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Astaxanthin determination in marine biological samples: an overview

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Abstract

Astaxanthin (AX) (3,3'dyhydroxy-β,β- carotene-4,4´dione) is a pigment that belongs to the family of the xanthophylls, the oxygenated derivatives of carotenoids whose synthesis in plants derives from lycopene. AX, which may not be synthesized *de novo* by animals, is one of the main pigments in marine ecosystems found in crustacean, and many fish species. In pelagic marine food webs, copepods are the main producers of AX, being also the principal components of Antarctic Krill pigment. Thus, in the aquaculture industry AX is present in feed formulations for salmonids and other farmed fish species, where represent an important cost of the feeds.

Depending in their origin, AX can be found in association with other compounds. It may be sterified in one or both hydroxyl groups with different fatty acids such as palmitic, oleic, estearic, or linoleic. It may also be found free, that is, with the hydroxyl groups without sterification; or else, forming a chemical complex with proteins (carotenoproteins) or lipoproteins (carotenolipoproteins). Synthetic AX is not sterified while found in algae is always sterified.

AX has an enormous commercial and industrial prospect. Therefore, due the growing demand for natural foods has been stimulated the search for natural sources of AX with potential for industrialization like microalgae, shrimp, krill, crab and langostilla between them. In the aquaculture context, the use of AX for the feeding industry is important not only from the standpoint of pigmentation to increase consumer acceptance but also as a necessary nutrient for adequate growth and reproduction of commercially valuable species.

Therefore, the accurate determination of AX forms in this kind of biological matrices is necessary which involves extraction prior to their determination. In this work, a review of the reported methods for the analysis of AX in marine organisms implied in the aquaculture industry (microalgae, shrimps, crabs and fishes) which are always based on LC coupled to different detectors like diode array (DAD) and mass spectrometry (MS) was done, and the different extraction and clean-up techniques currently employed discussed.

Abbreviations

APCI: Atmospheric pressure photoionization; APPI:Atmospheric pressure chemical ionization; ASAP: Atmospheric pressure solids analysis; AX: Astaxanthin; BHT: Butyl-hidroxy toluen; $CO_{2:}$ Carbon dioxide; ESI: Electrospray; FAB :Fast atom bombardment; GC: Gas chromatography; HPLC: High performance liquid Chromatography; EI :Electron impact; *H. pluvialis: Haematococcus pluvialis*; IL-SI: Ionic liquid based silicas; LC: Liquid chromatography; MALDI: matrix-assisted laser desorption/ionization; MS:Detection mass; MTEB;:methtyl tert- Butyl-Ether; PLE: pressurized liquid extraction; SC-C $O_{2:}$ supercritical carbon dioxide; SFE-CO2: supercritical carbon dioxide fluid extraction; SJ: *Saccaharina Japanica;* SPE: Solid phase extraction; SUPRAS: Supramolecular Solvent; UHPLC: Ultra High- performance liquid chromatography; UV-VIS: Ultraviolet visible.

1. INTRODUCTION

Pigments are chemical compounds that absorb light in the wavelength range of the visible region. Produced color is due to a molecule-specific structure (chromophore).

They can be classified by their origin. Natural pigments are produced by living organisms such as plants, animals, fungi, and microorganisms. Natural and synthetic pigments are organic and inorganic compounds.

Moreover, natural pigments can be classified by their structural characteristics as:

(a) Tetrapyrrole derivatives: chlorophylls and heme colors.

(b) N-heterocyclic compounds different from tetrapyrroles: purines, pterins, flavins, phenazines, phenoxazines, and betalains.

(c) Benzopyran derivatives (oxygenated heterocyclic compounds): anthocyanins and other flavonoid pigments.

(d) Quinones: benzoquinone, naphthoquinone, anthraquinone.

(e) Melanins.

(f) Isoprenoid derivatives: carotenoids and iridoids.

Within of isoprenoid derivatives, carotenoids are natural pigments synthetized by plants and some microorganisms. They are readily soluble in non-polar organic solvents such as acetone, diethyl ether, chloroform and hexane, whereas their oxygenated derivatives, xanthophylls, dissolve best in polar solvents such as alcohols.

Carotenoids are known to be indispensable cellular components in microorganisms, fungi, algae, higher plants, animals, and humans and are one of the most important natural marine pigment groups. They are also one of the main natural food colorants with widespread use (Goodwin, 1986).

Remarkable progress has been made in research regarding the structure and chemistry of carotenoids. The chemical structures of approximately 600 carotenoids, which are synthesized de novo in higher plants, mosses, algae, bacteria and fungi have been well known, although their functions in living cells remain unknown (Karnaukhov, 1990).

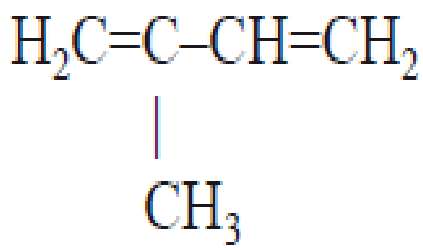

Figure 1. Isoprene structure

Carotenoids are isoprenoid polyenes formed by joining of eight C5-isoprene units (Figure 1) in a regular head-to-tail manner except in the center of the molecule where the order is tail to tail and the molecule is symmetrical. They are arranged in such a way that the two central methyl groups are in the 1,6 position, while the remaining methyl groups are in the 1,5 position relative to each other. A series of conjugated double bonds constitutes a chromatophore of variable length, resulting in characteristic yellow to red colors (Nelis, 1989).

All carotenoids can be considered as lycopene ($C_{40}H_{56}$).derivatives (Figure 2) by reactions involving: (1) hydrogenation, (2) dehydrogenation, (3) cyclization, (4) oxygen insertion, (5) double bond migration, (6) methyl migration, (7) chain elongation, (8) chain shortening (Goodwin, 1980).

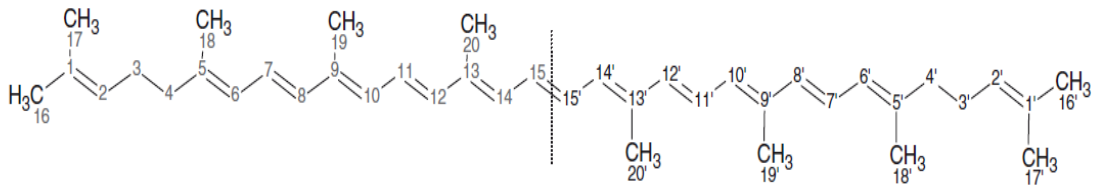

Figure 2. Structure of carotenoid lycopene with common numbering system (Namitha et al. 2010)

Carotenoids have been classified as:

(a) primary carotenoids: compounds required by plants in photosynthesis like β-carotene, violaxanthin, and neoxanthin and;
(b) secondary carotenoids: localized in fruits and flowers like α-cryptoxanthin, zeaxanthin, antheraxanthin, capsanthin, capsorubin,... (Lichtenhaler, 1987).

Also, carotenoids are classified by their chemical structure, as shown in Figure 3:

(1) carotenes that are constituted by carbon and hydrogen, such as, β-carotene and lycopene;

(2) oxycarotenoids or xanthophylls that have carbon, hydrogen and, additionally, oxygen. In this group, oxygen can be present as OH groups, as in zeaxanthin or as oxi-groups, as in canthaxanthin or in a combination of both, as in astaxanthin.

