrimp waste	Solvent extraction	Ethanol	17.8 (μg g^{-1})	70
Shrimp (*Pandalus borealis*)	Solvent extraction	Chloroform	147.7 (μg g^{-1})	71
Snow crab (*Chinoecetes opilio*)	Solvent extraction	Chloroform	119.6 (μg g^{-1})	71
Crawfish waste	Solvent extraction	Petroleum ether-acetone-water (15: 75: 10 v/v)	153.0 (μg g^{-1})	72
Haematococcus pluvialis	Supercritical extraction	CO_2, 70 °C, 50 MPa, 4 h	2304 (μg g^{-1})	73
Shrimp waste (*Litopenaeus setiferus*)	Oil extraction	Flax seed oil	62.3 (μg g^{-1})	74

has several advantages, such as increased selectivity by separating the analyte from the mixture and concentrating the analyte from a large sample volume. Therefore, solvent extraction techniques are used widely in the purification of bioactive compounds from natural products[29].

Supercritical extraction: Solvent extraction methods have problems such as time consuming, multiple extraction steps and using the large amounts of organic solvent. Organic solvent are also harmful to human health as well as the environment. The problems associated with traditional solvent extraction techniques have aroused growing interest in developing simpler, faster, more efficient methods for the extraction of carotenoids from foods and natural products[30,31]. Supercritical fluid extraction is a modern technology with increasing applications in the pharmaceutical and food processing industries[32]. The principle of the process consists of utilizing a supercritical fluid, whose physico-chemical properties are between those of a liquid and a gas[33]. Supercritical fluids have special properties, such as high diffusivity, low viscosity and low surface tension[34]. Supercritical fluids have been applied in to astaxanthin extraction from various marine products and developed optimal extraction condition. Ali-Nehar *et al.*[35] reported the maximum yield of astaxanthin was found in krill oil extracted at 25 MPa and 45 °C under condition, obtained 8.62 μg g^{-1} astaxatthin. Recently, Sánchez-Camargo *et al.*[36] reported the effect of ethanol addition as a co-solvent with supercritical fluid CO_2 on the astaxanthin extraction yields from freeze-dried red-spotted shrimp waste (*F. paulensis*). The astaxanthin extraction yields increased considerably with increasing proportion of ethanol in the ethanol + supercritical fluid CO_2 mixture when an ethanol percentage of 5 % was used. In addition, astaxanthin as well as fatty acid is also extracted[36]. Supercritical carbon dioxide extraction is initial design costs expensive. However, supercritical carbon dioxide extraction have a lot of advantage such as non-toxic, non-flammable, non-explosive, cost-efficient, readily available and easy to remove from the extracted materials[37]. Because of these advantages, there has been a lot of research.

Oil extraction: Carotenoids are a group of oil soluble pigments. Anderson used soybean oil for the extraction of carotenoids from shrimp waste in 1975. Spinelli and Mahnken developed a 3-stage counter current extraction method to recover astaxanthin containing oil form crab waste at 1978. No and Meyers developed an oil extraction method of carotenoids from crawfish waste with the production of chitin and chitosan in 1992[26]. Handayani *et al*. used palm oil as solvent for extract astaxanthin from giant tiger (*Panaeus monodon*) shrimp waste[24]. Many extraction methods were developed for the separation of astaxanthin from red cyst *Haematococcus* cells such as organic solvent extraction, acid or base treatment and spray drying. However, this method has disadvantage such as high energy consumption and multiple separation steps. Therefore, Kang *et al*.[38] to overcome these disadvantages used vegetable oil for direct extraction of astaxanthin from *Haematococcus* culture. Table-2 lists the amount of astaxanthin extracted from several vegetable oils, such as sunflower oil, groundnut oil, gingelly oil, mustard oil, soybean oil, coconut oil, rice bran oil and cod liver oil. Astaxanthin extracted with an organic solvent is difficult used in cosmetic or food although removing organic solvent due to remaining organic solvent. Therefore, the high advantage is safety of astaxanthin when oil extraction of astaxanthin is used as food and cosmetic additive. The addition of the extract to oil made a significant increase in the oxidation stability of sample at low temperature and a higher amount of the extract resulted in a higher inhibitory effect on the peroxide formation[37].