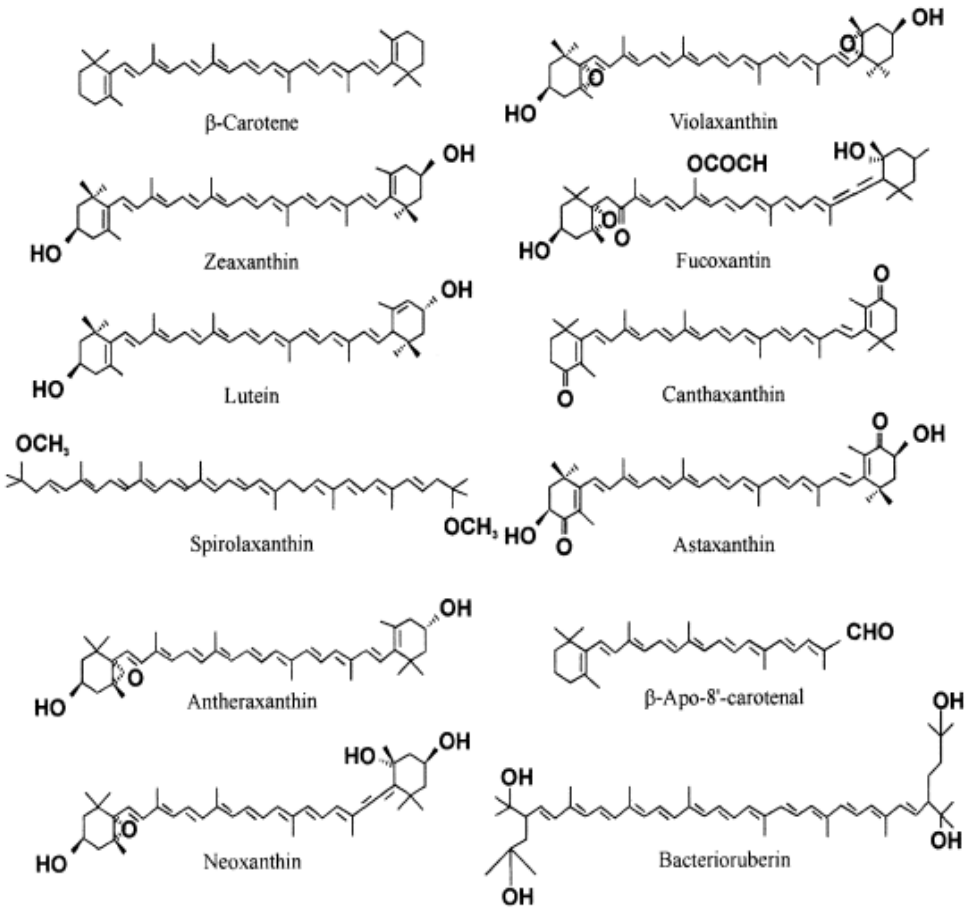

Figure 3. Structure of common carotenoids (Delgado- Vargas et.al, 2000)

Although it is generally accepted that animals are unable to synthesize carotenoids *de novo*, they apparently are able to modify dietary plant carotenoids. The carotenoids are either dissolved in fats or combined with proteins in the aqueous phase. In living organisms, these photosensitive compounds play important roles, such as coloring the body, tissues, or biological fluids, and therefore allowing chromatic adaptation to environments, and also by assuming various physiological functions, as in respiration and vision . These compounds are precursors of vitamin A and retinoid compounds required for morphogenesis (Altamirano et al., 1992; Ananboranich et al., 1995). Furthermore in industry, carotenoids are used in nutrient supplementation, for pharmaceutical purposes, as food colorants and fragrances and in animal feed (Bakker et al., 1997).

Astaxanthin (AX) is one of the main pigments included in crustacean, salmonids (Sanchez-Silva et al, 2012), and other farmed fish feeds (Hou et al. 2010). Also, it is the principal components of Antartic Krill pigment (Huang et al. 2013). In pelagic marine food webs, copepods are the main producers of AX (Rønsholdt et al., 2001; Hanne et al, 1998; Sheehan et al., 1998; Dissing et al., 2001).

The poliene system gives carotenoids its distinctive molecular structure, their chemical properties and their light-absortion characteristics. Each double bond from the poliene chain may exist in different configurations, as geometric isomers cis or trans. Cis-isomers are thermodynamically less stable than the trans isomers (Higuera-Ciapara et al. 2006)**.**

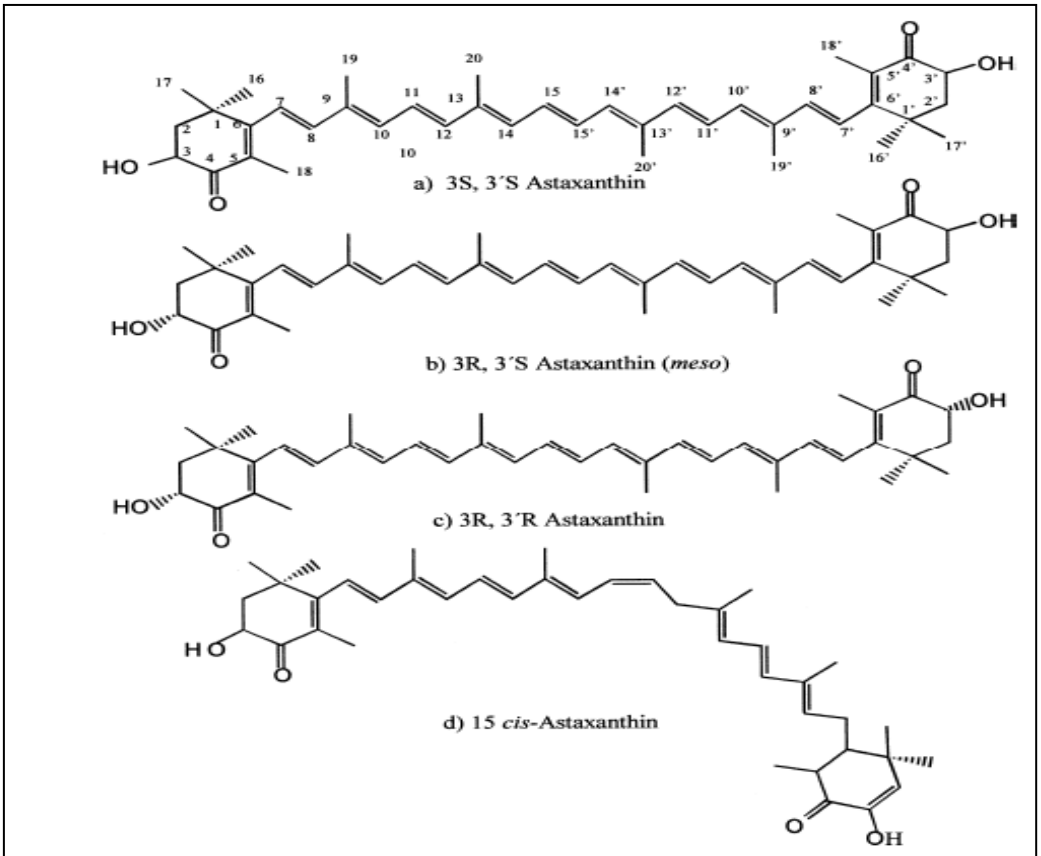

Figure 4. Astaxanthin configurational isomers (a–c) and a geometric cis isomer (d) (Turujman et al., 1997; Osterlie et al., 1999).

AX may present three configurational isomers: two enantiomers (3R, 3´R and 3S, 3´S) and a meso form (3R, 3´S). From all these isomers, the 3S, 3´S is the most abundant in the nature (Turujman et al 1997) (see Figure 4).

AX is distinguished by their capacity to interact with chemical reactive species of oxygen known as singlet oxygen and free radicals. Its main role is to provide the desirable reddish-orange color in organisms. In addition to its effect on color, one of the most important properties of AX is its antioxidant properties which has been reported to surpass those of β-carotene or even α-tocopherol (Liu et al., 2007). Also, it is a photoprotector in zooplankton (Snoeijs et al, 2013).

Depending in their origin, AX can be found in association with other compounds. It may be sterified in one or both hydroxyl groups with different fatty acids such as palmitic, oleic, estearic, or linoleic. It may also be found free, that is, with the hydroxyl groups without sterification; or else, forming a chemical complex with proteins (carotenoproteins) or lipoproteins (carotenolipoproteins). Synthetic AX is not sterified, while found in algae is always sterified. Crustacean AX, on the other hand, is a mixture of the three forms previously described (Yuan et al., 1997).