Surfactant extraction: Traditionally, extraction method of astaxanthin required multiple organic solvent extraction steps, evaporation and vacuum dehydration. Overcome these disadvantages used surfactant. Astaxanthin can be extracted using a surfactant. Hydrophobic property astaxanthin were solubilization inside micelle and can be extracted in aqueous solution. Micelle is an aggregate of surfactant molecules dispersed in a liquid colloid. A typical micelle in aqueous solution forms an aggregate with the hydrophilic "head" regions in contact with surrounding solvent, sequestering the hydrophobic single-tail regions in the micelle centre. Astaxanthin were scavenging as inside hydrophobic part when added in micelle solution. Micellar solution, the particle size is very small and stable in thermodynamics and easy to manufacture can be sterilized by filtration. Therefore, drug delivery system and many applications in cosmetics research is in progress[39,40]. Kim *et al.*[41] investigate effect of surfactants, pH and ionic strength extracted using the surfactant from *Phaffia rhodozyma*. Astaxanthin obtained 396 μg g^{-1} using Tween 20 as surfactant. When extracted with chloroform obtained amount of astaxanthin is 533 μg g^{-1}. Astaxanthin than the surfactant in an organic solvent is extracted much more. However, surfactant extraction method has many advantages. Because of these advantages, there has been a lot of research[41].

Purification of astaxanthin

Saponification and hydrolysis: Astaxanthin exists mainly as an astaxanthin ester of various fatty acids. Astaxanthin was saponified for the hydrolysis of the astaxanthin esters. The saponification of astaxanthin can cause destruction and structural transformations[42]. The general process for the astaxanthin ester hydrolysis of astaxanthin is as follows. Known volumes (*e.g.*, 0.1 mL) of freshly prepared sodium hydroxide solutions in methanol at different concentrations were mixed with aliquots (*e.g.*, 0.5 mL) of the pigment extract at a ratio of 1:5 (v/v) under a nitrogen atmosphere. The hydrolysis reaction of astaxanthin esters was carried out in the dark under nitrogen at ambient temperature (22 °C). The reaction mixtures were sampled and analyzed by HPLC to monitor the progress of hydrolysis during saponification until the end of the hydrolysis reaction[43]. The contents of the carotenoids were also determined simultaneously using the above HPLC method to measure the possible losses of carotenoids during saponification.

The astaxanthin ester hydrolysis reaction is as follows:

Astaxanthin - Fatty acid + NaOH → Astaxanthin + Fatty acid - Na

when NaOH is added, the hydroxyl anion attacks the carbonyl group of the astaxanthin ester[44,45]. Zhou *et al*.[46] investigate the solvent extraction and saponification was applied to obtain free astaxanthin from *Saccharina japonica*. 29.30 μg g^{-1} of astaxatthin was extracted using the organic solvent. After

subsequent saponification, amount of astaxanthin was increased to 37.26 μg g^{-1} [46]. Usually, Crustacean byproducts are hydrolyzed using alkaline aqueous solutions however this has caused environmental problems due to liquid waste containing alkali. Therefore, enzymatic hydrolysis developed for recovery of the astaxathin fraction from crustacean byproducts[47]. The hydrolysis of astaxanthin esters is the most common method for purifying astaxanthin.

Fermentation: Crustacean byproducts need to deproteinization-demineralization treatments due to including protein (47.7 %) and calcium (26.8 %). The industrial production of astaxanthin from crustacean byproducts is using the high concentration alkali and acid solutions as chemical treatments. This mean high energy consumption and degradation of astaxanthin as well as the use of large amount water. Bacterial fermentations and lactic acid fermentations were using for reduce chemical treatment as eco-friendly processes and enhances the astaxanthin recovery as it is a very sensitive compound[48]. Many studied the optimization of astaxanthin production by bacterial fermentation such as sugar-feeding strategy pH control strategy[49]. Hu *et al*.[50] evaluated the effects of pH on astaxanthin formation in batch fermentation by *Phaffia rhodozyma*. The optimal pH for astaxanthin formation was around[50]. Normally, lactic fermentation method was used to deproteinize crustacean byproducts. In this way, the protein and calcium content of crustacean byproducts were decreasing up to 50 %. Astaxanthin is stabilized through lactic fermentation by acid production. In addition, astaxanthin is extract from stabilized fermented waste and fermentation broth by solvent extraction[49].