The characteristic red colour of wild Atlantic salmon muscle is caused to the presence of AX and are the most used carotenoids for muscle pigmentation of salmonid fishes. It is unestirifed in the muscle, the geometrical all-E isomer predominating (>90% of total AX) and it believed to be bound non-specifically to the hydrophobic pocket in the actomyosin protein-complex by weak interactions (Birkeland et al., 2004).

On the other hand, synthetic AX is an identical molecule to that produced in living organisms and it consists of a mixture of isomers. It is the main carotenoid used worldwide in the aquaculture industry. Since 1990, Roche began a large scale production of synthetic AX and practically fulfilled the world market for the pigment, estimated at 150–200 million dollars.

In fact, Food and Drugs Administration (FDA) authorizes the use of AX as a food additive to intensify the colour red fish. Besides it was authorized by the European Union as input E 161. The use of AX in fishes the aquaculture industry is important from the standpoint of pigmentation and consumer appeal but also as an essential nutritional component for adequate growth and reproduction. It is directed toward the muscle pigmentation and it has been proposed in the development salmonid eggs and

other marine fishes. Some of the studies have revealed a higher survival rate in diets supplemented with AX (Meyers, 2000).

AX is also used in cosmetic, medical applications as antioxidant activity and food and due to the attractive red, this pigments is used as a colorant in the meat, added fish meal and poultry and may be beneficial to consumer health. In fact, it has enormous commercial and industrial prospects. Therefore, due the growing demand for natural foods and the high cost of synthetic pigments has been stimulated the search for natural sources of AX with potential for industrialization.

Although natural sources of AX are numerous, nearly all are found in very low concentrations. Only a few sources of microbial origin can compete economically with synthetic AX: the green microalgae *Haematococcus pluvialis* and the red yeast *Phaffia rhodozyma.* Manufacturing methods have been reviewed for this type of compound by different authors. Several small companies have been founded (Igene, Aquasearch, and Cyanotech) at the end of the pass century trying to compete with Roche by offering AX from natural sources although their production was limited (McCoy, 1999). By far, *Haematococcus pluvialis* provides the most concentrated natural source of AX known, from 10000 to 40000 mg/kg, in addition to other important carotenoids suchas lutein and canthaxanthin, among other.

In this sense, recent advances in photobioreactor technology has been a fundamental tool to achieve commercial feasibility in the production of AX from microalgae (Olaizola, 2000) as it has allowed the development of culture methods with AX concentration varying from 1.5 to 3% on a dry weight basis (Lorenz et al.,2000)

Several AX products currently marketed are derived from *H. pluvialis* microalgae and are being manufactured. These products may contain between 1.5 and 2.0% in dry weight of AX and are utilized as pigments and nutrient for aquatic animals and also in the poultry industry for the pigmentation of broilers and egg yolk (Cyanotech, 2000).

On the other hand, the red yeast *Phaffia rhodozyma* has been widely studied due to its capacity in producing AX (Flores-Cotera et al., 2001) or in assays testing salmonid pigmentation with diets containing *Phaffia*, with a similar efficiency to that achieved using synthetic AX (Whyte et al., 2001). Currently, the yeast is marketed in a fine powder form as a natural source of AX, protein and other nutrients and utilized as an ingredient in salmonid feed. It is manufactured by natural fermentation in a carefully controlled environment thus effectively obtaining a product with a high percentage of free AX (8,000 μg/g) (Igene, 2003).

The potential utilization of shrimp, krill, crab and langostilla by products to induce pigmentation of cultured fish has been tested too (Coral et al., 1997). They are generated during processing operations of recovering or conditioning of the edible portion of crabs, shrimp and lobster. The carotenoid content in shrimp and crab by products varies between 119 and 148 μg/g. AX is mainly found free or sterified with fatty acids.

The use of AX as pigmenting agent in aquaculture species has been well documented through many scientific publications for more than two decades ; Choubert et al., 1993; Coral et al., 1998; Bowen et al., 2002; Kalinowski et al., 2007; García Romero et al., 2010, 2013).

Currently, the synthetic form of both pigments represents the most important source for fish and crustacean farming operations. AX is widely preferred due to the higher color intensity attained. Additionally, AX is deposited in muscles more efficiently probably due to a better absorption in the digestive tract . Buttle et al. (2001) found that the absorption of AX is species dependent and it is related with the optical and symmetry isomerism of AX on the various tissues of salmonids. Moreover, *cis* isomers tend to preferentially accumulate in the liver, while *trans* ones do so on muscle and plasma (Bjerkeng et al., 1997; Bjerkeng, 2000). Also, studies undertaken on rainbow trout have shown that the distribution of R/S optical isomers found in faeces, blood, liver, and muscle resembled that of the overall content of the supplied diet (Osterlie et al., 1999). In spite of the fact that AX is widely used with the purpose of attaining a given pigmentation, it has many other important functions in fish related

mainly to their reproduction: acceleration of sexual maturity, increasing fertilization and egg survival, and a better embryo development (Scabini et al., 2010). It has also been demonstrated that AX improves liver function, it increases the defense potential against oxidative stress (Nakano et al., 1995) and it has a significant influence on biodefense mechanisms (Amar et al., 2001). Several other physiological and nutritional studies have been performed in crustaceans, mainly on shrimp, which have suggested that AX increases tolerance to stress, improves the immune response, acts as an intracellular protectant, and has a substantial effect on larvae growth and survival (Darachai et al., 1999; Betancor et al., 2011).

According to the above information, the use of AX in the aquaculture industry is important not only from the standpoint of pigmentation to increase consumer acceptance but also as a necessary nutrient for adequate growth and reproduction of commercially valuable species. Thus, in the aquaculture industry AX is present in feed formulations for salmonids and other farmed fish species, where represent an important cost of the feeds. Moreover, last trends in feed formulation and European regulatory rules force time by time to include natural instead of synthetic AX sources. For that, the accurate determination of carotenoids, including AX, in this kind of biological (natural) matrices is necessary to know how much AX could provide and how much AX have been assimilated for the fishes in order to reduce as possible the cost of the diets.

Since the known physiological impact of AX in live animals, nowadays the published literature is very extensive in this matter but notably not enough to understand deeply the physiological fate of AX in marine organisms , and nothing about observed changes in carotenoid profiles between wild specimens and same species but under culture conditions. To better understand carotenoid AX and it importance in fish physiology it is necessary to study changes in carotenoid profiles and sterifications when animals are submitted or growth in different conditions. Any advance regardless these changes in carotenoid content and profiles in fish tissues may us to know about their relative physiological interest which by the way directly impact in the feed industry.

Unfortunely, the analysis methods for AX content are not standardized although FDA has accepted a method by liquid chromatography (LC) analysis (method 21 CFR 73.185) and it is used by different international agencies. In any case, this determination necessarily involves the extraction of AX from the marine organisms prior to their determination.

In this work, an intense review of the reported methods used for the analysis of AX in marine organisms implied in the aquaculture industry (microalgae, yeats, crabs and fishes) which are always based on LC coupled to different detectors like diode array (DAD) and mass spectrometry (MS) was done, and the different extraction and clean-up techniques currently employed discussed.

2. ANALYTICAL PROCEDURES

An important aspect to be considered when dealing with the determination of organic compounds from natural complex matrices, an extraction step prior to their determination is required. In general, the extraction of carotenoids must be carried out very quickly, avoiding exposure to light, oxygen, high temperatures and to prooxidant metals, such as iron or copper, in order to minimize autooxidation and isomerization. To avoid oxidation of AX, several methods are usually employed to improve the stability of this pigment in the matrix of study. The addition of antioxidants, such as ascorbic acid, pyrogallol has been recommended to prevent carotenoid losses during the extraction procedure.

In this sense, the first problem arises in the standard preparation. It is necessary to prepare it in a non polar dissolvent, like chloroform, sonicate in a ultrasonic bath during 15 min and add butyl-hidroxy-toluen (BHT) like antioxidant reactive. An aliquot (10 mL) of this solution is transferred into a 100 mL volumetric flask and combined with isohexane until 100 mL. This is the stock AX solution for measurement at 470 nm on a spectrophotometer and for injection onto the HPLC. This AX standard must be prepared every 6 weeks. The concentration of AX stock is calculated according to the following equation (L. Robaina and F. Strachan, personal communication):

C = absorption x 10000/ 2100 (mg/L).