Other methods: Solid-phase extraction (SPE) is used extensively as a sample preparation method for purifying the target compounds and for applications, such as the removal of toxic or active constituents. Solid-phase extraction became well known in the early 1970s because it minimizes the weakness of liquid-liquid extraction[51,52]. A normal solid-phase extraction cartridge is composed of a short column (open syringe barrel) packed with a sorbent[53]. Solid-phase extraction is based on the distinct transfer of compounds absorbed and desorbed during elution between the sorbent material and mobile phase. Retention is related to the hydrophobic, polar, ion exchange interactions between the target compounds and surface of the sorbent[54]. Currently, ionic liquid-based silica (IL-Si) has excellent chemical and physical properties and has recently been used as a solid phase extraction sorbent. According to the chemical structure of astaxanthin, the hydrophobic, dipole-dipole and π-π interactions between astaxanthin and IL-Si might have some excellent effects to increase the separation efficiency of solid phase extraction. Zhou *et al*.[46] optimization of SPE condition for astaxatnin purify from *Saccharina japonica*.

Separation astaxanthin: Various astaxanthin stereoisomers such as *trans* (three configurational isomers: two enantiomers 3R, 3R'; 3S, 3S'; and a *meso* form 3R, 3S') form and *cis* form are found in nature that differ in the configuration of the two hydroxyl groups on the molecule. Separate and quantitate of stereoisomers astaxanthin is necessary as studies of astaxanthin's health benefits proliferate[55,56]. Merete *et al*.[57] used HPLC, Vis-spectra, MS and 1H NMR for detected astaxantin.

Separation of all-*trans* astaxanthin: Separation of *trans*-astaxanthin from nature source reported through many studies. Almost studies are determination of only one pick as *trans*-astaxanthin after various extractions. Generally, astaxanthin is determined by spectrophotometry or chromatography. *trans*-Astaxanthin can be analyzed easily using HPLC. The HPLC analysis was performed with a commercial C_{18} column (4.6 × 150 mm, 5 μm). The mobile phase was dichloromethane/methanol/acetonitrile/water (5.0: 85.0: 5.5: 4.5, v/v) used as isocratic elution in room temperature, the flow-rate was set at 0.5 mL/min, the UV wavelength was set at 476 nm and the injection volume was 10 μL. Under these conditions, *trans*-astaxanthin was determined in the 10-11 min[58]. Astaxanthin exists as two enantiomers (3R, 3R' and 3S, 3S') and a *meso* form (3R, 3S'). Yuan *et al*. determined two enantiomers *trans*-astaxatnhin (3R, 3R' and 3S, 3S') and astaxanthin-ester using commercial C_{18} column (4.6 × 150 mm, 5 μm). The mobile pahse was 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) used as gradient elution and photodiode array detector from *Haematococcus pluvialis*. However, *meso*-astaxanthin can not detected[59]. Rezanka *et al*.[60] analyzed the enantiomers astaxanthin and *meso* form astaxanthin used the chiral stationary phase from the snow alga. Enantiomers separation is difficult using the commercial column. Therefore, chiral stationary phase used for enantiomer astaxanthin. Chiral stationary phases are designed to separate enantiomeric compounds. They can be bonded to solid supports or created *in situ* on the surface of the solid adsorbent or they can be surface cavities that allow specific interactions with one enantiomeric form[61].

Separation of isomer astaxanthin: HPLC is the technique of choice for the analysis of carotenoids. Table-3 compares some of the methods reported in the literature for the determining isomers of astaxanthin in different matrices by chromatography. High-performance liquid chromatography (HPLC) coupled with a photodiode array detector or more commonly with an ultraviolet-visible (UV-visible) detector used[62]. Astaxanhin separation is difficult in natural extracts using only a single chromatography run. A few are considered ideal for the simultaneous separation of astaxanthin, isomers of astaxanthin, astaxanthin esters, other carotenoids and chlorophylls in the natural extract[63]. The symmetry C_{18} column was selected and applied to the separation of astaxanthin isomers and esters in natural extracts. Typically, dichloromethane, acetonitrile, methanol and water are used as the mobile phase. The concentration of the mobile phase is related to the retention factor. The mobile phase composition and gradient process to separate the isomers of astaxanthin is suitable. Yuan and Chen, previously used a mobile phase containing dichloromethane (6.5 %), methanol (82 %), acetonitrile (7.5 %) and water (4 %) to separate *trans*-astaxanthin, lutein and the *cis*-isomers of astaxanthin[63]. In addition, derivatization was used to separate the astaxanthin stereoisomers. These include normal-phase separations on a Sumipax OA-2000 column[64,65]. In addition, a chiral stationary phase was used to separate the isomers of astaxanthin. HPLC with chiral stationary phases (CSPs) is one of the most powerful tools for enantiomeric separation. A few chiral stationary phases have been developed specifically to separate primary

TABLE-3
SEPARATION OF ASTAXANTHIN ISOMER

Source	Compounds	Mobile phase	Analysis column	Detector and condition	Reference
Standard	(3S,3'S), (3S,3'R), and (3R,3'R)-Astaxanthin	Isocratic: methyl tert-butylether/ acetonitrile (50:50, v/v)	Chiralpak IC chiral stationary phase (250 × 4.6 mm)	UV, 476 nm	56
Standard	*trans*-Astaxanthin, 9-*cis*-astaxanthin, 13-*cis*-astaxanthin	Isocratic: methanol/ dichloromethane/ acetonitrile/ water, (85:5:5.5:4.5, v/v)	C_{18} column (250 × 4.6 mm)	UV, 476 nm	57
Haematococcus pluvialis	*trans*-Astaxanthin, 9-*cis*-astaxanthin, 13-*cis*-astaxanthin, (3R,3'R)-*trans*-astaxanthin	Gradient: solvent A, (dichloromethane/ methanol/ acetonitrile/ water (5.0:85.0:5.5:4.5, v/v); solvent B, (dichloromethane/ methanol/ acetonitrile/ water, 22.0:28.0:45.5:4.5, v/v)	Beckman Ultrasphere C_{18} column (250 × 4.6 mm, 5 µm)	Photodiode array detector, 250 to 700 nm	59
Shrimp by-products	*trans*-Astaxanthin, 9-*cis*-astaxanthin, 13-*cis*-astaxanthin	Isocratic: acetonitrile/ chloroform/ methanol/ water/ propionic acid (71:22:4:2:1 v/v)	Waters Symmetry^{MR} C_{18} column	UV, 474 nm	75
Egg Yolk	*trans*-Astaxanthin, 9-*cis*-astaxanthin, 13-*cis*-astaxanthin	Gradient: solvent A, methanol/ methyl t-butyl ether/ water (81:15:4 v/v), solvent B, methanol/ methyl t-butyl ether/ water (6:90:4 v/v)	YMC carotenoid (250 × 4.6 mm, 3 µm)	UV-vis, 450 nm	76
Shrimp Shells *(Parapenaeopsis hardwickii)*	*trans*-Astaxanthin, 9-*cis*-astaxanthin, 13-*cis*-astaxanthin	Gradient: methanol/ dichloromethane/ acetonitrile (90:5:5, v/v) and water	C_{18} column (250 × 4.6 mm, 5 µm)	UV, 480 nm	77

amine enantiomer. The first of these is based on chiral crown ethers, as originally developed by Cram *et al.*[65,66]. Furthermore, the analysis times were normally 0.5 h or longer. Recently, Grewe *et al*. reported the reversed-phase separation of all-*trans*-astaxanthin on a coated cellulose, *tris* (3, 5-dimethylphenyl-carbamate) CSP (Chiralcel OD-RH)[56], which is very important for the purification of *trans*-astaxanthin.