Although it is necessary to develop appropiate, fast, cost-effective and environmentally friendly extraction processes to isolate the compounds of interest, sampling and storage are also important steps prior to sample preparation (Rodriguez et al., 2006).

Respect the sampling and storage, microalgae cultures have to grow in 8- cm-wide glass reactors containing 1 L of modified Bold's basal medium supplemented with KNO_3 and subjected to continuous stirring by bubbling air through the mixture at a constant flow rate. Pure CO_2 has to supply every 30 s at 10-min intervals to the air stream to provide inorganic carbon and to maintain the pH at 8. Reactors are maintained in a culture chamber at 24±2 °C, with a 16:8 h light:dark photoperiod using fluorescent light at a photosynthetic photon flux density of 400 μmol photons per square meter per second. After the cells had reached the late exponential phase, biomass was harvested by centrifugation (7000 rpm for 5 min at 10 °C), frozen at −20 °C, freeze dried at −40°C for 48 h, and stored under dry and dark conditions until further use (Castro-Puyana et al., 2013).

Shrimps are harvested and transported on ice to keep them frozen for do not lose their properties. Some authors used waste shell (carapace) of shrimps because they are a rich source of AX. López-Cervantes et al. (2006) and other as Sanches-Silva et al. (2012) used raw and cooked shrimps. These are peeled and the head, exoskeleton and meat (peeled shrimp) were analyzed independently. Sachindra et al. (2005) process the samples by removing the head and body shell and the yield of meat, head and carapace are determined by weighing.

Samples of marine crab are transported from the landing centres and collected and transported to the laboratory under iced condition (-20ºC) until analysis (Coral-Hinostroza et al., 2001; Vilasoa-Martíınez et al., 2008; Sachindra, 2005). Crabs are processed by separating the meat from body and the claws. The gills, viscera etc., were discarded and the yield of meat and shell is determined by weighing (Sachindra, 2005).

The defrosting can be performed in different ways. The sample was thawed in a water bath temperature at 60-70°C. Then, the shells are dried in an oven at 55°C until a final dry weights constant. Pulverized samples were obtained, homogenized and stored in bottles of topaz and stored in the freezer (Vilasoa et.al., 2008).

However, Felix-Valenzuela et al., (2001) dried the samples directly at 75ºC during 4 h in a convection oven until constant weight is obtained and stored them in a polyethylene bag at room temperature until the AX extraction.

With respect to the fishes, they are collected to allow the animals to go through rigor mortis. They are then weighed, selected and packed individually in plastic bags to reduce the risk of physical changes. The individual fish are cut in three pieces (head, middle and tail) of equal length, for the analysis of AX. The middle portion (fillet) is cut in turn as shown in Figure 5 with or without skin and stored in cold (RØnsholdt et al., 2001) After thawed steaks, they are ground and mixed homogeneously and stored in sealed plastic containers at -70º/-80°C until analyzing (Johston et al., 2006).

Figure 5. Cuts in a fillet salmon

2.1 Preparation and extraction methods in microalgae.

Microalgae constitute a complex and heterogeneous group of organisms characterized by being photosynthetic organisms that possess simple reproductive structures (Herrero et al., 2012). The huge diversity of microalgae makes them an almost unlimited resource for discovery of bioactive compounds. Microalgal

biotechnology has advanced considerably, and it is possible to commercially produce some carotenoids through aquaculture. For instance, *Dunaliella salina* is able to accumulate high amounts of β-carotene when subjected to particular growth conditions, including high salinity (Zhu et al., 2008), whereas *Haematococcus pluvialis* is the major producer of AX under environmental stress, being able to selectively accumulate this carotenoid up to 5 % of its dry weight (Yuan et al., 2002).

AX occurs in algae like *H. pluvialis* in three main forms: free (5%), monoesters (70%) and diesters (25%). Monoesters and diesters could be hydrolyzed to free AX by saponification (Yuan et al., 2002). Therefore, organic solvents were used to extract AX and saponification was included to increase the content of free AX.

To optimize the saponification process, addition of NaOH was necessary for the hydrolysis of AX esters, but it could result in degradation of AX because AX is an alkali-labile carotenoid (López-Cervantes et al., 2006). Yuan et al., (1998) indicated that the high temperature favored the rate of hydrolysis of AX esters, but on the contrast, the degradation of AX was also promoted at the same time. According their results, the optimal saponification of AX and its esters were hydrolyzed by 0.075 mol/L NaOH/methanol solution at 40ºC for 30 min.

Traditionally, natural compounds have been obtained by method of conventional extraction with organic solvents. In this sense, Zou et al. (2011) applied conventional solvent extraction and saponification to obtain free AX from *Saccharina japonica* (*SJ*), a species of marine algae extensively cultivated in East Asia. In this study, grinding with mortal and a pestle was chosen to break SJ cell wall. During the extraction processes, dry SJ powder was extracted by different solvents (methanol, water, ethyl acetate, ethanol, n-hexane, acetone and dichloromethane) at room temperature. After 12 h of immersion and stirring, methanol and dichloromethane were proved to be better than the other solvents. High solubility of AX in dichloromethane (approximately 30 g/L) might facilitate the extraction of AX, but dichloromethane solution could not be completely separated from the SJ cell debris by centrifugation in this study. However, complete separation could be achieved when dichloromethane was mixed with methanol. Therefore, the extracted amount of AX with different ratio of

methanol/dichloromethane was investigated and the maximum extracted amount (29.3 μg/g) was obtained at the ratio of 25/75 (methanol/dichloromethane, v/v) after 5 h extraction.

Important step after extraction and previous to the determination is the solid-phase extraction (SPE). SPE has emerged as a powerful tool for chemical isolation and it can be used as extraction methods of liquid samples and/or as clean-up step previous to the determination. SPE could be performed using conventional sorbents or ionic liquid based silicas (IL-Si). They have excellent chemical and physical properties and have recently been used as SPE sorbents (Li et al., 2005) According to the chemical structure of AX (Yuan et al., 2002), the hydrophobic, dipole–dipole, and π–π interactions between AX and IL-Si exhibit some excellent effects to increase the separation efficiency of SPE. For that, 10 mL AX solution were selected to load onto the SPE cartridges containing SilprEMIm like IL-Si sorbents without a leak and then allowed to equilibrate for 6 h. Washing and elution were investigated to optimize selective extraction. Initially, washing solvents of different polarities (water, ethanol, acetonitrile, and n-hexane) were investigated. Water was found to be the most suitable. Most interferences were eliminated with 3 mL of water without loss of target compounds. Ethanol was the best to elute AX from the stationary phase (Zou et al., 2011).

Another extraction method is the pressurized liquid extraction (PLE). It uses solvents categorized as generally recognized as safe to extract carotenoids from different microalgae such as *H. pluvialis*, *D. salina*, *Chlorella vulgaris*, and *Spirulina platensis* has been demonstrated (Jaime et al., 2010; Plaza et al., 2012). This extraction technique is based on the extraction using temperature and pressure that maintain the extraction solvent in the liquid state during the extraction process (Mendiola et al., 2007). High pressure forces the solvent into the matrix, facilitating the extraction, whereas high temperature promotes higher analyte solubility (by increasing the solubility and mass transfer rate) and decreases the viscosity and the surface tension of the solvents, improving the extraction rate (Kaufman et al., 2002). In the liquid state, it is possible to obtain higher extraction yields in a shorter time using significantly smaller volumes of extraction solvents than in conventional extraction methods.

Castro-Puyana et al. (2013) optimized the extraction of carotenoids, including AX, from *Neochloris oleoabundans* using PLE with food-grade solvents such as ethanol and limonene. Experimental factors, including the extraction temperature and the solvent composition, were optimized using a factorial design.