Conclusion

Astaxanthin has unique molecular structure that gives it special chemical and biological properties, such as antioxidant and as a potential protective treatment for cancer and cardio-vascular disease. Protections against a range of diseases by astaxanthin are likely to involve antioxidant mechanisms including the prevention of oxidative damage and cellular necrosis or apoptosis induced by oxidative stress. The most important outcome of this paper is the extraction and separation of astaxanthin. Many reported extraction methods, such as solvent extraction, supercritical fluid extraction and oil extraction, were assessed. The structure of astaxanthin isomers make them difficult to separate using normal techniques. Consequently, there has been considerable research in the separation of astaxathin isomers, such as 13-*cis* astaxanthin, 15-*cis* astaxanthin and *trans*-astaxanhin. Generally, astaxanthin is determined by spectrophotometry or chromatography. In particular, HPLC is the technique of choice for the analysis of carotenoids. HPLC with chiral stationary phases is one of the most powerful tools for enantiomeric separation.

ACKNOWLEDGEMENTS

This study was supported by Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology (2014002046).

REFERENCES

1. V. Mann, M. Harker, L. Pecker and J. Hirschberg, *Nat. Biotechnol*., **18**, 888 (2000).
2. B. Renstrøm, H. Berger and S. Liaaen-Jensen, *Biochem. Syst. Ecol*., **9**, 249 (1981).
3. G. Hussein, U. Sankawa, H. Goto, K. Matsumoto and H. Watanabe, *J. Nat. Prod*., **69**, 443 (2006).
4. M. Grung and S. Liaaen-Jensen, *Biochem. Syst. Ecol*., **21**, 757 (1993).
5. J.P. Yuan, J. Peng, K. Yin and J.H. Wang, *Mol. Nutr. Food Res*., **55**, 150 (2011).
6. R.T. Lorenz and G.R. Cysewski, *Trends Biotechnol*., **18**, 160 (2000).
7. I. Higuera-Ciapara, L. Felix-Valenzuela and F.M. Goycoolea, *Crit. Rev. Food Sci. Nutr*., **46**, 185 (2006).
8. G. Britton, *J. Fed. Am. Soc. Exp. Biol*., **9**, 1551 (1995).
9. X. Liu and T. Osawa, *Biochem. Biophys. Res. Commun*., **357**, 187 (2007).
10. G. Hussein, H. Goto, S. Oda, U. Sankawa, K. Matsumoto and H. Watanabe, *Biol. Pharm. Bull*., **29**, 684 (2006).
11. N. Anarjan, H. Mirhosseini, B.S. Baharin and C.P. Tan, *Food Chem*., **123**, 477 (2010).
12. T. Tanaka, H. Makita, M. Ohnishi, H. Mori and K. Satoh, *Cancer Res*., **55**, 4059 (1995).
13. D.L. Narayanan, R.N. Saladi and J.L. Fox, *Int. J. Dermatol*., **49**, 978 (2010).
14. S. Hama, K. Takahashi, Y. Inai, K. Shiota, R. Sakamoto, A. Yamada, H. Tsuchiya, K. Kanamura, E. Yamashita and K. Kogure, *J. Pharm. Sci*., **101**, 2909 (2012).
15. D. Chu, Y. Xie, S. Hong and X. Yan, *Asian J. Chem*, **22**, 2635 (2010).
16. H. McNulty, R.F. Jacob and R.P. Mason, *Am. J. Cardiol*., **101**, S20 (2008).
17. H.P. McNulty, J. Byun, S.F. Lockwood, R.F. Jacob and R.P. Mason, *Biochim. Biophys. Acta*, **1768**, 167 (2007).
18. L.J. Goldstein and S.M. Brown, *Annu. Rev. Biochem*., **46**, 897 (1997).
19. T.J. Maher, www.nhir.com/tests/astaxanthin.pdf (2000).
20. K.L.H. Carpenter, C. Van Der Veen, R. Hird, I.F. Dennis, T. Ding and M.J. Mitchinson, *FEBS Lett*., **401**, 262 (1997).
21. M. Orosa, J.F. Valero, C. Herrero and J. Abalde, *Biotechnol. Lett*., **23**, 1079 (2001).
22. J. Yang, H. Tan, R. Yang, X. Sun, H. Zhai and K. Li, *Agric. Eng. Int. CIGR J.*, **13**, issue No. 2 (2011).
23. S.D. Santos, T.B. Cahú, G.O. Firmino, C.C.M.M.B. de Castro, L.B. Carvalho Jr., R.S. Bezerra and J.L.L. Filho, *J. Food Sci*., **77**, H141 (2012).