The first step used to achieve efficient extraction from the microalgae was to break the cell wall to induce the lysis and this can hinder the extraction and availability of compounds and obtain the greatest quantity of carotenoids (Sarada et al. 2006; Cerón et al., 2008). Among different methods used, the highest yield of dry weight mass from *N. oleoabundans* (calculated as dry weight/initial weight expressed as a percentage) was obtained using freezing–thawing and cryogenic grinding as the pretreatment prior to PLE. The optimum conditions predicted by the model were 112 °C as the extraction temperature and 100 % ethanol as the extraction solvent (Castro-Puyana et al., 2013).

Nevertheless, possible changes in the physicochemical properties of extracts can alter their functionality, so the extraction processes should be performed at suitable and mild conditions. Supercritical carbon dioxide fluid extraction (SFE-CO_2) is an advanced technology that has a low environmental impact because it entails no residue of harmful solvents being nontoxic and noncorrosive and have easy separation from extracts (Macías-Sánchez et al., 2005). With an operating temperature and pressure that are above the critical values, supercritical fluids have their own special physicochemical properties between those of a liquid and gas, such as high diffusivity, high compressibility, low viscosity, and low surface tension (Felix-Valenzuela et al., 2001). This allows the fluid to diffuse easily through the natural solid matrix, and thus achieve better quality extraction of the natural compounds compared to the conventional liquid solvents. Furthermore, since CO_2 has a low critical temperature and pressure (31.1ºC and 1084.86 psi), the extracts would not suffer thermal degradation during the process, making it an ideal solvent for bioactive constituents (Macmudah et al., 2006).

Pan et al. (2012) used SFE-CO_2 to extract AX from *H. pluvialis* and demonstrated that the addition of ethanol to SC-CO_2 was the most effective in terms of solubility and extraction yield with regard to SC-CO2 fluid (Macmudah et al., 2006; de la Fuente et al., 2006). The batch extraction yield decreased with increases in the

amount of *H. pluvialis*, flow rate, static extraction time, extraction pressure and temperature (above 66.0ºC), due to the absorption effect of the ethanol modifier and thermal degradation of AX. The best conditions determined from this work were *H. pluvialis* weight 21.67 g/LCO_2-flow rate 6.0 NL/min, extraction time 20 min, extraction pressure 4500 psi, volume of ethanol modifier added 60 mL, extraction temperature 50ºC and modifier composition 99.5%.

2.2. Sample preparation and extraction methods in shrimps

In general, conventional extraction methods are used to extract AX from shrimps samples. However, each author used different organic solvents to perform the extraction. The most of the methods reported to extract AX from shell matrix employ edible oils, hydrochloric acid or organic solvent (Sachindra et al., 2005a).

In this sense, the extraction conducted by Simpson et al. (1985) consisted in the addition of acetone to the homogenised samples to extract the pigment. The acetone extracts were combined and separated in phase with petroleum ether (40-60°C). The petroleum ether extract was washed several times with 0.1M of NaCl solution to remove traces of acetone, then dried with sodium sulphate, filtered and washed again and evaporated with nitrogen gas. The resulting concentrate was collected in petroleum ether.

López-Cervantes et al. (2006) measured the AX content in samples of lipid fractions the shrimps. An aliquot of 5 mL of different solvents (methanol, n-hexane, ethanol, acetonitrile and methylethylcetone) was added and immediately shaken for 20 s. Then, the samples were sonicated for 5 min to complete extraction, followed by centrifugation for 15 min at 425 rpm. Best results were obtained with n-hexane although they do not use it because it is incompatible with the mobile phase. Methanol was selected in this study.

Sánchez-Silva (2012) prepared the shrimps samples according to the method described by López- Cervantes et al. (2006) with light modifications. In this case, about 0.1–0.25 g of shrimp by-products were weighted, 5 mL of methanol was added and it

was thoroughly mixed in a vortex for 1 min. Afterwards, samples were kept in an ultrasonic bath for 10 min to allow complete extraction, followed by filtration through 125mm diameter filter paper from Whatman, and then filtered through a 0.2 mm pore size PTFE syringe filter.

2.3. Sample Preparation and extraction methods in crabs.

In the same sense, conventional extraction methods are used to extract AX from crab samples with different organic solvents to perform the extraction although Felix-Valenzuela et al. (2000) used supercritical CO_2/ethanol extraction to extract AX from crab samples obtaining good results.

In general, the extraction procedure is carried out with acetone. Sachindra et al. (2005b) and López et al. (2006) obtained carotenoids, including AX, from 25 g of samples which were extracted using acetone and phases separated with petroleum ether. The petroleum ether extract is dried with sodium sulphate, filtered, flushed with nitrogen and then evaporated under vacuum at 40º C using a rotary flash evaporator. The resulting carotenoid concentrate was taken up in petroleum ether

.

Another authors made some changes using organic solvents too (Vilasoa – Martínes et al., 2008). In this case, one gram of dry sample with three portions of 5 mL of acetone was centrifuged at 2000 rpm for 5 min. Organic phase was separated and the resulting solution that containing the carotenoids, including AX, is evaporated to dryness on a rotary evaporator system. Obtained extract is dissolved in 1mL of methanol-dichloromethane-hexane (50:25:25), filtered and injected for analysis.

2.4. Sample Preparation and extraction methods in fishes.

For extraction of the pigments in the sample fishes, different authors have used conventional solid-liquid extraction, using organic solvents. Sheehan et al. (1998) and Hanne et al. (1998) made the process with acetone. Three grams of each sample were homogenised with an equal weight of anhydrous sodium sulphate and extracted with three 10 mL aliquots of acetone. The combined extracts were filtered, the solvent

evaporated under nitrogen (< 50°C) and the residue redissolved in 5 mL n-heptane. This solution was then transferred onto a silica S-Pak cartridge like clean-up SPE step. The cartridge was eluted with l0m1 of 20% diethyl ether in n- heptane and 10 mL ethanol. The ethanol eluate was evaporated under nitrogen and the residue was redissolved in 1 mL of methanol.

Rønsholdt et al. (2001) used ten grams of sample and were mixed with 14 mL of demineralized water in a 250 mL centrifuge bottle (high-density propylene). Fifty millilitres of methanol were added and the sample was mixed at 2200 rpm for 30 s. Then, 25 mL of $CHCl_3$ was added and the sample was mixed for 30 s. Finally, 25 mL of demineralized water was added and mixed for 30 s. The sample was subsequently centrifuged at 2900 rpm for 10 min at 103ºC in a cooling centrifuge. After centrifugation, the methanol phase (top) was sucked out, the protein layer was gently tilted and the chloroform phase (bottom) containing the oil and carotenoids was decanted and was protected from light by wrapping in aluminium foil. Percentage recovery of carotenoids was determined by adding 2 mL AX standard solution (40 mg/L) .

Barua (2001) presented an extraction that is carried out with ethyl acetate: methanol (1:1), followed by ethyl acetate, and finally, hexane. Pooled supernatants were evaporated to dryness under nitrogen atmosphere, redissolving the carotenoid residue in a volume of hexane.

Bikerland et al. (2004) did similar extraction that another authors. The sample was extracted with chloroform using an ultra-turrax macerator (13000 rpm) for 1 min. After settling for 10 min in the dark, the sample was centrifuged. The water-phase was decanted off, and the extraction procedure was repeated twice. In an effort to assure complete extraction of AX from the protein extracts, the chloroform phase was subjected to a third extraction. The extract was filtered into an HPLC sample vial through a 0.45-mm syringe filter.

Other authors utilized 10-20 grams of fish muscle crumbles into a beaker and swam 5 g of hydrated magnesium sulfate. After, 40 mL of acetone is added and mixed

using a homogenizer. Then, the mixture is filtered and the remaining residue was resuspended in 40 mL of acetone and followed by homogenizing. This process is repeated several times, until it becomes colorless. To remove excess water, 20 mL of ethanol is evaporated, and the remaining oil was dissolved in mobile phase (86:14 hexane / acetone) and analyzed (Johnston et al., 2006).