24. A.D. Handayani, Sutrisno, N. Indraswati and S. Ismadji, *Bioresour. Technol*., **99**, 4414 (2008).
25. D. Ruen-ngam, A. Shotipruk and P. Pavasant, *Sep. Sci. Technol*., **46**, 64 (2010).
26. A.P. Sánchez-Camargo, H.A. Martinez-Correa, L.C. Paviani and F.A. Cabral, *J. Supercrit. Fluids*, **56**, 164 (2011).
27. M. Gimeno, J.Y. Ramírez-Hernández, C. Mártinez-Ibarra, N. Pacheco, R. García-Arrazola, E. Bárzana and K. Shirai, *J. Agric. Food Chem*., **55**, 10345 (2007).
28. S.Y. Kim, E. Cho, J.M. Yoo, M.J. In and H.J. Chae, Korean *J. Biotechnol. Bioeng*., **23**, 546 (2008).
29. H. Liu and P.K. Dasgupta, *Anal. Chem*., **68**, 1817 (1996).
30. H.G. Daood, V. Illés, M.H. Gnayfeed, B. Mészáros, G. Horváth and P.A. Biacs, *J. Supercrit. Fluids*, **23**, 143 (2002).
31. M. Careri, L. Furlattini, A. Mangia, M. Musci, E. Anklam, A. Theobald and C. von Holst, *J. Chromatogr. A*, **912**, 61 (2001).
32. R. Mendes, B. Nobre, M. Cardoso, A. Pereira and A. Palavra, *Inorg. Chim. Acta*, **356**, 328 (2003).
33. L. Felix-Valenzyela, I. Higuera-Ciapara, F. Goycoolea-Valencia and W. Argüelles-Monal *J. Food Process Eng*., **24**, 101 (2001).
34. K.L. Chiu, Y.C. Cheng, J.H. Chen, C.J. Chang and P.W. Yang, *J. Supercrit. Fluids*, **24**, 77 (2002).
35. A. Ali-Nehari, S.B. Kim, Y.B. Lee, H.Y. Lee and B.S. Chun, *Korean J. Chem. Eng*., **29**, 329 (2012).
36. A.P. Sánchez-Camargo, M.Â.A. Meireles, A.L.K. Ferreira, E. Saito and F.A. Cabral, *J. Supercrit. Fluids*, **61**, 71 (2012).
37. L. Wang, B. Yang, B. Yan and X. Yao, *Innov. Food Sci. Emerg. Technol*., **13**, 120 (2012).
38. C.D. Kang and S.J. Sim, *Biotechnol. Lett*., **30**, 441 (2008).
39. M.M. Barth, C. Zhou, K.M. Kute and G.A. Rosenthal, *J. Agric. Food Chem*., **43**, 2876 (1995).
40. R.P. Bagwe, J.R. Kanicky, B.J. Palla, P.K. Patanjali and D.O. Shah, *Crit. Rev. Ther. Drug Carrier Syst*., **18**, 77 (2001).
41. Y.B. Kim, K. Ryu, G.B. Lim and E.K. Lee, *Korean J. Biotechnol. Bioeng*., **17**, 176 (2002).
42. E.A. Johnson and G.H. An, *Crit. Rev. Biotechnol*., **11**, 297 (1991).
43. B. Madhava Rao and K. Gajanan, *Asian J. Chem*, **12**, 221 (2000).
44. J.P. Yuan and F. Chen, *J. Agric. Food Chem*., **47**, 31 (1999).
45. F.X. Cunningham and E. Gantt, *Plant Cell*, **23**, 3055 (2011).
46. J. Zhou, W. Bi and K.H. Row, *J. Food Sci*., **76**, C441 (2011).
47. H.D. de Holanda and F.M. Netto, *J. Food Sci*., **71**, C298 (2006).
48. N. Bhaskar, P.V. Suresh, P.Z. Sakhare and N.M. Sachindra, *Enzyme Microb. Technol*., **40**, 1427 (2007).