Reported extraction methods use large volumes typically 150–170 mL (Johnston et al., 2006; Rønsholdt et al., 2001;) of toxic, flammable and environmentally damaging organic solvents, they are laborious and time-consuming and invariably involve multi-step extractions. Furthermore, sometimes solvent evaporation is required (Johnston et al., 2006) and the fish fats interfere in the photometric determination and their contribution to the absorbance measurements must be subtracted to obtain accurate results (Rønsholdt et al., 2001).

An alternative to organic solvents is the use of supramolecular solvents (SUPRASs) for the effective, rapid and inexpensive extraction of AX from farmed salmonid fishes obtaining acceptable results (Caballo et al., 2012). SUPRASs is made up of biosurfactant, tetrahydrofurane (THF) and water and they are water immiscible liquids made up of surfactant aggregates dispersed in a continuous phase (usually water), which are produced by two well-defined self-assembly processes occurring on two scales, molecular and nano. First, surfactants aggregate above a critical concentration and then, the generated nanostructures self-assemble under the action of a external stimuli (e.g. temperature, pH, electrolyte, a non-solvent for the surfactant aggregate) and separate as an immiscible liquid in equilibrium with a surfactant-lean phase. These solvents constitute an advantageous alternative for the extraction of organic compounds in a wide polarity range from both liquid (e.g., García-Fonseca, Ballesteros-Gómez, Rubio and Pérez-Bendito, 2008) and solid samples (e.g., Moral et al, 2009). In this case, precision of the method was 3.3%, and recoveries were near to 100%.

3. Determination

To perform the chemical characterization of the compounds obtained after extraction, it is necessary to use advance analytical tools to identify each compound. Among the high-performance chromatographic methods available, gas chromatography (GC) is unsuitable for the analysis of AX because of the inherent instability and low volatility of this molecules. Therefore, HPLC using absorption and mass detection (MS) techniques is currently the most common chromatographic method used for its analysis. Improvements in chromatographic performance using ultra high-performance liquid chromatography (UHPLC) have recently been reported (Swartz 2005). This technique uses narrow-bore columns packed with very small particles (below 2 μm) and mobile phase delivery systems operating at high back-pressures. While in conventional HPLC, the maximum back-pressure is in the region of 35–40 MPa depending on the instrument, back-pressures in UHPLC can reach up to 103.5 MPa. Thus, UHPLC offers several advantages over conventional HPLC, such as faster analyses (shorter retention times), narrower peaks (giving increased signal-to-noise ratio) and greater sensitivity (Sanches Silva et al., 2012).

3.1 HPLC

Normal and reversed phase systems, in isocratic or gradient elution modes, have been used to analyze carotenoids, included AX. Cromatographic optimization consists of choosing the appropriate mobile phases, flow rate, injection volume, and the column temperature. The difference obtained between the different methods is that the time of extraction of AX will do differently, depending on the optimized conditions.

Most of the separations of these compounds reported in the literature involve reversed-phase HPLC using C_{18} and C_{30} columns. In general, polymeric C_{30} phases provide better separations of carotenoid geometric isomers than C_{18} ones (Khachik et al., 1997; Furr et al., 2004) although C_{18} columns are the most used. Various mixtures of solvents have been used with these reversed-phases, including water, methanol, acetonitrile, 2-propanol, acetone, ethyl acetate, tetrahydrofuran, t-butyl methylether (MTBE), dichloromethane (DCM) and chloroform (Felt et al., 2005). These solvents

have already been used in isocratic (e.g., Yuan et al., 1997; López-Cervantes et al., 2006) or gradient modes (e.g., Lin et al., 2005).

The selection of the wavelength to determine AX is based on the maximum absorbance in the UV–vis scan, which occurred at 480 nm. Yuan et al., (1997) also used this same wavelength to determine AX by HPLC-DAD. However, other wavelengths have been used to determine this compound, such as 440nm (Sachindra et al., 2005), 450nm (Lacker et al., 1999; Vilasoa-Martínez et al., 2008), 470nm (Refsgaard et al., 1998; Sheehan et al., 1998) or 476nm (López-Cervantes et al., 2006, Zou et al., 2011). One of the reasons for these differences in wavelengths is the use of other solvents .

Felix–Valenzuela (2000) determined AX in extracts obtained from crabs using supercritical fluid extraction (SFE). They utilized HPLC with UV-VIS detector and a reverse phase C_{18} ultrasphere column. Elution was carried out using a mobile isocratic phase prepared with 5.5% dichloromethane, 85% methanol, 5% acetonitrile and 4.5% water. Flow rate was 1 mL/min and detection was performed at 480 nm (Yuan et al., 1998**)** at room temperature.

Coral (2002) used two isocratic HPLC systems with different columns to determine AX in crabs: in system I used acetone and n-hexane as mobile phase (20:80, with 1.5 mL/min) and system II used a mobile phase of acetone in n-hexane (14:86, with 1.2 mL/min). Detection wavelength was set to 470 nm or 491 nm for the analysis of AX dicamphanates.

Sachindra et al. (2005) obtained concentrated carotenoids extracts of shrimps in petroleum ether and they was subjected to TLC using activated silica gel G plates to separate AX monoester (Rf 0.50) and diester (Rf 0.76). They were scraped off the developed plates, suspended in acetone, filtered and concentrated. AX extract was subjected to determination with HPLC with UV/vis detector using a μBondapack C_{18} column of 30 cm. Samples were introduced in acetone (20 μL) and eluted for 15min with a concave mobile phase gradient of 80–100% methanol in water at a flow rate of 2.0 mL/min and the eluate absorbance was measured at 440 nm.

In the same way, López-Cervantes et al. (2006) used a HPLC system with diode array detector (DAD) with a SS Exil ODS column to determine AX in shrimps. The mobile phase consisted of the isocratic mixture of water:methanol:dichloromethane:acetonitrile (4.5:28:22:45.5) at a flow rate of 1.0 mL/min at ambient temperature. Detection and identification were performed at 476 nm.

Vilasoa-Martínes (2008) used a HPLC system with DAD and fluorescence detectors to determine AX in crabs. The separation was performed on a thermostatized ODS2 column. AX was determined at 450 nm. The fluorescence detector was set at λ_{ex} 280nm and λ_{em}331nm. Chromatographic analysis was performed in gradient using a mobile phase consisting of methanol as solvent A, acetonitrile as solvent B and hexane–dichloromethane (50:50) as solvent C. The flow rate was varied between 0.8 and 2mL/min. Method precision was 6% and limit of detection detection limit for AX was 0.01 µg/mL. The confirmation was made by LC-MS, as described below.

Holtin et al. (2009) used a HPLC system with a diode array detector at 455 nm to separate all forms of AX in *Haematococcus pluvialis* extract The separations were performed with a C_{30} column and a C_{18} cartridge was used as precolumn. The separation of the algae extract was achieved using a mobile phase composition of methanol/tert-

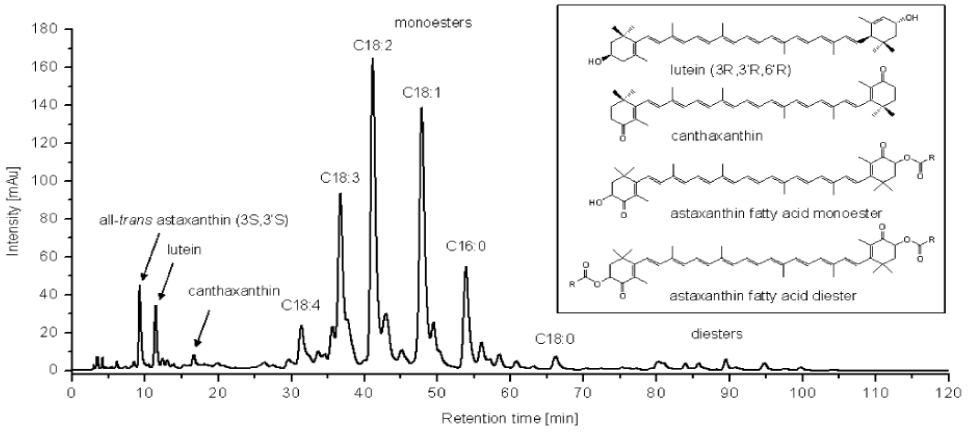

Figure 6. HPLC chromatogram (DAD, 455 nm) of the *H. pluvialis* extract and the chemical structures of the carotenoids (R = saturated or unsaturated alkyl chains) in the extract (Holtin et al., 2009)

butyl-methyl-ether (MTBE)/ water [83:15:2 v/v/v (A) and 8:90:2 v/v/v (B)]. The elution proceeded isocratically at 100% A for 20 min and was followed by a linear gradient to 40% A until 160 min, with a flow rate of 1 mL/min. Obtained chromatogram is shown in Figure 6.

As discussed above, Zou et al. (2011) determined AX in *Sacarina japonica* using a commercial C_{18} column and a mobile phase of dichloromethane/methanol/acetonitrile/water (5:85:5.5:4.5) in a isocratic elution, with a detection with DAD at 476 nm. Flow-rate was set at 0.5 mL/min. Each sample was repeatedly injected 5 times for evaluating the precision and accuracy of analysis. Calibration curves were constructed in the range of 0.78 to 50 μg/mL. Method precision, as repeatability, was evaluated on the basis of the relative standard deviation (RSD) of AX, with determination in 12 replicates of the same sample prepared on the same day. The result was 0.32%, which was sufficient for routine analysis of AX. Determination of the detection limit for AX was 61 ng/mL. Comparing with another results, approximately, 104 to 398 ng/mL (López-Cervantes et al., 2006), this confirmed that these values are of acceptable precision and accuracy to detrmine different forms of AX in these kind of samples.

As a final example, Castro-Puyana (2013) quantified AX monopalmitate and AX dipalmitate in *Neochloris oleoabundans* by LC–DAD-detection using a C_{30} reversed-phase column and the mobile phase was a mixture of methanol–MTBE–water (90:7:3 v/v/v) (solvent A) and methanol–MTBE (10:90 v/v) (solvent B) eluted according to different gradients. The flow rate was 0.8 mL/min, the injection volume was 10 μL, and detection was at 450 nm. Standards were dissolved in hexane–acetone (1:1 v/v).

Table 1 compares some of the methods found in the literature for the determination of AX in different matrices by LC coupled with DAD detection.

Table 1. Comparison of different LC methods to determine AX.

Matrix	Extraction tecnhique	Extraction solvent	Mobile phase	Analytical column	Reference
Neochloris oleoabundans	Liquid–liquid extraction	Acetone and 0.1%(w/v) butylated hydroxytoluene	Reverse phase Solvent A: methanol-MTBE-water (90:7:3 v/v) Solvent B: Methanol-MTBE (10:90 v/v)	C_{30}	Castro-Puyana et al. (2013)
Raw and smoked salmon	Liquid–liquid extraction followed by solid-phase extraction	Acetone (liquid–liquid extraction); elution solvent (solid-phase extraction), 20% diethyl ether in heptane	Isocratic: 20% ethyl-acetate and 80% methanol–water (9 + 1)	C_{18}	Sheehan et al. (1998)
Farmed Atlantic Salmon	Bligh–Dyer method for extraction of fat	Mixture chloroform–methanol (1:2)	Isocratic: N heptane–acetone (86:14, v/v)	LiChrosorb Si_{60} (100mmx3mm x5 μm)	Refsgaard et al. (1998)
Phaffia rhodozyma	Supercritical fluid extraction	n-Hexane–ethanol (1:1, v/v)	Isocratic: acetone–hexane (82:18, v/v)	Luna-Silca column	Lim et al. (2002)
Chlorococcum sp.	Liquid–liquid extraction	n-Hexane–ethanol (1:1, v/v)	Gradient: solvent A: dichloromethane–methanol–acetonitrile–water (5:85:5.5:4.5, v/v) Solvent B: dichloromethane–methanol–acetonitrile–water (22:28:45.5:4.5, v/v)	C_{18}	Li et al. (2001)

Table 1 (continued)

Matrix	Extraction tecnhique	Extraction solvent	Mobile phase	Analytical column	Reference
Haematococcus lacustris	Liquid–liquid extraction	Methanol–dichloromethane (3:1, v/v)	Gradient: methanol–dichloromethane–water (80.5:17:2.5, v/v/v) and water	C_{18}	Yuan and Chen (1997)
Indian shrimps	Liquid–liquid extraction	Acetone	Gradient: 80–100% methanol in water at a flow rate of 2.0 mL/min	C_{18}	Sachindra et al. (2005a)
Crabs	Liquid–liquid extraction	Acetone	Gradient: 80–100% methanol in water at a flow rate of 2.0 mL/min	C_{18}	Sachindra et al. (2005b)
Spear shrimp shells (Parapenaeopsis hardwickii)	Liquid–liquid extraction or supercritical fluid extraction	Acetone (liquid–liquid extraction	Gradient: methanol–dichloromethane–acetonitrile (90:5:5, v/v/v) and water	C_{18}	Lin et al. (2005)
Shell of Chioecetes opilio	Liquid–liquid extraction	Acetone	Gradient: solvent A:, Methanol. Solvent B: acetonitrile; Solvent C: hexane–dichloromethane (50:50)	ODS2	Vilasoa-Martínez et al. (2008)

3.2 UHPLC

UHPLC is a promising tool for carotenoid analysis, including AX. As indicated above, one of the main differences between HPLC and UHPLC columns is the particle size of the stationary phase. Smaller particles make the column more efficient and they tend to allow solutes to transfer into and out of the particle more quickly because their diffusion path lengths are shorter. Thus, the solute is eluted as a narrow peak because it spends less time in the stationary and stagnant mobile phase where band broadening occurs. A higher resolution between analytes can be expected. This technique can also greatly reduce the run time, thereby avoiding the risk of degradation, a process caused by the high sensitivity of these compounds to physical and chemical factors.

Sánches-Silva et al. (2012) developed and optimized a method to determine AX by UHPLC with a DAD method in shrimp constituents (exoskeleton, head and meat) of both raw and cooked shrimp and in fermented shrimp waste. Moreover, the method was also used to evaluate the migration of astaxanthin from active packaging into food simulants. Separation and quantification were performed with a UPLCW BEH analytical column of 5 cm. Mobile phases were (A) ACN–methanol (containing 0.05 M ammonium acetate)–DCM (75:20:5, v/v/v); (B) ultrapure water. AX detection was monitored at 480 nm. The analytical method was validated according to US Food and Drug Administration (Food and Drug Administration and Center for Drug Evaluation and Research, 1994) guidelines. The method presented good intra-assay precision (RSD = 3.6%) and it also presented good inter-assay precision (RSD = 4.7%), evaluated by analysis of three independent samples in three consecutive days. The limit of detection was 0.054 μg/mL and the limit of quantification was 0.16 μg/mL. The limit of detection was lower than the one found by López-Cervantes et al. (2006), which used the same mobile phase solvents. Recovery was determined using the standard addition procedure and the result was satisfactory (90.5%) for 8 mg of added astaxanthin.

3.3 MS for identification and quantification.

In HPLC, UV–VIS instruments are the most common detectors used to identify AX. However, given that the UV–vis spectra of many carotenoids are similar and a

number of structurally related molecules coelute, many researchers have complemented the identification of carotenoids using other detection methods like NMR and IR, between them. Among those, mass detectors have shown great advantages for the analysis of these substances, including the elucidation of their structure on the basis of the molecular mass and their fragmentation pattern. These properties facilitate the quantification of individual carotenoids that coelute.

The fragment pattern observed in the carotenoid mass spectra depends on the ionization technique, including electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI), atmospheric pressure chemical ionization (APCI) and more recently, atmospheric pressure photoionization (APPI) and atmospheric pressure solids analysis probe (ASAP) and the composition of the mobile phase used.