49. Z.C. Hu, Y.G. Zheng, Z. Wang and Y.C. Shen, *Food Technol. Biotechnol*., **45**, 209 (2007).
50. Z.C. Hu, Y.G. Zheng, Z. Wang and Y.C. Shen, *Enzyme Microb. Technol*., **39**, 586 (2006).
51. C.W. Huck and G.K. Bonn, *J. Chromatogr. A*, **885**, 51 (2000).
52. Y. Li, Y. Peng, Y. Ma, Q. Hu and G. Yang, *Asian J. Chem*., **21**, 4593 (2009).
53. C.F. Poole, A.D. Gunatilleka and R. Sethuraman, *J. Chromatogr. A*, **885**, 17 (2000).
54. N. Masque, R.M. Marcé and F. Borrull, *Trends Analyt. Chem*., **17**, 384 (1998).
55. M. Guerin, M.E. Huntley and M. Olaizola, *Trends Biotechnol*., **21**, 210 (2003).
56. C. Wang, D. Armstrong and C.D. Chang, *J. Chromatogr. A*, **1194**, 172 (2008).
57. M. Grung, P. Metzger and S. Liaaen-Jensen, *Biochem. Syst. Ecol*., **22**, 25 (1994).
58. W. Bi, M. Tian, J. Zhou and K.H. Row, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*., **878**, 2243 (2010).
59. J.P. Yuan and F. Chen, *J. Agric. Food Chem*., **46**, 3371 (1998).
60. T. Rezanka, L. Nedbalová, I. Kolouchová and K. Sigler, *Phytochemistry*, **88**, 34 (2013).
61. W.H. Pirkle, J.M. Finn, J.L. Schreiner and B.C. Hamper, *J. Am. Chem. Soc*., **103**, 3964 (1981).
62. R. Frassanito, G. Flaim, I. Mancini and G. Guella, *Biochem. Syst. Ecol*., **34**, 843 (2006).
63. J.P. Yuan and F. Chen, *Food Chem*., **68**, 443 (2000).
64. S. Abu-Lafi and S.A. Turujman, *Enantiomer A*., **2**, 17 (1997).
65. G.D.Y. Sogah and D.J. Cram, *J. Am. Chem. Soc*., **101**, 3035 (1979).
66. L.R. Sousa, G.D.Y. Sogah, D.H. Hoffman and D.J. Cram, *J. Am. Chem. Soc*., **100**, 4569 (1978).
67. N.M. Sachindra and N.S. Mahendrakar, *Bioresour. Technol*., **96**, 1195 (2005).
68. X. Hou, Y. Li, G. Wu, L. Wang, M. Hong and Y. Wu, *J. Chromatogr. Sci*., **48**, 22 (2010).
69. C.D. Kang and S.J. Sim, *Biotechnol. Prog*., **23**, 866 (2007).
70. C.H. Yoon, H.S. Bok, D.K. Choi and K.H. Row, *Korean Chem. Eng. Res.*, **50**, 545 (2012).
71. F. Shahidi and J. Synowiecki, *J. Agric. Food Chem*., **39**, 1527 (1991).
72. S.P. Meyers and D. Bligh, *J. Agric. Food Chem*., **29**, 505 (1981).
73. P. Thana, S. Machmudah, M. Goto, M. Sasaki, P. Pavasant and A. Shotipruk, *Bioresour. Technol*., **99**, 3110 (2008).
74. J. Pu, P.J. Bechtel and S. Sathivel, *Biosystems Eng*., **107**, 364 (2010).
75. R.E. Armenta and I. Guerrero-Legarreta, *J. Agric. Food Chem*., **57**, 6095 (2009).
76. L.A. Walker, T. Wang, H. Xin and D. Dolde, *J. Agric. Food Chem*., **60**, 1989 (2012).
77. W.C. Lin, J.T. Chien and B.H. Chen, *J. Agric. Food Chem*., **53**, 5144 (2005).