APCI has been used to successfully ionize not only carotenes but also carotenoid esters , thereby demonstrating the suitability of this approach to ionize carotenoids with different polarities. A highly promising technique to ionize nonpolar compounds, such as carotenoids, is APPI. This method has recently been introduced as a new ionization method for LC–MS and can be considered complementary to the other two atmospheric pressure ionization (API) techniques, namely ESI and APCI although research is required to test the effectiveness of this technique to ionize diverse carotenoids.

Thus, although some carotenoids show the same or a very similar fragmentation pattern (meaning that their structures are similar and therefore they might coelute), differences between the intensities of their fragments have been reported. These differences can be used to distinguish the molecules. Moreover, these differences can provide an insight into the predominant carotenoid when coelution occurs. In addition, LC–MS has been used not only to characterize carotenoids but also to quantify them. The latter is possible because of the low detection limits and wide linear dynamic range values exhibited by the mass detectors.

In this sense, Vilasoa-Martínez et al. (2008) confirmed AX forms in crabs with a LC–MS system which have a ODS2 column and mobile phases: A (methanol–

acetonitrile, 14:86 v/v) and B (hexane–dichloromethane, 50:50 v/v) with a gradient elution and different flow rates Detector operated under the following conditions: atmospheric pressure chemical ionization positive (APCI+); probe temperature 450ºC, cone voltage (+) 20V, drying gas nitrogen at 425 L/h, APCI gas nitrogen at 175 L/lh.

In the same manner, Holtin et al. (2009) developed a gradient method, described previosuly, to separate and determine the composition of the complex astaxanthin extract by HPLC-MS using an APCI interface and an ion trap. The detection was performed using APCI in the positive ionization mode. The voltage of the corona needle was set to 4 kV. Nitrogen was used as the drying gas as well as the carrier gas at a flow rate of 5 L/min, with a nebulizer pressure of 65 psi. The ionization chamber temperature was set to 450°C and the dry gas temperature was held at 350°C. The compound stability was set to 80% and the trap drive level to 70%. In this study, mono acid esters of AX are the most common carotenoids found in the microalgae *Haematococcus pluvialis*. Here, in addition to minor amounts of palmitinic acid (C16:0), single and polyunsaturated fatty acids of the C18 family (C18:1, C18:2 and C18:3) were found to be the principally occurring fats. In addition to the all-trans compound, the 9- and 13-cis isomers were found to be the most common configurations in the algal extract.

Castro-Puyana et al. (2013) characterized the extracts obtained from *Neochloris oleoabundans* with a LC equipped with a diode-array detector as discussed above and directly coupled to an ion trap mass spectrometer via an APCI interface, in positive ionization mode using the following parameters: capillary voltage,−3.5 kV; drying temperature, 350 °C; vaporizer temperature, 400 °C; drying gas flow rate, 5 L/min; corona current (which sets the discharge amperage for the APCI source), 4,000 nA; nebulizer gas pressure, 60 psi. A range from m/z 150 to m/z 1,300 was acquired. Combining the data obtained from the analysis of the extracts by LC-DAD and LC-MS, authors were able to conduct, for the first time, a tentative identification of different carotenoids, including different AX forms (monoesters and diesters), present in *N. oleoabundans* extracts under certain growth conditions.

Tandem mass spectrometry (MS/MS) provides many advantages for the analysis of carotenoids. LC–MS/MS offers added selectivity and specificity to the simple LC–

MS systems. This more selective detection method reduces interference by impurities in the extract and allows the following: (a) a minimal sample clean-up (leading to a high sample throughput); (b) distinguishing between carotenoids that coelute; (c) information about structural isomers; and (d) a decrease in overall analysis time. Thus, using LC–MS/MS, it is possible to distinguish between structural isomers. In this sense, Rivera et al. (2011) have verified the use of transitions for improving the selectivity of carotenoid analysis. They observed that antheraxanthin and astaxanthin coelute under the chromatographic conditions used in the UHPLC analysis. However, these carotenoids were distinguished using the specific transitions found for each carotenoid using APCI. Antheraxanthin was identified using the MS/MS transitions 585.3 > 93.1 and 585.3 > 105.2, while astaxanthin presented the transitions 597.6 > 147.1 and 597.6 > 579.4. Neither compound showed the corresponding transitions of its counterpart species. Thus, the MS/MS transitions allow the individual quantification of these substances in spite of the fact that they show the same chromatographic retention time. MS/MS spectra have also proven especially valuable for confirming the presence of specific components.

However, carotenoid analysis is sometimes difficult with soft ionization techniques such as ESI because these molecules fail to ionize efficiently with these systems. Consequently, the mass spectra present poor structural information with a lack of molecular ions. Moreover, the multiple fragments observed often do not provide any valuable information about the structural characteristic of the compound. However, carotenoid ionization can be improved by adding chemical compounds that facilitate ionization like ammonium acetate, acetic acid and halogen-containing eluents.

Qualitative analysis of carotenoids can be used for many purposes, among these to (a) obtain a rapid overview of the carotenoids present in a sample; (b) study carotenoid compositions in their natural environment; (c) study the conformational changes of carotenoids; and (d) classify samples.

4. Conclusions and future trends.

Microalgae, shrimp and crab species can be considered as a novel potential source of natural carotenoids, including AX, which can be used in aquaculture feeds. A trend for increasing natural carotenoid sources, mainly from marine origin, in aquaculture diets may force the industry to better determine AX in the natural sources and its role in fish physiology, in order to growth towards sustainability of the resources and the industry. Research is being conducted to study the morphological and physiological changes associated with accumulation of carotenoids and the effect of the growth conditions on the overproduction of them and how act in aquaculture fishes.

For that, AX determination in marine organisms is very important. In fact, one of the most critical steps in the determination of AX is the sample preparation due to their physical-chemical characteristics. Study of new extraction and clean-up procedures are necessary. In this sense, future trends in this field have to be oriented toward the development of new extraction protocols for clean-up of marine organism samples.

Regarding the current instrumentation, several techniques can be used to improve the separation and detection of different AX isomers and their derivatives. HPLC and more recently, UHPLC are used for their separation. Both chromatographic systems are usually linked to DAD detectors and to MS and MS/MS detectors. The latter provides more confirmative information, thereby allowing the analysis of coeluting compounds. Qualitative or semi-quantitative analysis can be carried out using a large number of spectroscopic and mass spectrometric methods (e.g. MS-TOF). This variety of detection techniques will contribute to extending information about carotenoids, including AX forms, such as their distribution in their natural environment and the type of chemical changes they undergo in marine organisms.

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Alumna: Tania Darias Hernández

Para la elaboración de este trabajo he realizado las siguientes actividades:

a) Estudio bibliográficos sobre el conocimiento, la determinación, la importancia, las aplicaciones de astaxantina en diferentes matrices biológicas,
b) Parte experimental, realizando algunas extracciones convencionales e investigando la preparación del patrón de astaxantina. Además, he trabajado con un sistema UHPLC.

Durante el periodo en el que he realizado las actividades mencionadas anteriormente he tenido una relación fluida con mis compañeros de laboratorio y con mis directoras, sintiéndome parte integrada del grupo de investigación de Análisis Químico Medioambiental (AQMA).

Algunos aspectos positivos del Trabajo Fin de Máster (TFM) son el haber aprendido cuál es el comportamiento de la astaxantina, para qué sirve y la importancia que tiene, trabajar en el laboratorio aprendiendo cómo hacer las extracciones de astaxantina de algunos peces y manejar el UHPLC. Además, me he sentido implicada en el trabajo realizado y tengo una buena relación con el grupo AQMA.

Algunos aspectos negativos del mismo han sido la demora en la llegada del patrón de astaxantina, las complicaciones en determinar dicho compuesto y no obtener los resultados esperados, inicialmente, para realizar el trabajo experimental que habíamos programado en un primer momento.

Realizar este trabajo me ha supuesto tener una visión global de lo qué es el trabajo de investigación, aportándome un conocimiento importante sobre el tema elegido para mi TFM. El final de este trabajo me ha causado mucho interés sobre las futuras tendencias que puede tener este campo, quedándome con ganas de continuar trabajando y ampliando mis conocimientos.